# Lessons learned: Natural killer cell education as a determinant of the anti-viral functional potential of natural killer cells

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Submitted August 2012

A thesis submitted to McGill University in partial fulfillment of the requirements

of the degree of Doctor of Philosophy

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# Abstract

A vaccine against the human immunodeficiency virus (HIV) is urgently needed. Recent estimates predict that 34 million people are currently infected with HIV. Attempts to induce potentially protective cytotoxic T-lymphocytes or broadly neutralizing antibodies by vaccination have either proven unsuccessful or failed to elicit the desired immune responses. However, the recent RV144 vaccine trial that provided partial protection against HIV infection appears to have induced antibodies that can utilize cells of the innate immune system, such as natural killer (NK) cells, to mediate antibody-dependent cellular cytotoxicity (ADCC) against HIV-infected cells. This potential mechanism of protection corroborates recent epidemiological and functional studies demonstrating that HIV-exposed seronegative individuals (HESN) and HIV-infected slow progressors (SP) have higher functioning NK cells and carry certain allelic combinations of killer immunoglobulin-like receptors (KIR) and their major histocompatibility complex class I (MHC-I or HLA-I) ligands that confer NK cells with enhanced functionality. Cumulatively, these observations suggest that understanding the conferral of functional potential during NK cell ontogeny could be important for designing anti-HIV vaccine constructs.

In particular, data from HESN and SP have demonstrated that allelic combinations of *KIR3DL1* and its *HLA-Bw4* ligand are associated with protective outcomes in the context of HIV. Although previous work has demonstrated that

interactions between these receptor ligand combinations during NK cell development confers NK cells with functional potential, the exact mechanism of the protective outcomes in the context of HIV remain unknown. For example, KIR3DL1$^{+}$ NK cells have been demonstrated to be hypofunctional in the presence of autologous HIV-infected T cells. This suggests that if KIR3DL1$^{+}$ NK cells are providing protection through mediating function, they require additional activating signals. As previously published data has demonstrated that activation through CD16a by antibody constant regions can overcome KIR-mediated NK cell inhibition and lead to ADCC of allogeneic cells, we hypothesized that ADCC could overcome inhibitory signalling and allow KIR3DL1$^{+}$ NK cells to respond to autologous anti-HIV ADCC target cells.

The data presented in this thesis reaffirms that HIV protective *KIR3DL1/HLA-Bw4* allelic combinations confer enhanced functional potential upon NK cells. The results presented demonstrate that *KIR3DL1/HLA-Bw4* combinations educate NK cells for enhanced anti-HIV ADCC against autologous target cells, and that this educational advantage is maintained after stimulation with function-conferring cytokines, such as IL-15. Furthermore, allelic combinations of *KIR3DL1/HLA-Bw4* that are protective in the context of HIV are shown to confer the highest ADCC functional potential.

These data suggest that the education of NK cells by allelic combinations of *KIR3DL1/HLA-Bw4* could explain some of the protection observed in individuals with these combined genotypes. However, as we also observed an

abrogation of this education-conferred functional advantage in HIV-infected individuals, we propose that the mechanisms of NK cell-mediated protection differ between uninfected HESN and infected SP.

# Résumé

Il existe un urgent besoin de développer un vaccin dirigé contre le virus de l'immunodéficience humaine (VIH). Des données récentes estiment que 34 millions de personnes sont présentement infectées par le VIH. Les tentatives d'induire une protection potentielle par les vaccins qui cible les réponses des lymphocytes T cytotoxiques ou les anticorps neutralisants ont soit été non fructueuses ou n'ont pas donné les résultats escomptés en ce qui concerne la réponse immunitaire. Par contre, la récente étude de vaccin RV144 ayant engendré une protection partielle contre l'infection par le VIH semble avoir induit des anticorps qui utilisent les cellules du système immunitaire inné, comme les «natural killer» (NK), afin de médier la cytotoxicité cellulaire dépendante des anticorps (ADCC) contre les cellules infectées par le VIH. Ce mécanisme potentiel de protection corrobore avec les récentes études épidémiologiques et fonctionnelles démontrant que les individus séronégatifs exposés au VIH (HESN) et les progresseurs lents infectés par le VIH (SP) ont des cellules NK avec un potentiel fonctionnel plus élevé et portent plus fréquemment certaines combinaisons alléliques pour les récepteurs «killer immunoglobulin-like» (KIR) et leurs ligands CMH-1 (ou HLA-1) encodés par le complexe majeur d'histocompatibilité de classe I qui confèrent aux cellules NK un potentiel fonctionnel plus élevé. Cumulativement, ces observations suggèrent qu'une meilleure compréhension du processus de développement du potentiel

fonctionnel des cellules NK qui se produit lors de leur ontogenèse serait importante pour la conception de vaccins anti-VIH.

En particulier, des données provenant de HESN et de SP ont démontré que la combinaison allélique *KIR3DL1* avec son ligand *HLA-Bw4* est associée avec la protection dans le contexte du VIH. Bien que des études ultérieures aient démontré que les interactions entre ces récepteurs et leurs ligands pendant le développement des cellules NK confèrent un potentiel fonctionnel aux cellules NK, le mécanisme exact de ce potentiel protecteur dans le contexte du VIH demeure inconnu. Par exemple, il a été établi que les cellules NK KIR3DL1$^{+}$ sont hypofonctionnelles en présence des cellules T autologues infectées par le VIH. Cela suggère que si les cellules NK KIR3DL1$^{+}$ profèrent la protection par le biais de leurs fonctions, elles requièrent des signaux activateurs additionnels. Des résultats publiés auparavant ont démontré que l'activation des cellules NK par le biais du récepteur CD16a, qui reconnait la région constante des anticorps, peut annuler la suppression des cellules NK venant des signaux intracellulaires émis par les récepteurs KIRs. L'activation des cellules NK via CD16a induit la cytotolyse des cellules allogéniques via l'ADCC. Sur la base de ces observations nous émettons comme hypothèse que l'ADCC pourrait dominer sur la signalisation inhibitrice venant de certains KIR et permettre aux cellules NK KIR3DL1$^{+}$ de répondre aux cellules cibles autologues infectées par le VIH via le mécanisme de l'ADCC.

Les données présentées dans cette thèse réaffirment que la combinaison allélique VIH protective *KIR3DL1/HLA-Bw4* confère un potentiel fonctionnel plus élevé aux cellules NK. Les résultats exposés démontrent que les cellules NK venant d'individu portant la combinaison *KIR3DL1/HLA-Bw4* ont reçu une éducation qui se manifeste comme une réponse ADCC anti-VIH contre les cellules cibles autologues plus accrus que ceux venant de sujets portant d'autre combinaison KIR/HLA. Cet avantage éducationnel est maintenu suite à la stimulation avec des cytokines, telle que l'IL-15, qui sont capable d'améliorer la fonction des cellules NK. De plus, une certaine combinaison allélique de *KIR3DL1/HLA-Bw4*, qui est la plus protectrice dans le contexte de l'infection par le VIH, est capable de conférer au cellules NK le potentiel fonctionnel ADCC le plus élevé.

Ces données suggèrent que l'éducation des cellules NK par la combinaison allélique *KIR3DL1/HLA-Bw4* pourrait expliquer une part de la protection observée chez les individus possédant ces génotypes. Par contre, comme nous avons aussi observé une suppression de cet avantage fonctionnel conféré par l'éducation des cellules NK chez des individus infectés par le VIH, nous proposons que les mécanismes de protection médiés par les cellules NK diffèrent entre les HESN non infectés et les individus infectés SP.

# Preface

**Chapter 2: Mind the gap: lack of association between KIR3DL1*004/HLA-Bw4-induced natural killer cell function and protection from HIV infection**

Matthew S Parsons, Salix Boulet, Rujun Song, Julie Bruneau, Naglaa H Shoukry, Jean-Pierre Routy, Christos M Tsoukas, and Nicole F Bernard.

Published in The Journal of Infectious Diseases. 2010 Nov; 202 (Suppl 3): S356-S360.

**Contributions of authors:** MSP, SB, and RS performed experimental work. MSP analyzed the data and prepared the manuscript. JB, NHS, JPR, and CMT are responsible for the cohorts from which clinical samples, behavioural and clinical information were obtained. NFB designed the research and prepared the manuscript.

**Chapter 3: HIV infection abrogates the functional advantage of natural killer cells educated through KIR3DL1/HLA-Bw4 interactions to mediate anti-HIV antibody-dependent cellular cytotoxicity**

Matthew S Parsons, Leia Wren, Gamze Isitman, Marjon Navis, Ivan Stratov, Nicole F Bernard, and Stephen J Kent.

Published in The Journal of Virology. 2012. April; 86 (8): 4488-4495.

**Contributions of authors:** MSP designed the research, conducted the experiments, analyzed the data, and prepared the manuscript. LW, GI, and MN

provided assistance with the experimental work. IS and SJK provided access to clinical samples. SJK and NFB prepared the manuscript.

**Chapter 4: Influence of Cytokines on HIV-Specific Antibody-Dependent Cellular Cytotoxicity Activation Profile of Natural Killer Cells**

Leia Wren*, Matthew S Parsons*, Gamze Isitman, Robert J Center, Anthony D Kelleher, Ivan Stratov, Nicole F Bernard, and Stephen J Kent.

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Published in PLoS One. 2012. 7(6): e38580

**Contributions of authors:** MSP and LW designed the research, performed the experiments, analyzed the data, and prepared the manuscript. GI provided assistance with experiments. ADK, IS, and NFB provided reagents, materials, and analysis tools. SJK designed the research, provided clinical samples, and prepared the manuscript.

**Chapter 5: The AIDS-protective allelic combination of HLA-B*57 and highly expressed variants of KIR3DL1 confers natural killer cells with robust antibody-dependent cellular cytotoxicity functional potential**

Matthew S Parsons, Pilar Zanoni, Benjamin Tallon, Sofia Miconiatis, Julie Bruneau, Christos M Tsoukas, and Nicole F Bernard.

Prepared for submission as a letter to the editor to AIDS.

**Contributions of authors:** MSP designed the research, performed the experiments, analyzed the data, and prepared the manuscript. PZ, BT, and SM

provided assistance with experiments. JB and CMT are responsible for the clinical cohorts from which clinical samples were obtained. NFB designed the research and prepared the manuscript.

# Acknowledgements

First and foremost, I would like to thank my parents for installing in me a value for education and pushing me to never stop striving for knowledge. This thesis is dedicated to you.

To my supervisor, Dr. Nicole Bernard, I wish to thank you for giving me the opportunity to complete my doctoral training in your laboratory. The training program you have established, along with your expertise, availability, and kind personality, have not only made this thesis possible, but made the journey to completing this body of work both rewarding and enjoyable.

To the members of the Bernard laboratory, I wish to thank you for your friendships and assistance, as well as making the lab an enjoyable place to work. In particular, I wish to thank Benjamin Tallon, Pilar Zanoni, and Carlos-Melendez Pena for your valuable comments regarding the work presented in this thesis, as well as the many “sociables” that we shared.

I would also like to thank my Master’s supervisor, Dr. Michael Grant, for your continued support throughout my PhD. In particular, I thank you for the valuable feedback you provided on the discussion of this thesis.

Lastly, I would like to thank Dr. Stephen Kent for offering me space within your laboratory at the University of Melbourne for six months of training

during my PhD. The training I received in your lab was very productive and valuable.

# List of Abbreviations

Ab – Antibody

ADCC – Antibody-dependent cellular cytotoxicity

AIDS – Acquired immune deficiency syndrome

BnAbs – Broadly neutralizing antibodies

CFSE - Carboxyfluorescein succinimidyl ester

CMV – Cytomegalovirus

$Cr^{51}$ – Chromium

CTL – Cytotoxic T-lymphocyte

DC – Dendritic cell

EBV – Epstein-Barr virus

EC – Elite controller

Env – Envelope

Fc – Antibody constant region

Gag – Group specific antigen

HAART – Highly active antiretroviral therapy

HCV – Hepatitis C virus

HESN – HIV-exposed seronegative

HIV – Human immunodeficiency virus

HHV8 – Human herpes virus 8

HLA-I – Human leukocyte antigen class I

HLA-Bw4*80I – HLA-Bw4 with isoleucine at position 80

HLA-Bw4*80T – HLA-Bw4 with threonine at position 80

Hmz - homozygote

HPV – Human papilloma virus

ICS – Intracellular staining

IDU – Intravenous drug users

IFNγ – Interferon gamma

IgA – Immunoglobulin alpha

IgG – Immunoglobulin gamma

IL-2 – Interleukin-2

IL-10 – Interleukin-10

IL-12 – Interleukin-12

IL-15 – Interleukin-15

IN – Integrase

ITAM – Immunoreceptor tyrosine-based activation motifs

ITIM – Immunoreceptor tyrosine-based inhibitory motifs

KIR – Killer immunoglobulin-like receptors

LRC – Leukocyte receptor complex

LTNP – Long term non-progressors

LTR – Long terminal repeats

mAb – Monoclonal antibody

MCMV – Murine cytomegalovirus

MHC-I – major histocompatibility complex class I

NADM – Non-AIDS defining malignancies

NCR – Natural cytotoxicity receptors

NK cells – Natural Killer cells

PBMC – Peripheral blood mononuclear cells

PIC – Pre-integration complex

Pol – Polymerase

PR - Protease

Pr55 – Gag precursor polyprotein

Rev –Regulator of viral gene expression

RF-ADCC – Rapid fluorescence ADCC assay

RT – reverse transcriptase

SHIV – Simian-human immunodeficiency virus

SIV – Simian immunodeficiency virus

SP – Slow progressor

STI – Structured treatment interruption

Tat – Transcriptional activator

TGF-β – Transforming growth factor beta

TNFα – Tumor necrosis factor alpha

TSG101 – Tumor susceptibility gene 101

UNAIDS – United Nations AIDS

Vpr – Viral protein R

# Chapter 1: Introduction

## 1.1 Acquired immune deficiency syndrome (AIDS)

AIDS was first observed in 1981 as a severe immunodeficiency that caused morbidity and mortality in young homosexual men within urban areas in the United States of America, such as New York City, Los Angeles, and San Francisco[1-3]. At that time, the syndrome was explained as the onset of low $CD4^+$ T cell counts in individuals with no known cause for immunodeficiency. These low counts left the affected individuals open to infection with opportunistic pathogens such as the *Pneumocystis jirovecii* fungus and the *Toxoplasma gondii* parasite, which cause pneumocystis pneumonia and toxoplasmosis, respectively[4;5]. Furthermore, individuals with AIDS were also observed to be more susceptible to developing cancers caused by oncogenic viruses, such as human herpes virus 8 (HHV8), Epstein-Barr virus (EBV), and human papilloma virus (HPV)[6].

With the continuous collection of laboratory and epidemiological data on AIDS, it quickly became apparent that the syndrome was not just affecting homosexual men in urban American areas. Indeed, shortly after it was initially observed, AIDS was noted in intravenous drug users (IDU)[7], heterosexual Africans[8;9], hemophilia patients[10], as well as babies born to mothers within risk groups[11]. The identification of these clusters of individuals with AIDS led researchers to hypothesize that AIDS was being caused by an infectious agent transmitted through blood and blood products. Evidence supporting this

contention was provided in 1984, when the human immunodeficiency virus (HIV) was associated with the development of AIDS[12-15].

HIV/AIDS still represents a global health issue; by the end of 2010, United Nations AIDS (UNAIDS) statistics predicted that 34 million people were currently living with HIV infection, 2.7 million new infections occur every year, and 1.8 million people die annually from complications related to AIDS[16]. The current epidemic is characterized by declining but high rates of infection in sub-Saharan African countries (bearing an estimated 68% of the global HIV/AIDS burden), growing rates of infection in Eastern Europe and Central Asia, and stabilized low infection rates in Western and Central Europe and North America[17].

## 1.2 HIV

Discovered in 1983 by the laboratory of Dr. Luc Montagnier[18], HIV was associated with the development of AIDS in 1984 by a series of papers published in the journal *Science* by the laboratory of Dr. Robert Gallo[12-15]. The characteristics of HIV were recently reviewed by Dr. Jay Levy[19]. HIV is classified within the *Retroviridae* family of viruses and within the Lentivirus genus. Characteristic of a prototypic retrovirus, HIV carries its 9.8kb genome in the form of RNA and utilizes a viral enzyme, reverse transcriptase (RT), to convert this RNA into DNA. HIV virions carry the RNA genome along with viral RT, protease (PR) and integrase (IN) enzymes inside a protein viral core, which is shielded by a lipid

bilayer envelope (Env). Spikes consisting of two non-covalently associated viral glycoproteins (i.e., gp120 & gp41) penetrate the viral Env, and are essential for the virus to infect host cells that express CD4, such as T cells, monocytes, and macrophages[20;21]. Models of an HIV virion and the HIV genome are represented in Figures 1 and 2.

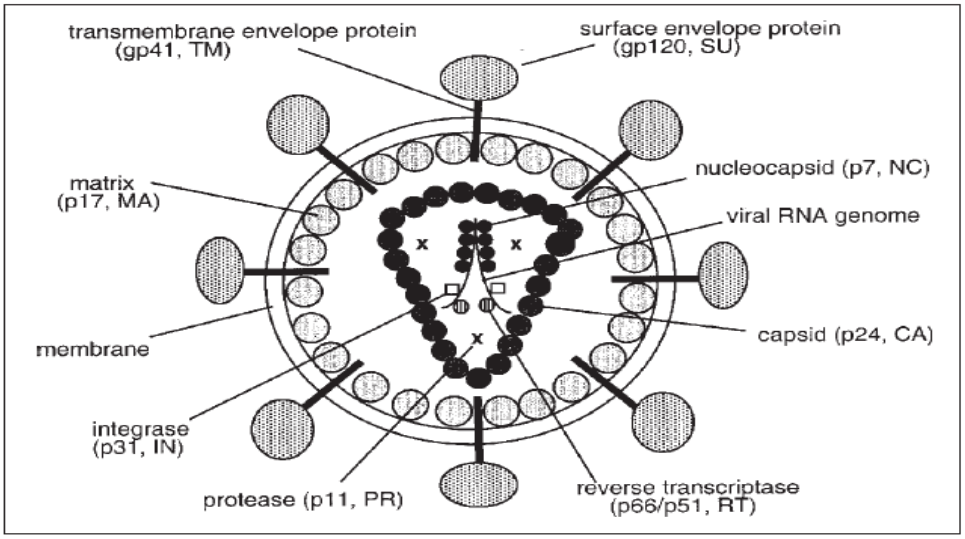


**Figure 1[22]. Schematic representation of a mature HIV-1 particle.** (Reprinted with permission from Springer Science: Somat Cell Mol Genet, 26: 13-33, 2001).

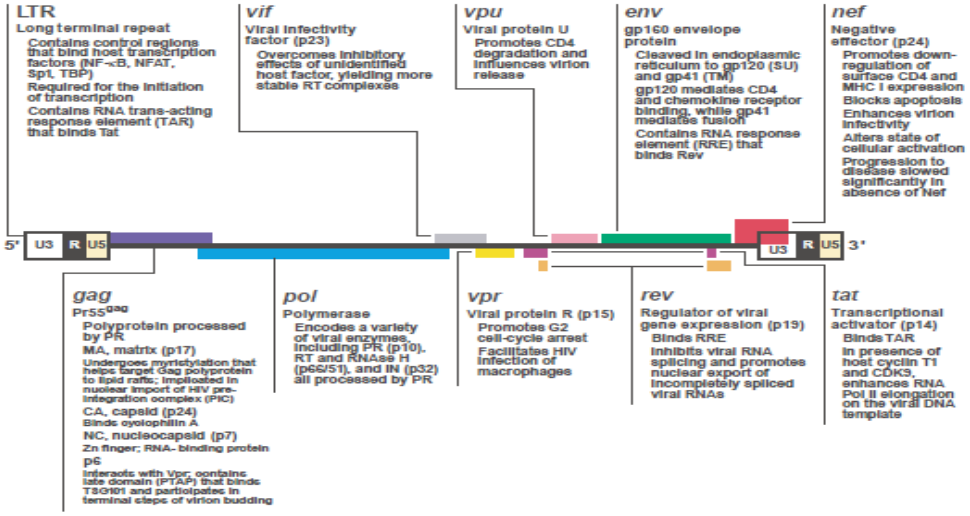


**Figure 2[23]. HIV genome and function of encoded proteins.** (Reprinted with permission from MacMillan Publishers Ltd. Nature Medicine, 8: 673-680, 2002).

Prior to initiating infection, HIV needs to be transmitted from an infected individual to a new host. The transmission of HIV occurs through the contact of virus-containing body fluids from an infected individual with a mucosal membrane of the uninfected individual[24]. As HIV can be found in blood and blood products, semen, vaginal fluids, and breast milk, transmission can occur through several routes. HIV transmission most commonly occurs through sexual intercourse, with intercourse amongst heterosexuals accounting for the majority of HIV infections worldwide. Indeed, in Sub Saharan African countries heterosexual transmission accounts for 80% of HIV infections[25]. Alternatively, within occidental countries, such as the United States of America, intercourse amongst men who have sex with men may account for up to 61% of HIV infections[26]. HIV is also transmitted through the sharing of contaminated needles

amongst IDU and within the context of medical practice, as well as through blood transfusions utilizing blood from infected individuals[24]. Lastly, HIV-infected mothers can transmit the virus to their children through the processes of childbirth or breastfeeding.

Upon transmission to a new host, HIV initiates infection by interacting with cells carrying the CD4 receptor in combination with one of the viral co-receptors, such as CCR5 or CXCR4[27]. The non-covalently associated complex of viral Env glycoproteins, gp120 and gp41, mediates the interaction between virions and cells. Once the virion encounters the potential host cell, gp120 interacts with the CD4 receptor. This causes a conformational change in gp120, which liberates a region within the glycoprotein that can bind a cellular co-receptor. Depending on the viral tropism, the co-receptor binding site will engage the CCR5 or CXCR4 co-receptor. This is followed by the interaction of gp41 with the cell membrane, which initiates the fusion of the viral and cellular membranes, leading to the viral core being released into the cellular cytoplasmic environment. The HIV entry process is depicted in Figure 3.

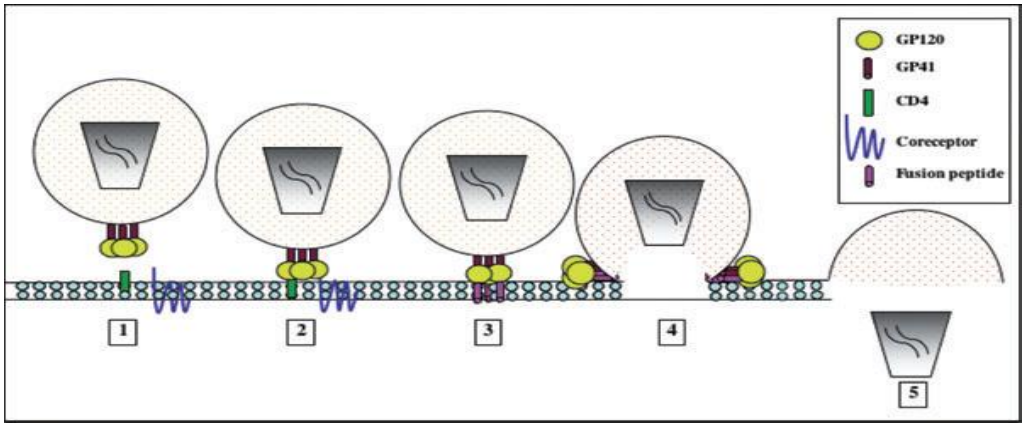


**Figure 3[27]. HIV entry.** (Reprinted with permission from John Wiley and Sons, Inc. Cellular Microbiology, 7: 621-626, 2005).

Once inside the cell, dissolution of the viral capsid occurs. It is uncertain if dissolution occurs prior to the conversion of viral RNA to DNA by RT, or after reverse transcription and prior to the migration of viral DNA into the nucleus[28]. Irrespective of the timing of capsid dissolution, viral entry is followed by the transcription of the viral RNA into DNA through the process of reverse transcription[22;28]. Reverse transcription is performed by the viral RT enzyme, which is a heterodimeric enzyme that has both DNA polymerase and RNase H activity. Reverse transcription is initiated when self $tRNA^{lys}$ binds to the viral primer binding site and serves as a primer that allows the RT enzyme to build a small DNA fragment towards the 5' end of the viral RNA. This short DNA sequence briefly associates with a short region of viral RNA, forming a DNA/RNA hybrid. Next, the RNase H activity of RT allows for the degradation of the short RNA region, and the short DNA fragment is free to interact with the 3' end of the viral RNA genome. The short DNA fragment can move to the 5' end of the viral

RNA because there are conserved sequences within the viral long terminal repeats (LTR) on both ends of the RNA genome. Once bound to the 3' end of the RNA genome, the short DNA fragment serves as a primer for the RT DNA polymerase activity, and results in the synthesis of a single DNA strand encoding the viral genome. Next, the RT utilizes its RNase H activity to degrade the viral RNA, except for two polypurine rich areas, one in the center of the genome and another at the 3' end of the genome. These maintained polypurine-rich regions of RNA can then bind to the single strand of DNA and serve as primers for another round of RT-mediated DNA polymerase activity, resulting in the second strand of viral genomic DNA. As the DNA is built from two independent sites a DNA flap is created in the center of the viral DNA. The process of reverse transcription is depicted in Figure 4.

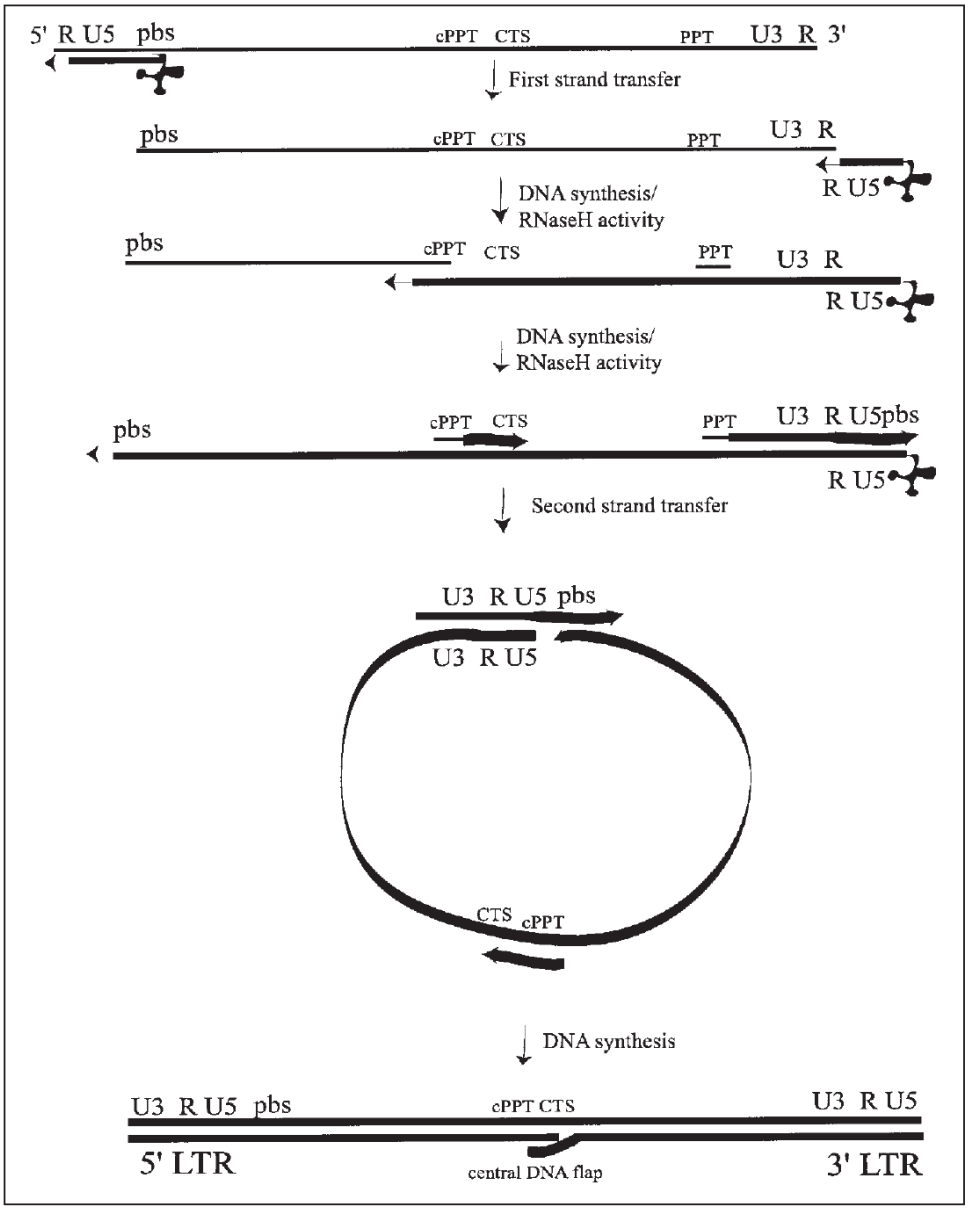


**Figure 4[22]. HIV reverse transcription.** (Reprinted with permission from Springer Science: Somat Cell Mol Genet, 26: 13-33, 2001).

Reverse transcription is not only essential for creating the provirus from which future virions will be produced; it is also an important event for introducing variation into the viral population. Indeed, the RT enzyme lacks a

proof reading mechanism. As such, high levels of mutation ($3.4x10^{-5}$ mutations per base pair per cycle) can be introduced during this process[29]. Although these mutations can frequently be deleterious[30], they may also serve to introduce alterations that allow escape from immunological and/or pharmacological pressure.

After reverse transcription, the viral DNA is contained within a pre-integration complex (PIC) along with IN, matrix, Viral protein R (Vpr), and RT[22;28]. The PIC is too big to move through nuclear pores; as such it needs to be actively transported into the nucleus of the cell. Transportation is thought to be driven by nuclear transport signals provided by matrix, IN, Vpr, and/or self-proteins associated with the viral DNA flap. Once inside the nucleus, the viral IN enzyme cleaves the 3' termini of both strands of viral DNA, creating sticky ends that allow the DNA to be integrated into active gene sites in the genomic DNA of the cell. The newly integrated provirus will serve as a source of future infectious virions.

After the provirus has been integrated within the host cell genome, cellular activation will stimulate viral replication. This occurs due to the movement of transcription factors into the nucleus and their interactions with the HIV LTR. The transcription factor most frequently involved in stimulating HIV replication is the NF-κB transcription factor, which moves to the nucleus upon T-cell activation[22;31]. Interaction of NF-κB with the HIV LTR initiates the transcription of viral mRNA, which is spliced into smaller fragments and moved

across the nucleus into the cytoplasmic environment. Here, the mRNA is translated into viral regulatory proteins, such as Regulator of viral gene expression (Rev) and Transcriptional activator (Tat). Tat is involved in driving further viral replication. Rev is responsible for assisting the movement of unspliced and partially spliced mRNAs across the nucleus, where they can be translated into larger proteins, such as the group specific antigen (Gag) proteins that form the viral core, polymerase (Pol) proteins that assist with viral assembly, and Env proteins that are necessary for the new virions to be infectious. Once the viral proteins are translated, newly produced Env precursor gp160 is cleaved by cellular proteases to create gp120 and gp41, which non-covalently associate at the cell surface[22;27]. Next, Gag precursor polyproteins (Pr55) accumulate at the inner surface of the cellular lipid bilayer. This Gag precursor contains three domains that are essential for viral particle formation. First, the membrane targeting (M) domain allows Pr55 to associate with negatively charged phospholipids and targets the precursor the cell membrane. Next, the interaction (I) domain mediates interactions between Gag monomers. Lastly, the late (L) domain contains a motif that interacts with the tumor susceptibility gene 101 (TSG101), a protein important for cellular vacuole formation. Other components of Gag also ensure correct viral particle formation. For example, nucleocapsid interacts with the viral RNA and matrix interacts with the cytoplasmic tail of gp41. Cumulatively, these properties of Gag ensure that viral particles released from the cell contain all the essential components for virions

to be infectious. It should be noted, however, that viral particles are not infectious immediately upon release, as the viral capsid has not yet been appropriately cleaved. Indeed, upon the release of viral particles HIV PR cleaves Gag and GagPol combinations to create the viral capsid, which will surround the viral enzymes and RNA genome. Once the capsid is formed the virus is considered mature and is infectious.

The accumulation of data regarding the transmission mechanisms of HIV and the mechanisms of viral infection, replication, and assembly has informed strategies for how to prevent HIV infection and interfere with viral replication. The next section of this literature review will discuss some of these medical and social interventions. As well, the reasons why these interventions are not fully protective or desirable will be addressed, pointing to the need for a prophylactic HIV vaccine.

## 1.3 Measures to prevent HIV infection

Understanding the routes of HIV transmission has informed the design of several education and social programs directed at preventing the transmission of HIV. These programs have included condom distribution[32], provision of anti-retroviral drugs to mothers prior to childbirth and during breastfeeding[33;34], the distribution of clean needles to IDU[35], and the development of safe injection sites[36]. Although these programs have demonstrated efficacy for reducing HIV

transmission rates and/or reducing risk behaviors, groups advocating socially conservative or conspiratorial perspectives have reduced the efficacy and/or acceptance of these programs.

Examples of resistance to HIV prevention programs include religious groups, such as the Catholic Church, fighting condom distribution and safe sex education under the guise that birth control is immoral[37]; Conservative governments, such as the current Harper Government, that attempted to shut down a Vancouver safe injection site with proven efficacy[38]; as well as, the former President of South Africa, Thabo Mbeki, who did not allow HIV infected mothers to access anti-retroviral drugs, as he had accepted Dr. Peter Duesberg's hypothesis that HIV is not the cause of AIDS[39-41].

Several programs exist to reduce new HIV infections. It is important to note that the efficacy of preventative programs depend on how they are implemented. Despite education, some individuals continue to put themselves at risk and contract new infections. Cumulatively, these factors limit the efficacy of preventative programs, and highlight the need for a prophylactic vaccine.

## 1.4 Anti-retroviral drugs

Although new HIV infections continue to occur, understanding the HIV infection and replication processes has allowed for the development of an array

of highly effective anti-retroviral drugs. These drugs target several steps in infection and the replication cycle, and dramatically reduce viral replication in infected individuals. Indeed, anti-retroviral drugs have been designed to block viral entry (i.e., entry inhibitors), inhibit RT (i.e., nucleoside analogue RT inhibitors & non-nucleoside RT inhibitors), inhibit the viral PR (i.e., PR inhibitors), and inhibit the viral IN (i.e., IN inhibitors)[42]. These drugs are generally administered for the remainder of the infected individual's life as a cocktail of at least three drugs from at least two classes, which is referred to as highly active antiretroviral therapy (HAART). Such treatment can inhibit viral replication to levels below what is detectable with current techniques and can allow partial restoration of the immune system.

The administration of these drugs as cocktails is required to prevent the development of drug resistance[42]. Monotherapy often results in the development of escape variants. This may be related to the ability of RT to introduce random mutations during viral replication[29]. Cocktail therapies, however, decrease the chances that drug-resistant viral variants will be selected, as several distinct escape mutations must arise together in a setting of suppressed viral replication to overcome drug pressure. Nevertheless, HIV drug escape does remain a complicating problem for successful treatment of HIV infection, as escape mutations are observed in viral isolates from 76% of treated viremic individuals[43]. Drug escape occurs for several reasons, including poor

adherence, suboptimal drug combinations, or differences between viral isolates[42;44].

Despite great advances in the pharmacological therapy of HIV infection, these drugs are far from a perfect solution to the global issue of HIV/AIDS. Chronic use of these drug cocktails has been associated with severe cardiovascular, liver, and kidney side effects[45-47]. Furthermore, although HAART can reduce viral replication and partially restore the immune system, the immune system does not return to normal in infected individuals. Indeed, successfully treated HIV-infected individuals remain more susceptible to an array of non-AIDS defining malignancies (NADM), compared to the general population[48].

Similar to preventative measures to thwart new infections, anti-retroviral drugs have great potential to contribute to the control of the global HIV epidemic. However, severe side effects, poor adherence, and the development of viral escape mutants can impede the efficacy of HAART. Furthermore, the inability of HAART to allow complete restoration of the immune system and/or reduce susceptibility to NADM, highlight the desirability of preventing HIV infection as compared to merely treating existing infections. As for the problems associated with preventive measures to reduce new HIV infections, the issues surrounding HAART indicate the need for a prophylactic HIV vaccine.

## 1.5 Successful immune responses against HIV

In order to design an effective prophylactic vaccine against HIV it is essential to understand what constitutes a successful immune response against the virus. Although the nature of such immune responses have been very difficult to elucidate, several groups of individuals have been identified that could provide clues about successful anti-viral responses. These groups include individuals that have been repeatedly exposed to HIV without contracting HIV infection, known as HIV exposed seronegative (HESN) individuals, as well as individuals that despite being HIV infected do not progress to AIDS, known as slow progressors (SP). Furthermore, laboratory simian primate infection models have provided a system in which to conduct proof of principle studies.

## 1.6 HESN

Numerous cohorts of HESN have been assembled and studied worldwide. These cohorts are heterogeneous in terms of the routes through which they were exposed, as well as the immunological correlates of protection that they exhibit. HESN cohorts exposed through intravenous drug use, male homosexual intercourse, heterosexual intercourse, and childbirth and/or breastfeeding have been identified[49]. Furthermore, immune responses as varied as adaptive cell-mediated immunity, adaptive humoral immunity, and innate immune responses have been associated with the observed protection[50].

As adaptive immune responses are easiest to induce through immunization, many groups have attempted to identify adaptive immune responses that associate with the protection observed in HESN. It has been reported that both $CD4^+$ and $CD8^+$ T cell mediated HIV specific immunity is present in HESN[51;52]. Based on the rationale that these responses are protective, investigators have attempted without success to vaccinate rhesus macaques against pathogenic simian immunodeficiency virus (SIV) by inducing adaptive T cell responses[53]. The failure of central memory T cell responses to prevent infection may be due the size of the challenge dose or the fact that it takes too long for these primed T cells to proliferate and differentiate into effector cells that can kill cells infected with SIV and prevent the establishment of a progressive infection. Therefore, the presence of adaptive T cells responses in HESN may represent a marker of previous exposure to HIV rather than the presence of a response able to protect against HIV infection. Adaptive humoral responses may also play an important role in the protection of HESN exposed at mucosal sites via sexual contact. Mucosal immunoglobulin A (IgA) antibodies (Abs) directed against the gp41 component of the HIV Env appear to confer this protection[54]. Although the exact mechanism of protection is unknown, these Abs are capable of both neutralizing the infection of $CD4^+$ T cells and preventing the epithelial transcytosis of infectious virions.

Other recent work has identified innate immune responses that may be responsible for the observed protection in HESN. For example, IDU HESN have

been shown to exhibit enhanced natural killer (NK) cell functionality, as compared to the non-drug using general population[55]. Furthermore, several allelic combinations of the NK cell killer cell immunoglobulin-like receptor (KIR) family and their major histocompatibility complex class I (MHC-I or HLA-I) ligands, which have been demonstrated to confer enhanced NK cell functionality, occur at higher frequencies in HESN[56-59].

The observation of immune responses associated with HESN status provides several clues for designing an effective prophylactic vaccine against HIV infection. These observations suggest that a broad array of immunological responses may be beneficial, as both adaptive and innate immune responses appear to be capable of preventing HIV infection.

## 1.7 Slow disease progressors (SP)

Similar to HESN cohorts, SP are a heterogeneous group of individuals. SP include long term non progressors (LTNP) and Elite Controllers (EC). Using the definition of maintaining CD4 counts >450 cells/$mm^3$ for seven or more years without treatment LTNP make up 5% of HIV-infected individuals[60]. These individuals can have a detectable viral load or be aviremic. Individuals infected for at least one year and able to spontaneously control viremia to undetectable levels are called EC, and they make up less than 1% of HIV infected individuals[61-63]. In the following sections we will use the term SP to refer to either one of

these subsets of HIV infected individuals. The immunological responses associated with non-progression are numerous, and as observed in HESN span both adaptive and innate immune responses.

Adaptive immune responses associated with SP status have mostly been observed in the cell-mediated arm of the adaptive immune system. One of the most frequently cited associations between immunity and SP status is the presence of polyfunctional $CD8^+$ cytotoxic T-lymphocytes (CTL)[64;65]. Furthermore, potentially protective CTL responses have been linked with the carriage of certain HLA-I alleles, such as HLA-B*57 and HLA-B*27[66-68]. As such, it appears as though the ability of CTL to mediate potentially protective responses is at least partially dependent upon the ability of autologous HLA-I molecules to present viral peptides.

Adaptive humoral responses have also been suggested to play a role in the lack of disease progression in some SP. Higher levels of neutralizing Abs have been observed in some SP cohorts[69]. However, other studies have demonstrated that Abs capable of neutralizing a wide array of HIV isolates, known as broadly neutralizing Abs (BnAbs), also develop in individuals who progress to AIDS[70]. As such, it has been suggested that although the presence of BnAbs prior to infection may protect against HIV infection, they may not be capable of delaying disease progression. Other studies have demonstrated that SP have higher levels of Abs capable of recruiting cells of the innate immune system, such as NK cells, which can respond to Ab coated cells and mediate chemokine and cytokine

synthesis and/or cytolysis, a response known as Ab-dependent cellular cytotoxicity (ADCC)[71].

Coinciding with the high levels of ADCC-competent Abs present in SP, these subjects also exhibit innate immunological correlates of protection. Indeed, SP carry NK cells with a higher functional potential than NK cells from individuals that progress to AIDS[72;73]. Although the driving force behind the higher NK cell functionality in SP has not been elucidated, these individuals have been demonstrated to carry a higher frequency of certain allelic combinations of KIR and their HLA-I ligands that can confer heightened NK cell functional potential[56;74].

Similar to HESN, the immunological correlates of protection in SP provide several clues as to how to design prophylactic immunological therapeutics. Again, the data suggest that both adaptive and innate immune responses could potentially be harnessed for the purpose of vaccine design.

## 1.8 Proof of principle of successful anti-HIV immune responses

Clinically important groups such as HESN and SP provide insights into immune responses that may be protective against HIV infection or disease progression. Interestingly, many of these immune correlates of protection have

been validated in simian primate models of HIV infection, as well as in passive transfer experiments involving HIV-infected individuals.

The most commonly utilized simian primate species for modeling HIV infection are the rhesus macaques. These animals can be infected with SIV, which can cause a condition similar to AIDS within six months to two years[75]. To make the model more appropriate for the evaluation of immune responses against HIV, a chimeric version of SIV has been created that contains genes from HIV. This chimeric virus is known as the simian-human immunodeficiency virus (SHIV)[76]. As research in HIV-infected humans has suggested that CTL responses are important for reducing viral replication and that BnAbs may be ideal for preventing HIV infection, the rhesus macaque SHIV model has been used to evaluate these immune responses. Indeed, the SHIV model has provided proof of principle that CTL can reduce viral burden and that BnAbs can prevent infection with SHIV. Studies demonstrating the efficacy of CTL have involved depleting the $CD8^+$ CTL population from SIV or SHIV-infected macaques and measuring the effect of this depletion on viral dynamics. These studies have repeatedly shown that viral replication increases following the depletion of CTL[77;78]. Studies of the potential efficacy of BnAbs as a prophylactic to HIV infection have repeatedly demonstrated that the passive administration of BnAbs can prevent SHIV infection upon viral challenge[79;80].

Studies to evaluate the potential of BnAbs have also been conducted in HIV-infected humans. These studies have involved the passive administration of

BnAbs to patients undergoing structured treatment interruptions (STI)[81]. These studies demonstrated that although these patients exhibited lower viral replication for a longer period than patients not receiving BnAbs, the passive transfer of BnAbs ultimately failed to control the replication of virus in already chronically infected individuals. In summary these observations suggest that BnAbs can prevent the establishment of infection but cannot attenuate an already established infection.

Cumulatively, proof of principle studies established adaptive immune responses as attractive targets for prophylactic HIV vaccines. As such, HIV vaccine attempts to date have mostly focused on inducing adaptive immune responses. In the next section of this literature review, previous HIV vaccine trials will be described. As well, the role of the failures and obstacles of these trials in driving attempts to harness innate immune responses will be discussed.

## 1.9 HIV vaccines

Vaccines designed for many other pathogens have exclusively targeted the adaptive immune system. Data from HIV infected humans and simian primate models suggest an important role for adaptive immune responses in controlling viral replication. Therefore, the three major (phase IIb or III) HIV vaccine trials completed to date have focused on inducing adaptive cellular and/or humoral immune responses.

The first HIV vaccine trial attempted to induce a protective Ab-based immune response. The vaccine, termed AIDSVAX, consisted of recombinant gp120 immunogens, and was tested in America and Thailand[82;83]. The format of the vaccine tested on homosexual men and high risk women in America consisted of recombinant Env glycoproteins from two subtype B isolates; whereas, the format of the vaccine tested on IDU in Thailand consisted of recombinant Env glycoproteins from one subtype B and one subtype E isolate. Despite its ability to induce detectable anti-HIV Abs, AIDSVAX was demonstrated to be ineffective at preventing HIV infection or altering disease progression. Indeed, amongst the nearly 5,000 participants in the Phase II trial in America, the infection rate in the placebo group (5.8%) was similar to that observed in the vaccinated group (5.7%). Similarly, amongst the 2,546 participants in the Thailand trial, 105 infections were observed in the placebo group and 106 in the vaccinated group.

The observations from the AIDSVAX trial, in conjunction with the accumulation of evidence that CTL responses are associated with protection from progression to AIDS in LTNP and primate models, led to the development of the second major HIV vaccine trial, the STEP trial. Sponsored by Merck, the STEP trial consisted of three immunizations with an adenoviral vector that contained the Gag/Pol/Nef HIV genes[84;85]. Participants for this trial included 3000 internationally recruited HIV-uninfected men and women who were considered at high risk for contracting HIV. The trial was started in 2005 and was halted in

2007 prior to its scheduled end date, as it was apparent that the vaccinated group was more susceptible to infection with HIV than the placebo group.

A third HIV vaccine trial was conducted in Thailand. This trial, referred to as RV144, included 16,402 participants who received a prime-boost vaccination protocol over six months[86]. The priming immunogen, known as ALVAC, consisted of a canarypox virus that carried the Gag/Env/Pol genes from HIV given to study participants six times. However, for the last two administrations of ALVAC, the previously failed AIDSVAX immunogen that consisted of recombinant gp120 from subtype B and subtype E isolates was also administered. Post vaccine analysis revealed that the vaccine had a modestly protective effect, as demonstrated by the observation that vaccinated individuals were 31.2% less likely to contract HIV infection than individuals that received the placebo.

Exactly how the RV144 vaccine provided protection has been an intense area of research. Contrary to the expectations that any protective HIV vaccine would have to induce potent CTL or BnAbs[87], the RV144 immunization protocol induced only weak CTL responses and narrowly directed neutralizing Abs[86]. Instead, protection in vaccinated individuals was associated with two distinct types of non-neutralizing Abs. Protected individuals have been demonstrated to carry higher levels of plasma Abs of the immunoglobulin G (IgG) subtype that recognize the variable 1 and variable 2 regions of HIV Env and low levels of plasma Abs of the IgA subtype that recognize the HIV Env[88].

As breadth of neutralization mediated by vaccine induced IgG does not appear to be able to explain the protection mediated by RV144, several hypotheses have been put forward to explain the observed protection. One such hypothesis is that the vaccine induced Abs capable of utilizing cells of the innate immune system, such as NK cells, to mediate ADCC against early infected cells[89]. Indeed, the RV144 regimen has been demonstrated to induce ADCC-competent Abs[90]. Despite this observation, the primary analysis of the immune responses associated with protection from infection did not demonstrate ADCC to be a significant factor[88]. However, a sub-analysis revealed that the level of ADCC in vaccinated individuals with low IgA was significantly associated with protection from infection, leading the authors to suggest that anti-Env IgA could interfere with the ability of anti-Env IgG to induce anti-viral ADCC and mask the effect of the effector function.

The observation that anti-viral ADCC may be associated with vaccine induced protection from HIV infection is an interesting observation that coincides with the demonstration that such responses are higher in SP[71;88]. Furthermore, that an NK cell mediated response can protect from HIV infection is consistent with the observation of heightened NK cell functionality and the presence of function-conferring allelic combinations of KIR/HLA in both SP and HESN[55-58;74]. The results of the RV144 trial, as well as the observations that strong NK cell responses are associated with protective outcomes in the context of HIV, suggest that the vaccine-induced adaptive immune responses may not be

sufficient to protect against HIV infection. Indeed, vaccines that stimulate the innate immune system, or harness the potential of innate immune cells, such as NK cells, through utilization of the adaptive immune response may be necessary. As such, the design of an effective HIV vaccine may require a comprehensive understanding of the ontogeny and functional regulation of NK cells. In the next section of this literature review our current understanding of NK cell ontogeny, functionality, and regulation will be discussed.

## 1.10 NK cells

NK cells are large granular lymphocytes that compose 10-15% of the peripheral blood lymphocytes[91]. These lymphocytes are not only found in the blood, but also populate organs[92]. The functionality of these cells is a central component of the innate immune system, and has been implicated in controlling viral infections[93], mediating tumor immune surveillance[94], and potentially contributing to or protecting against autoimmune disorders[95]. NK cells have also been demonstrated to contribute to initiating adaptive immune responses. Indeed, NK cells engage in "cross-talk" with dendritic cells (DC), which can result in the reciprocal activation of both cells[96]. Ultimately, this results in stronger NK cell-mediated immunity and the ability of DC to initiate an adaptive T cell response. A recent study has demonstrated that in the context of infection with the hepatitis C virus (HCV), increased NK cell degranulation is positively

associated with the magnitude of the CTL response that develops[97]. Finally, NK cells, within humans, have also been confirmed to play an important role in the reproductive process[98]. Through cell-to-cell interactions, uterine NK cells cause fetal extra-villous trophoblasts to invade the spiral arteries. This enables the creation of large vessels to supply the placenta. In fact, NK cell biologist Dr. Peter Parham has recently suggested that the unique role of NK cells in human reproduction has led to an evolutionary compromise, in which NK cell immune fitness is traded off for reproductive fitness. Regardless of the role of NK cells in human reproduction, it is abundantly clear that NK cells from humans and other species can mediate numerous immunological effector functions that could be beneficial for controlling infections.

Several lines of evidence suggest NK cells can directly clear or contribute to controlling viral infections. Most convincingly, mice that carry the activating Ly49h receptor are able to clear infection with murine cytomegalovirus (MCMV)[99]. This protection is conferred by the ability of Ly49h to recognize an MCMV protein, m157, and mediate cytolysis of infected cells[100]. Similarly, a recent case study demonstrated that human NK cells, in the absence of T cells, control cytomegalovirus (CMV) infection[93]. Correlational data suggests the ability of NK cells to control viral replication applies to a variety of other viruses. Indeed, human NK cells can lyse cells infected with HHV8, which has been implicated as the causative agent of Kaposi's sarcoma[101]. The potency of NK cell functionality in HIV-infected individuals is associated with suppressing of HHV8 so that

Kaposi's sarcoma does not develop. Furthermore, heightened NK cell functionality is linked to protection from infection with several viruses, including HIV and HCV, and has been demonstrated to inhibit the replication of these viruses *in vitro*[55;102-105]. Given the ability of NK cells to clear or control viral infections naturally, and the potential to therapeutically harness NK cell functional potential, it is important to understand how NK cell effector functions are controlled.

Upon encountering appropriate target cells NK cells can mediate a variety of effector functions, including degranulation of cytolytic granules and/or the secretion of cytokines and/or chemokines[106]. The activation of NK cells to mediate such effector functions is determined by signals received through an assortment of activating and inhibitory receptors expressed on the NK cell surface[107]. Inhibitory receptors generally recognize MHC-I (aka HLA-I) ligands, which are expressed on healthy nucleated cells, and prevent unwarranted autoaggressive NK cell functionality. Receptors with activating potential can recognize MHC-I ligands, as well as several viral ligands or self-ligands that are upregulated upon viral infection or transformation. As many viral infections and transformation events simultaneously induce the downregulation of MHC-I on the cell surface[108;109], the specificity of activating receptors allows NK cells to preferentially direct their effector functions towards infected or transformed cells that need to be eliminated. It is the cumulative signal received upon ligation of these NK cell receptors that determines whether effector functions will be

triggered, and which will be observed[106]. Although a cumulative stimulatory signal will induce the NK cell to mediate effector functions, the exact effector functions triggered are determined by the magnitude of the stimulatory signal. Fauriat et al. (2010) recently demonstrated that the stimulatory threshold for chemokine production is reached first. The stimulatory threshold for degranulation is slightly higher, and this is superseded by the threshold for cytokine (i.e., IFNγ & TNF-α) production (Figure 5). It should be noted, however, that not all NK cells will respond to the same putative target cell stimulus with a similar response pattern. This is because the expression of NK cell receptors occurs in a stochastic and stable fashion, creating NK cell clonal lineages expressing unique constellations of receptors[110]. To better understand how this can affect NK cell behavior, it is essential to next review the receptors that can be expressed by NK cells and how these receptors function.

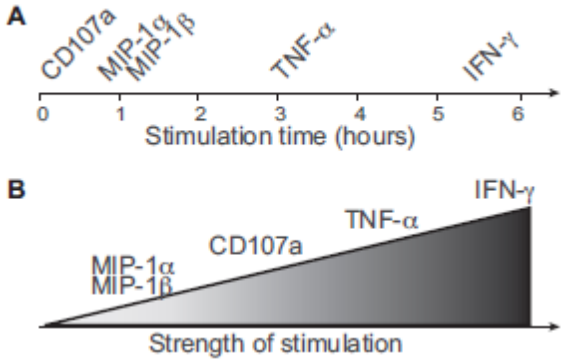


**Figure 5[106]. Activation thresholds for different NK cell effector functions.**

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## 1.11 NK cell receptors

Activating and inhibitory NK cell receptors recognize a variety of self and foreign ligands, which include MHC-I ligands, stress induced self-ligands, as well as some viral ligands[107]. In general, inhibitory receptors mediate strong interactions with constitutively expressed MHC-I ligands; whereas, MHC-I specific receptors with activating potential mediate weaker interactions with MHC-I[98]. Other activating receptors generally recognize viral ligands or stress induced ligands, which are upregulated after viral infection or cell transformation[100;111]. These patterns of recognition allow NK cells to spare healthy autologous cells that exhibit normal MHC-I expression patterns, while allowing NK cells to mediate effector functions against infected or transformed cells that have downregulated MHC-I and upregulated stress induced ligands.

The ability of activating and inhibitory receptors to transmit signals upon ligation is determined by the characteristics of their transmembrane regions and cytoplasmic tails[112]. Inhibitory receptors are characterized by cytoplasmic tails that contain immunoreceptor tyrosine-based inhibitory motifs (ITIM). Upon ligation of the receptor the tyrosine within this motif becomes phosphorylated and recruits phosphatases, such as SHP-1, SHP-2, and SHIP, which can decrease the phosphorylation of signaling molecules important for propagating activation signals. Activating receptors do not contain signaling motifs in their cytoplasmic

tails. Instead, these receptors are characterized by positively-charged residues in their transmembrane region. This allows them to associate with adapter proteins that carry activation signaling motifs, known as immunoreceptor tyrosine-based activation motifs (ITAM). Several adaptor proteins carrying ITAMs associate with activating receptors, including DAP-10, DAP-12, CD3ζ, and FCεRI-γ (Figure 6). Although the signaling mechanism upon ligation of inhibitory NK cell receptors is fairly consistent, the adaptor proteins and signaling pathways utilized by activating NK cell receptors is receptor dependent.

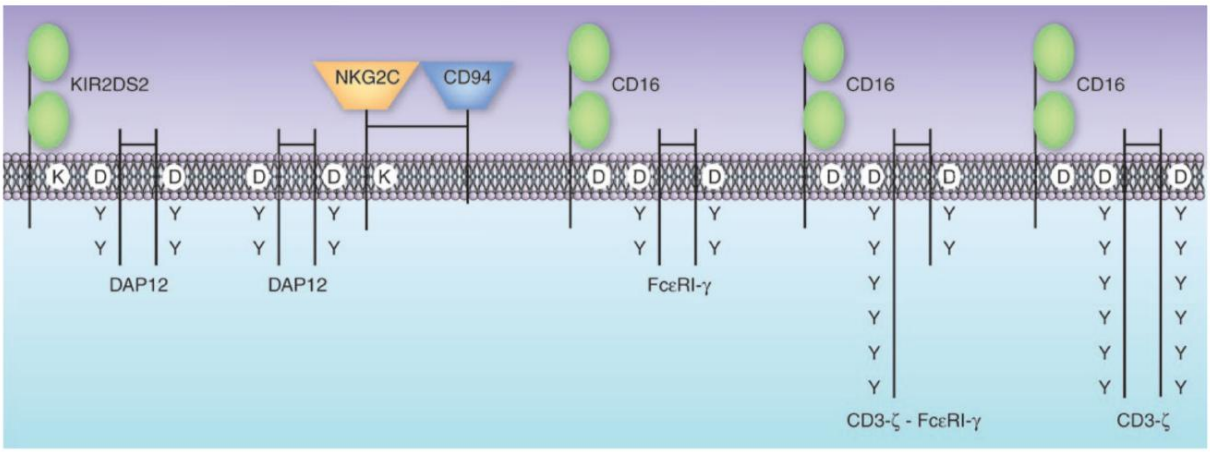


**Figure 6[112]. Association of activating NK cell receptors with ITAM-containing adapter proteins.** (Reprinted with permission from MacMillan Publishers Ltd. Nat Immunol 9: 495-502, 2008).

Despite great similarities in the signaling mechanisms utilized by NK cell receptors, the receptors expressed by human NK cells exhibit great diversity. NK

cell receptors include the evolutionary conserved lectin-like receptors (i.e., NKG2A, NKG2C, & NKG2D)[107], natural cytotoxicity receptors (NCR) (i.e., NKp30, NKp44, & NKp46)[113], and FCγRIIIa (i.e., CD16a)[114], which recognizes the constant region of IgG bound to cell-surface targets and allows NK cells to recognize infected or transformed cells with assistance from the adaptive immune system. Human NK cells also express the KIR, which have undergone dramatic evolution within simian primates[115]. Each of these receptor families modulates important, yet distinct, features of NK cell behavior. As such, understanding these receptors is important for understanding how NK cells normally function, as well as how to harness the potential of NK cells for therapeutic benefit.

## 1.12 Lectin-like CD94/NKG2 receptors

The NKG2A and NKG2C genes are located within the "NK complex" on chromosome 12, and they encode a functional activating and inhibitory receptor, respectively[116-118]. These receptors represent evolutionary conserved receptors that are present in simian primates, as well as evolutionary older species such as mice[107]. Furthermore, these receptors are not polymorphic within humans. Both NKG2A and NKG2C are lectin-like receptors that are expressed on the cell surface as heterodimers, in combination with CD94[119]. The CD94 molecule has no signaling capacity. The inhibitory activity of NKG2A depends on the ITIM in the cytoplasmic tail of the receptor; whereas, the activating capacity of NKG2C relies on its association with an ITAM-containing DAP-12 homodimer[107;118] .

Despite the distinct functional sequelae of ligating NKG2A and NKG2C, both receptors recognize a common ligand, the non-classical MHC-I molecule HLA-E[120]. The HLA-E gene is found on chromosome 6, within the HLA complex[121]. HLA-E differs from the classical HLA-A, B, and C molecules in terms of polymorphism observed at these loci. While the HLA-A, B, and C loci represent the most polymorphic loci in humans, only three alleles have been observed for human HLA-E. Expression of HLA-E is dependent on classical HLA-I molecules, as surface expression of HLA-E is stabilized by the binding of peptides from the leader sequences of classical HLA-I molecules within the HLA-E peptide binding groove[122]. Viral peptides can also be presented within the HLA-E peptide binding groove and stabilize expression, as has been observed for peptides from HIV p24[123].

Although both NKG2A and NKG2C recognize the constitutively expressed HLA-E, several mechanisms exist to prevent NKG2C-expressing NK cells from mediating autoaggressive effector functions. First, NKG2A recognizes HLA-E with a higher affinity than NKG2C, and the receptors can be expressed simultaneously such that the inhibitory signal dominates the activating one, precluding NK cell activation[107;124]. Secondly, NK cells often express NKG2C in combination with other inhibitory receptors that can recognize self MHC and prevent NK cell activation. These properties not only prevent NK cells from responding to self, but have also been taken advantage of by viruses that upregulate HLA-E, such as

HIV. Indeed, upregulation of HLA-E by HIV p24 has been shown to inhibit NK cells from targeting HIV-infected autologous cells[125-127].

## 1.13 NKG2D

The activating NKG2D receptor represents an evolutionarily conserved NK cell receptor. NKG2D receptors are found in many species, including humans and evolutionarily older species, such as mice, although they differ in terms of ligands and recognition by monoclonal Abs (mAb)[107]. The human NKG2D receptor is encoded within the NK complex on chromosome 12[128]. The receptor is expressed on the cell surface as a disulfide-linked homodimeric glycoprotein. NKG2D recognizes a group of MHC-I-like receptors, which includes MICA, MICB, ULBP1, ULBP2, ULBP3, and ULBP4[129]. These ligands are structurally similar to MHC-I, but lack the ability to present peptides. The ligands for NKG2D are not normally expressed on the cell surface, but are upregulated during periods of genotoxic stress that occur following cellular transformation or viral infection. Ligation of NKG2D by any of its ligands induces NK cell activation, due to the association of the NKG2D receptors with ITAM-containing DAP-10 adaptor proteins[130].

The activation of NK cells through NKG2D appears to be an important means for NK cells to target viral infections and cancers, as several viruses and tumors have developed means to evade NKG2D-mediated NK cell responses. For example, HIV proteins, such as Nef can downregulate the cell surface expression

of NKG2D ligands[131]. Furthermore, solid tumors can increase the level of soluble NKG2D ligands, which can decrease the level of cell surface NKG2D and impair the ability of NK cells to mediate functionality[132]. Cumulatively, the evasion of NKG2D-mediated functionality by tumors and viruses highlights the potential importance of NK cell-mediated immunity.

## 1.14 NCR

Three independent activating receptors, NKp30, NKp44, and NKp46, belong to the human NCR family of receptors[113]. One of these receptors, NKp46, is evolutionarily conserved as a functional version and is also found in mice. The human NKp44 and NKp30 receptors are encoded within the MHC region on chromosome 6. Human NKp46 is encoded within the Leucocyte Receptor Complex (LRC) on chromosome 19. The gene products for these receptors manifest as Ig-like glycoproteins, with NKp30 and NKp40 exhibiting one extracellular Ig-like domain and NKp46 exhibiting two extracellular Ig-like domains[133]. Similar to other activating receptors, all three of the NCR carry a positive charge within their transmembrane region. This charge allows NKp44 to associate with the ITAM-containing DAP-12 adaptor protein[134]. Alternatively, NKp30 associates with CD3$\zeta$ and NKp46 associates with both the ITAM-containing CD3$\zeta$ and FC$\varepsilon$RI adaptor proteins[135;136]. The activating potential of

these receptors has also been confirmed through studies that have demonstrated activation of NK cells upon Ab-mediated cross-linking of NCR[137].

Given the potential of NCR ligation to induce NK cell activation, it is important to understand the ligands for these receptors. Unlike other NK cell receptors, which recognize self and altered-self ligands, the NCR are capable of recognizing both altered-self and foreign ligands. For example, NKp30 can recognize the altered-self B7-H6 tumor antigen, as well as CMVpp65 tegument protein[138;139]. Similarly, NKp44 and NKp46 are capable of recognizing foreign antigens, such as influenza virus hemagglutinin, Sendai virus hemagglutinin and neuraminidase[140;141]. NKp46 has also been shown to interact with mycobacterium components on both infected cells and bacteria[142].

The NCR appear to play a role in Ab-independent NK cell-mediated effector functions. Some of the interesting features of these receptors are the versatility of their ligand recognition, as well as the fact that their ligand recognition specificity provides evidence that human NK cell receptors can evolve to specifically target ligands found on pathogens.

**1.15 FCγRIIIa or CD16a**

NK cell receptors that recognize the constant regions of Abs and allow NK cells to recognize and direct effector functions towards Ab-coated target cells are

common throughout evolution. The human low-affinity receptor for the constant region of IgG (i.e., FCγRIIIa or CD16a) is encoded on chromosome 1 and expressed on NK cells[143;144]. By means of a positively-charged transmembrane residue, CD16a associates with the ITAM-containing CD3ζ and FCεRI homodimers and/or heterodimers[112]. As such, ligation of CD16a by cell-bound Abs of the IgG1 and/or IgG3 subtypes can result in the activation of NK cells to mediate cytolysis, which is also known as ADCC[145]. Ligation and signaling through CD16a can also trigger secretion of cytokines and chemokines[106;146]. Following stimulation through CD16a, the receptor is downregulated on the NK cell surface for a brief period of time[147]. It should be noted that the ability of the CD16a receptor to mediate ADCC is influenced by a functional polymorphism at position 158. If this position is occupied by a valine the receptor exhibits higher affinity for the IgG constant region and induces higher ADCC than if the position is occupied by a phenylalanine[148;149]. Furthermore, it is apparent that differences in glycosylation patterns between IgG molecules can affect the interaction between IgG and the CD16a receptor. Indeed, Abs with low fucosylation induce higher ADCC[150]. Alternatively, sialylation of Abs has been associated with reduced ADCC[151]. Given its ability to utilize the humoral component of the adaptive immune system, CD16a represents an attractive target for harnessing the potential of the innate immune system through shaping the adaptive immune response.

The potential to utilize CD16a for therapeutic purposes has resulted in the design of several mAb therapies directed at cancers. Examples of such therapies include Rituximab, an anti-CD20 Ab used in the treatment of B-cell lymphomas, and Trastuzumab, an anti-Her-2 Ab used to treat Her-2-expressing breast cancers[152]. Although not the only Ab-dependent effector function elicited by these Abs, several animal models suggest that ADCC is important in the benefits associated with treatment with these mAbs. One convincing line of evidence supporting a role for ADCC in the protection mediated by mAb therapies is that the treatment is ineffective in mice without activating IgG receptors[153]. Studies in humans also support ADCC as an important effector function in the efficacy of these Ab therapies. For example, Rituximab has been demonstrated to be most efficacious in individuals who are homozygous for CD16a receptors containing a valine at position 158[154]. Furthermore, mAb therapies utilizing non-fucosylated Abs have been demonstrated to be more efficacious than those utilizing fucosylated Abs[150]. Collectively, data suggests that ADCC mediated by CD16a is an attractive effector function to exploit for therapeutic purposes. This has been clearly demonstrated for cancers, and the ability of ADCC to mediate cellular clearance suggests this mechanism could be relevant for viral infections.

## 1.16 KIR

The KIR family of receptors represents an extremely diverse set of cell-surface proteins that bind to classical and non-classical HLA-I molecules (Figure 7)[98;110]. Genes encoding KIR are located within the LRC on chromosome 19. In humans the KIR loci represent some of the most polymorphic sites outside of the HLA-A, B, and C loci. Although KIR encoding genes are found in species that are evolutionarily older, such as mice and seals, in mice these genes are encoded on the X-chromosome, and in both species there is a lack of diversity in KIR genes. Indeed, KIR diversity is a product of simian primate evolution. This is reflected by the gradual appearance of KIR specific for a broader array of HLA-I molecules in simian primates. For example, gibbons carry KIR that can recognize HLA-A and B-like MHC-I molecules. Higher simian primates, such as orangutans, gorillas, chimpanzees and humans, are the first species in which HLA-C-like MHC-I molecules appeared. As such, these species have additional KIR that can recognize HLA-C-like molecules[155]. Aside from diversity in the ligands recognized by KIR, there is also diversity between KIR in terms of the functional consequences of ligation. The functional diversity within KIR can be deduced from the structure of these receptors.

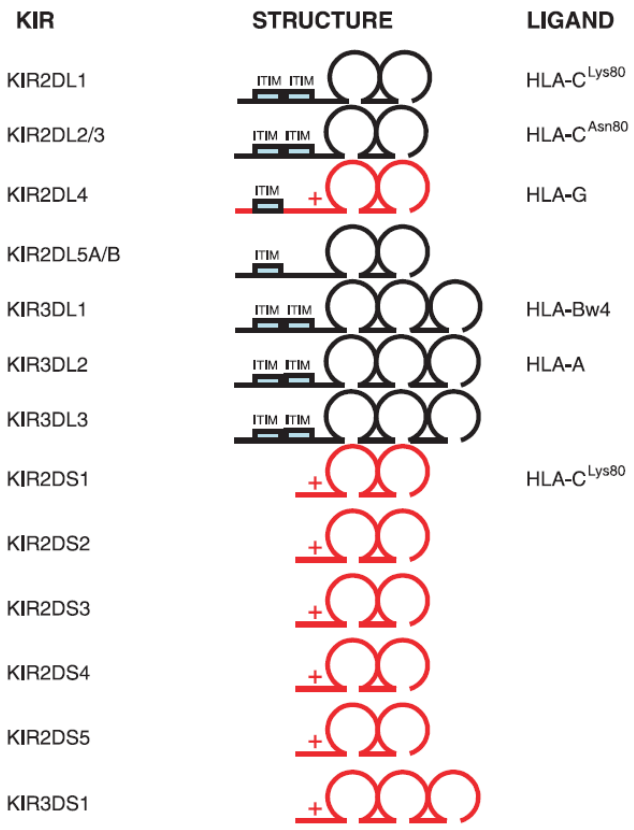


**Figure 7[110]. Receptor/ligand combinations of KIR and HLA-I.** (Reprinted with permission from CLOCKSS. Mol Interv 5: 226-240, 2005).

The human LRC encodes up to 15 KIR genes, of which 13 code for expressed KIR proteins and two are pseudogenes[156]. Expressed KIR genes manifest as Ig-like proteins, which express two to three extracellular domains[110]. The extracellular domains of all KIR are linked to a transmembrane domain that extends into the cytosolic environment as a cytoplasmic tail. If the receptor transmits an inhibitory signal upon ligation, the cytoplasmic tail is long and contains an ITIM. If the receptor is capable of transmitting an activating signal

upon ligation, the cytoplasmic tail is short. For activating KIR, there is a positive charge in the transmembrane region that allows the receptor to associate with ITAM-containing DAP-12 adaptor proteins[157]. These structural characteristics are reflected in the standard nomenclature for KIR[110]. The names for all genes/proteins for this receptor family start with the acronym "KIR". A number and the letter "D", which together represents the number of extracellular domains expressed on the protein, follow this acronym. For example, KIR2D is used for a protein with two extracellular domains, and KIR3D for a protein with three extracellular domains. The letter "S" follows the number of domains, if the receptor has a short cytoplasmic tail (i.e., KIR2DS). Alternatively, the letter "L" follows the number of domains, if the receptor has a long cytoplasmic tail (i.e., KIR2DL). Lastly, the standard nomenclature for KIR ends with a number that represents the order in which structurally similar KIR were discovered. As such, KIR2DL1 represents a gene that was discovered prior to KIR2DL2.

Given the extensive diversity in KIR structure, function and ligands, it is predictable that KIR impact the functionality of NK cells. As such, it is essential to understand how KIR expression is determined. Generally, the expression of KIR gene products occurs in a stochastic manner[110]. The main determinant of whether a particular KIR gene will be expressed appears to be the DNA methylation pattern of KIR promoters[158]. Hypermethylated promoters characterize unexpressed KIR genes, and hypomethylated promoters characterize expressed KIR genes. Demethylation of KIR promoters may be

inducible by the activation of NK cells by target cell interactions or the stimulation of NK cells with soluble factors, such as cytokines, as the activation of NK cells with MHC-I-devoid target cells or interleukin-2 (IL-2) has been shown to induce the expression of KIR3DS1[159]. Once KIR expression has been turned on, it appears to be maintained and passed forward to future cell generations in a clonal fashion[160]. Given these characteristics, it is expected that NK cells should accumulate cell surface-expressed KIR throughout their lifespan. Indeed, the laboratory of Dr. Patrice Debre has demonstrated the accumulation of higher numbers of expressed KIR as NK cells mature[161].

Although the vast array of potential KIR/HLA interactions could affect NK cell functionality in numerous ways, particular attention has been paid to KIR3DL1 and KIR3DS1. Unlike other activating and inhibitory forms of KIR, which are encoded by separate genes, *KIR3DL1* and *KIR3DS1* represent allelic versions of the same gene[162]. Products of *KIR3DL1* alleles interact with HLA-B and HLA-A molecules that contain a public epitope known as HLA-Bw4[163]. This public epitope is found in the $\alpha$1 domain of some HLA-A and B molecules at amino acids 77-83[164]. A polymorphism exists within HLA-Bw4 at position 80, which can be an isoleucine (HLA-Bw4*80I) or a threonine (HLA-Bw4*80T)[163]. KIR3DL1 has been shown to mediate a stronger interaction with HLA-Bw4*80I ligands. In fact, the interaction between KIR3DL1 and HLA-Bw4 is influenced by factors other than the HLA-Bw4 epitope, including polymorphisms outside the HLA-Bw4 epitope and the peptide bound within the peptide-binding groove[165;166]. Despite

evolving from KIR3DL1 and sharing 97% homology, KIR3DS1 does not interact with HLA-Bw4[167;168]. In fact, no ligand has been determined for KIR3DS1. Aside from being unique for encoding alleles for both activating and inhibitory receptors, the *KIR3DL1* locus has also attracted attention due to the extensive allelic polymorphisms that have been observed. As of this writing, upwards of 70 alleles have been observed for this locus[115]. Such allelic polymorphism has an impact on the functional consequences of receptor ligation, as well as the level of surface expression. Indeed, staining with the anti-KIR3DL1 mAb, DX9, demonstrates that KIR3DL1 alleles can be detected at the cell surface at high (*h) (e.g., *KIR3DL1*001, *002* & **008*,) or low (*l) (e.g., *KIR3DL1*005, *006*, & **007*) expression levels, or be unexpressed at the cell surface as in the case of the *KIR3DL1*004* allele[169;170].

The large amount of diversity at the *KIR3DL1, HLA-A*, and *HLA-B* loci make allelic combinations of *KIR3DL1* and *HLA-Bw4* interesting for disease associations. Indeed, combinations of *KIR3DL1* with *HLA-Bw4* alleles containing an HLA-Bw4*80I are associated with reduced susceptibility to the autoimmune disorder ankylosing spondylitis[171]. Several solid tumors have also been demonstrated to be more likely to occur in individuals who have more inhibitory *KIR3DL1/HLA-Bw4* pairs[172]. Despite these associations of *KIR3DL1/HLA-Bw4* combinations with protective outcomes in the cases of ankylosing spondylitis and poor outcomes in the case of solid tumors, several potent KIR3DL1/HLA-Bw4 interactions have been associated with protection from HIV infection and/or

disease progression[57;58;74]. These combinations will be discussed later, and their potential mechanisms of protection are the topic of this thesis.

## 1.17 NK cell regulation

From the information provided above on NK cell receptors, their ligands, and functionality, it is easy to see how NK cells minimize the possibility of damage to self and increase the chances of detecting abnormal autologous cells. Inhibitory NK cell receptors recognize constitutively expressed classical and non-classical MHC-I molecules, preventing NK cells from targeting healthy cells[107]. Alternatively, activating receptors recognize self-ligands that are induced by infection or transformation, IgG bound to foreign or tumor antigens expressed on the cell surface, or foreign antigens directly[129;141;148;173]. As viral infection and transformation decreases the expression of classical HLA molecules and increases the ligands for activating NK cell receptors, NK cells are designed to efficiently detect abnormal autologous cells. It is, however, possible to conceive of situations where such a simplistic concept of NK cell regulation would fail to protect healthy cells from NK cell-mediated attack. As NK cell receptor expression is determined in a stochastic fashion, it is possible that NK cells that lack inhibitory receptor expression could arise. Indeed, such NK cells have been detected in humans[174]. Given that stress-induced ligands for NKG2D can be upregulated on activated lymphocytes[175], it is possible that NK cells without

inhibitory receptors could become activated by non-infected and non-transformed autologous cells. The potential of normally observed NK cells to mediate responses against normal self raises the need for an extra level of NK cell ontogeny that determines the functional potential of NK cells in a manner that would minimize the chances of autoimmunity.

The idea that an ontological process prior to encountering putative target cells regulates NK cell functional potential is supported by evidence such as the following: if all NK cells were created with equal functional potential, it would be expected that all NK cells would mediate similar responses after receiving equal activating signals. However, NK cells are not equal in their ability to mediate effector functions upon receiving similar levels of activating signals. Stimulation of NK cells in both mice and humans demonstrates that great differences exist in the functional potential of NK cells within and between individuals[176-178]. Similarly to the need to maintain self-tolerance, these observations suggest that NK cell functional potential is determined during an ontological process.

In recent years there has been much research aimed at elucidating the ontological process that determines NK cell functional potential. Much of the data collected and theories proposed suggest that interactions of activating and inhibitory receptors with the normal self-environment tunes the functional potential of NK cells and prepares them for responding to abnormal autologous cells. Collectively, this process has been termed "NK cell education"[179], and the current understanding of this phenomenon will be discussed in the next section.

## 1.18 NK cell education

The functionality of NK cells is determined through a two-tier process. Prior to encountering putative target cells, where the cumulative signal received through NK cell receptors determines whether the NK cell will be able to mediate effector functions, interactions between unique constellations of activating and inhibitory receptors expressed on individual NK cells with the normal self-environment determine the functional potential of the NK cell[179]. As such, NK cells that express certain combinations of NK cell receptors that can interact with differing numbers of self-ligands will acquire distinct functional potentials. Evidence to date suggests that signals through inhibitory receptors, which recognize constitutively expressed self-ligands, increase the chances that an NK cell will mediate function upon encountering an appropriate target cell. The larger the number of inhibitory signals received during development the higher the chances of NK cell functionality when it encounters target cells[180]. Alternatively, signals through activating NK cell receptors, which recognize constitutively expressed self-ligands, decrease the chances that an NK cell will mediate function upon encountering an appropriate target cell[181]. Receiving such signals during the maturation phase down-modulates the magnitude of that NK cells' functional potential upon encountering targets. Cumulatively, these early interactions between activating and inhibitory receptors with normal constitutively expressed self-ligands have been termed NK cell education. Several

models have been proposed to explain how the signals are integrated to determine NK cell functional potential, including the arming, disarming, and rheostat models.

According to the arming model of NK cell education, NK cells initially exist as a hypofunctional lymphocyte subset[182]. Functional potential is then conferred upon these lymphocytes through the signals generated within the cell upon the ligation of inhibitory receptors binding to self-ligands. The disarming model offers an alternative view by proposing that NK cells initially exist as a functional lymphocyte subset. In contrast to the arming model, the disarming model does not predict that inhibitory receptor ligation confers functional potential to NK cells. Instead, inhibitory receptor ligation is predicted to be responsible for rescuing NK cells from anergy induced from extensive signaling received through activating receptor ligation. Finally, the rheostat model agrees with both the arming and disarming models in predicting that signals through both activating and inhibitory receptors are important for NK cell education[183]. In contrast to both of the other models, however, the rheostat model predicts that the signaling required for NK cell education has a cumulative effect, and is not a simple on/off switch. As such, the rheostat model predicts that a stronger cumulative inhibitory signal will increase the chances that an NK cell will mediate effector functions upon activation, as well as increase the magnitude of those functions. Furthermore, the model predicts that the intensity of the inhibitory signaling is associated with how many effector functions the NK cell can mediate

simultaneously. This model also predicts that the process of NK cell education is a continuous ontological process. It should be noted that although these three models offer subtly different explanations for NK cell education, they are not mutually exclusive. In fact, it is possible that each model explains different components of the education process.

The original evidence for NK cell education was provided by the laboratory of Dr. Wayne Yokoyama, which compared the function of NK cells isolated from MHC-I-deficient mice that carried an MHC-I-specific inhibitory receptor to that of NK cells isolated from mice that carried both the inhibitory MHC-I-specific receptor and the MHC-I molecule[176]. The study demonstrated that in order for NK cells to respond to Ab-mediated cross-linking of an activating receptor, the cells needed to come from MHC-I sufficient mice and express the inhibitory receptor. Furthermore, the Yokoyama lab demonstrated that signaling through the inhibitory NK cell receptor is necessary to confer NK cells with functional potential, as receptor mutants lacking cytoplasmic domains, and thus ITIMs, are incapable of conferring functional potential upon NK cells. The Yokoyama group referred to the process of inhibitory NK cell receptor/self-ligand interactions conferring functional potential upon NK cells as NK cell licensing. As explained by this group the phenomenon is compatible with the arming model of NK cell education.

Following the initial demonstration of NK cell education through inhibitory receptor ligation in mice, several labs published evidence of a similar

phenomenon in humans. Anfossi et al. (2006)[178] demonstrated that human NK cells lacking inhibitory KIR and NKG2A/CD94 exhibit hypofunctionality upon stimulation for direct or Ab-dependent activation. Furthermore, they demonstrated that both direct and Ab-dependent activation were mediated by higher numbers of NK cells expressing HLA-C specific inhibitory KIR, if they were obtained from individuals carrying the HLA-C ligand. Similarly, Kim et al. (2008)[177] demonstrated that KIR3DL1 expressing NK cells exhibit higher direct activation, when derived from individuals carrying the HLA-Bw4 ligand than from those carrying no ligand for this inhibitory receptor. Furthermore, Kim et al. demonstrated that the functionality of the KIR3DL1 expressing NK cells was related to the number of HLA-Bw4 alleles carried. Lastly, Parsons et al. (2010)[184] showed that the educational capacity of the KIR3DL1/HLA-Bw4 interaction extended to Ab-dependent activation as well. My colleagues and I demonstrated higher levels of cytotoxicity and IFNγ production in KIR3DL1 expressing NK cells isolated from HLA-Bw4 carriers than from individuals who did not carry this ligand for KIR3DL1. In summary, these studies demonstrate that the phenomenon of NK cell education applies to human NK cells, and that the arming model can explain at least some of this process.

Studies in both mice and humans have also provided evidence that ligation of activating receptors by constitutively expressed self-ligands can negatively impact NK cell functional potential. These findings support the notion that the disarming model can also explain some aspects of NK cell education.

This has also been seen in mice that carry Ly49h, an activating receptor that recognizes the m157 MCMV protein. In Ly49h carrying chimeric mice that constitutively express the m157 protein, Ly49h expressing NK cells become hypofunctional[185]. A similar process can occur in humans who carry the activating KIR2DS1 in combination with it ligand, HLA-C2. KIR2DS1 expressing NK cells from such individuals exhibit lower functionality than those isolated from individuals lacking the HLA-C2 ligand[181].

It should also be pointed out that several groups have provided evidence that processes predicted by the rheostat model contribute to NK cell education. For example the lab of Dr. Petter Höglund was one of the first groups to demonstrate that the intensity of the inhibitory signal received by NK cells during education can differentially tune the NK cell response. They showed that the cumulative number of inhibitory receptor/ligand combinations had an additive, and sometimes synergistic, effect on the ability of NK cells to mediate effector functions and the intensity of those effector functions[180;183]. They generated data suggesting that the intensity of the inhibitory signal received during NK cell education also determines the effector functions that the NK cell will be capable of mediating upon activation, as well as the ability to mediate these functions simultaneously. Their data demonstrates that lower level inhibitory signals will first confer NK cells with the ability to degranulate, as measured by expression of the CD107a marker. With the incorporation of higher inhibitory signals, NK cells gain the ability to produce cytokines, such as IFN$\gamma$ (Figure 8). The data obtained

in mice by the Höglund group has been shown to extend to humans by the Yokoyama group, which demonstrated that the cumulative number of inhibitory receptors, for which ligands were present within the donors, expressed on the NK cell surface was predictive of the ability of NK cells to mediate cytolysis and produce IFNγ[186]. These data demonstrate that the rheostat model explains some of the processes involved in NK cell education in both humans and mice.

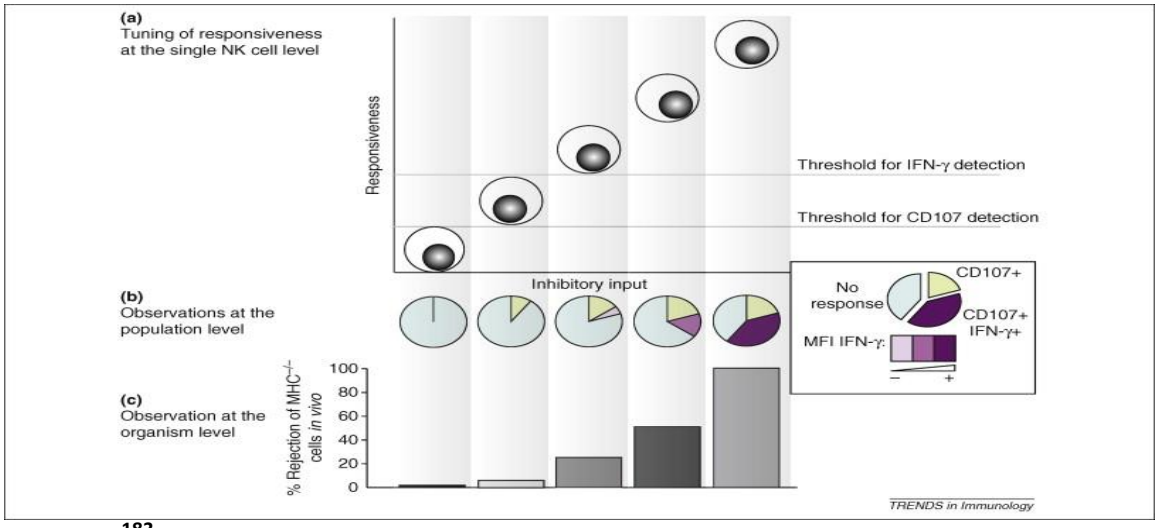


**Figure 8[183]. The "Rheostat Model" of NK cell education: Tuning through inhibitory receptors.** (Reprinted with permission from Elsevier Ltd. Trends Immunol 30: 143-149, 2009).

Further support for the rheostat model is provided by its ability to explain the educational development of NK cells as a continuous ontological process. That NK cells can be modified by a changing self-environment was recently demonstrated by transferring educated NK cells from animals carrying a ligand for an inhibitory receptor into animals lacking MHC-I, or transferring non-educated NK cells from MHC-I deficient animals into animals with MHC-I ligands

for the inhibitory receptors expressed on the NK cells[187-189]. These studies demonstrated that placing NK cells in a new MHC-I environment could re-educate the NK cells in a manner that would prevent destruction of the new "self-environment". These observations may also be relevant to changes in an autologous environment that is modified by viral infections that lead to modulations in MHC-I expression and the introduction of novel peptides for presentation on MHC-I. Decreased presence of MHC-I ligands could decrease the inhibitory signals received during NK cell education, and peptides that alter the interactions between inhibitory receptors and their ligands could influence NK cell education. As such, it can be seen that the rheostat model appropriately explains NK cell education as a continuous process, and this could be relevant for understanding the changes observed in NK cells during chronic viral infections.

Another potential factor for altering the educational status of an NK cell is exposure to cytokines. Numerous studies have demonstrated that treatment of NK cells with cytokines can alter the potential to mediate effector functions against HLA-devoid and ADCC targets. For example, IL-2, interleukin-10 (IL-10), interleukin-12 (IL-12), and interleukin-15 (IL-15) have been demonstrated to increase NK cell function, whereas transforming growth factor beta (TGF-β) has been shown to decrease NK cell function[190-195]. Treatment of murine NK cells with function-enhancing cytokines allows non-educated cells to mediate effector functions at a similar level as educated NK cells[176]. In humans, however, educated NK cells have been shown to maintain a functional advantage even

after exposure of all NK cells to function-enhancing cytokines[178]. As such, it appears as if the education of human NK cells is not influenced by treatment with cytokines.

The current perspective on NK cells is that they are regulated through a two-tier process. First, NK cells interact with the normal self-environment and acquire a degree of functional potential that will minimize the chances that these cells can mediate autoimmune responses[179]. Secondly, NK cells will encounter putative target cells[107]. If these putative targets provide a cumulative stimulatory signal to the NK cell, it will respond in a manner that is consistent with the functional potential it acquired during the educational process. These basic NK cell biology concepts are essential for understanding how certain combinations of NK cell receptors and their ligands can confer protective outcomes in the context of exposure or disease progression upon infection with viruses, such as HIV. In the next section of this review the behavior of NK cells during HIV infection, as well as the associations of NK cells with protection from infection and/or progression to AIDS will be discussed. This will be followed by a section addressing how our current understanding of NK cell education may be able to explain the role of NK cells in protective outcomes in the context of HIV.

## 1.19 The interaction between NK cells and HIV

Interactions between NK cells and HIV are responsible for variable and dynamic immune responses. Indeed, within different individuals NK cells have been implicated in determining outcomes such as protection from HIV infection, and slower time to AIDS[55;57;58;74;196]. In HIV infected individuals NK cells have been shown to be dysfunctional and may also play a role in CD4 decline[197;198]. The next sections of this literature review will focus on the dysfunction, pathogenesis and protection mediated by NK cells in the context of HIV infection.

## 1.20 NK cell dysfunction in HIV infection

The dysfunction observed in HIV infection is diverse, manifesting as changes in the patterns of receptor expression, NK cell subset distribution, as well as functionality. Altered receptor expression in HIV infection is associated with detectable viremia, and results in NK cells with phenotypes characteristic of NK cells that are less likely to become activated[198]. Indeed, HIV viremia is associated with an increased proportion of NK cells that express inhibitory receptors, including several inhibitory KIR[199]. Accompanying the increase in inhibitory NK cell receptors, NK cells from viremic patients also exhibit reduced surface expression of the three activating NCR compared to uninfected controls[200]. The relationship of these alterations to viremia is supported by the decrease in NK cells expressing inhibitory receptors and an increase in the

surface density of NCR after initiation of successful HAART that results in viral suppression[199]. Cumulatively, these phenotypic alterations in viremic individuals decrease the probability that the activating signals received by NK cells will overcome the inhibitory signaling received upon encountering putative target cells.

Contributing to the NK cell dysfunction in HIV infection is the altered distribution of NK cell subsets observed in infected individuals. In HIV-uninfected healthy controls, NK cells are usually divided into two distinct subsets[201]. These include NK cells that exhibit a $CD56^{dim}CD16a^{+}$ phenotype, which readily mediate cytolysis and secrete low levels of cytokines upon activation, and those that exhibit a $CD56^{bright}CD16a^{dim/-}$ phenotype, which are less likely to mediate cytolysis but secrete higher cytokine levels upon activation. Individuals infected with HIV, as well as those infected with other chronic viral infections such as HCV, carry a third NK cell subset that is $CD56^{-}CD16a^{+}$[202]. These "CD56null" NK cells mediate reduced functionality upon receiving activating signals[199]. The number of these cells is correlated with the level of viremia detected within infected individuals, and the frequency of these cells decreases after initiation of successful HAART.

HIV infection is also associated with a third level of NK cell dysfunction, which is the reduced functionality of NK cells from HIV-infected individuals upon receiving activating signaling. Indeed, stimulation of NK cells from HIV-infected individuals with HLA-I-devoid target cells, Ab cross-linking of activating receptors,

or through CD16a binding of IgG constant regions, which in turn recognizes ADCC target cells, results in lower levels of cytolysis and/or cytokine secretion than does stimulation of NK cells from uninfected subjects[199;203]. The degree of functional NK cell impairment in HIV infected individuals may be related to viral load measurements, as NK cell function can be partially restored with successful suppression of viremia with HAART. Although alterations in receptor expression and the redistribution of NK cell subsets in HIV infection most likely account for much of the altered functionality observed in infected individuals, several other factors, such as direct infection of NK cells, interactions with viral components, and plasma cytokine alterations may also contribute[145;204;205].

The observation of altered NK cell functionality, subset distribution, and receptor expression in viremic HIV-infected individuals suggests that NK cell dysfunction could be a contributing factor to uncontrolled viral replication and HIV disease progression. Furthermore, NK cell dysfunction, in combination with dysfunction in other immunological compartments, could contribute to the susceptibility of HIV-infected individuals to several opportunistic infections and malignancies.

## 1.21 Contribution of NK cells to HIV disease pathogenesis

Apart from NK cells being dysfunctional in the context of HIV infection, they have also been implicated in the destruction of uninfected $CD4^+$ T-

lymphocytes and thus may contribute to the CD4 decline characteristic of HIV disease. Beyond the initial loss of mucosal $CD4^+$ T cells during primary infection, a slow and continuous loss of $CD4^+$ T cells is characteristic of HIV disease[206]; its underlying mechanisms are an intense area of investigation. Some explanations include programmed cell death, direct cytopathic effects of HIV, and lysis by MHC-I unrestricted CTL[207-209]. Any theory proposed needs to account for the fact that most of the $CD4^+$ T cells dying in HIV infection are uninfected[210]. One hypothesis proposed to explain how NK cells destroy uninfected $CD4^+$ T cells postulates that uninfected $CD4^+$ T cells bind free gp120, which is then bound by anti-gp120-specific Abs able to bind CD16a on NK cells and mediate ADCC[145]. However, as yet there is no experimental evidence to support this hypothesis.

Another mechanism of NK cell mediated pathogenesis has been described by the laboratory of Dr. Patrice Debré. They showed that HIV Env gp41 has a conserved motif, known as 3S, which can interact with uninfected $CD4^+$ T cells to induce the expression of a ligand for the activating NKp44 receptor[211]. NK cells activated by uninfected autologous cells expressing the 3S induced NKp44 ligand can mediate the cytolysis of these healthy lymphocytes[197]. Dr. Debré's laboratory has demonstrated that the level of this cytolysis is associated with anti-3S Abs present in HIV-infected individuals or that have been induced in SHIV-infected macaques[212;213]. Furthermore, this research group has demonstrated HIV-infected viremic non-progressors are more likely than

progressors to carry Abs, or higher titers of Abs, capable of inhibiting 3S from inducing the NKp44 ligand[214].

In summary, in the context of HIV infection NK cells are not only dysfunctional but may also contribute to HIV disease pathogenesis. They may do this not only by exhibiting a reduced capability to respond to invading pathogens and/or malignancies, but also by impeding the ability of other components of the immune system to handle such challenges.

## 1.22 NK cell mediated protective outcomes in the context of HIV

Despite the potential for NK cell dysfunction and pathogenesis to contribute to the progression to AIDS, as well as the susceptibility of HIV-infected individuals to opportunistic infections and malignancies, NK cells have also been associated with protection from HIV infection and disease progression. Indeed, protection from HIV infection or slow time to AIDS has been associated with the co-carriage of certain alleles encoded by the *KIR3DL1/S1* and *HLA-B* loci[57;58;74;196]. NK cells from individuals with several putative receptor ligand combinations associated with protective outcomes in the context of HIV have a heightened NK cell functional potential[56].

Heightened NK cell functionality has been correlated with several protective outcomes in the context of HIV. For example, NK cells from an HESN

IDU cohort in Vietnam exhibited higher functionality upon activation with HLA-I-devoid target cells than NK cells from the general population[55]. Furthermore, HIV infected SP have a higher frequency of *KIR3DL1/HLA-B* combinations that endow NK cells with heightened functionality [56;74].

The factors responsible for the heightened NK cell functionality in these individuals have not yet been elucidated; however, a higher frequency of function-conferring combined allotypes of *KIR3DL1/S1* and *HLA-Bw4* has been observed in both HESN and SP[56;57;74]. Dr. Mary Carrington was the senior author on the first published epidemiological study indicating that KIR/HLA combinations could influence the outcome of HIV infection. They demonstrated that HIV-infected individuals that carried *KIR3DS1* in combination with an *HLA-Bw4*80I*, exhibited slower progression towards AIDS[196]. In a subsequent study the same group suggested that this allelic combination might also protect HIV-infected individuals from opportunistic infections[215].

Similar studies in HESN have observed that KIR3DS1 may also mediate protection from HIV infection. Work done in Dr. Nicole Bernard's laboratory demonstrated that homozygousity for *KIR3DS1* is detected at a higher frequency in HESN than in individuals that are susceptible to HIV infection[58]. While the Carrington lab found that the *KIR3DS1 HLA-Bw4*80I* combination appeared to be protective in terms of slow time to AIDS in those infected[196], data from the Bernard lab suggests that this genotype combination is not associated with protection from infection.

The data from both the Carrington and Bernard laboratories is made all the more interesting by results generated in Dr. Doug Nixon's laboratory demonstrating that KIR3DS1 expressing cells mediate enhanced functionality upon stimulation with HLA-I-devoid target cells[59]. This represents a potentially important observation, as HIV-infected $CD4^+$ T-lymphocytes have previously been demonstrated to downregulate HLA-I upon infection[108]. Indeed, it has been suggested that this reduced level of cell surface HLA-I on infected cells may allow NK cells to respond to infected cells in a fashion similar to how they respond to HLA-devoid target cells (Figure 9)[198].

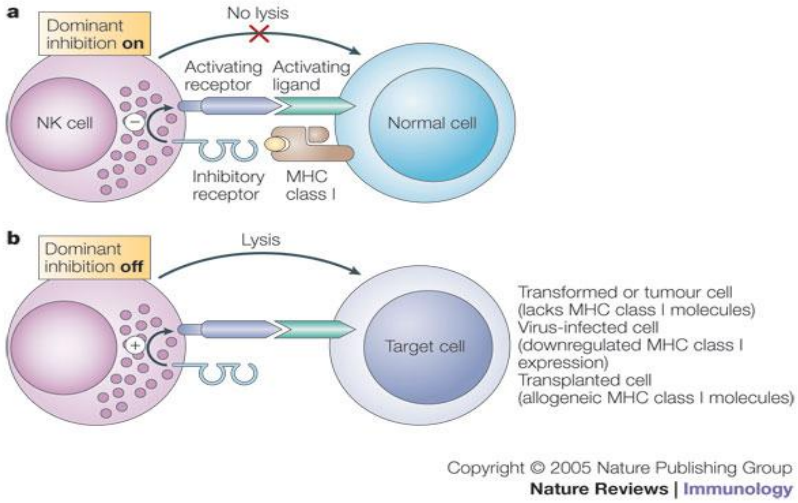


**Figure 9[198]. Hypothetical role of HLA-I downregulation on NK cell recognition of HIV-infected cells.** (Reprinted with permission from MacMillan Publishers Ltd. Nat Rev Immunol 5: 835-843, 2005).

Unfortunately, other studies have been unable to replicate and confirm Dr. Carrington's observation that *KIR3DS1/HLA-Bw4*80I* combinations are

associated with slower time to AIDS[216;217]. This may be because smaller group sizes with shorter follow ups were used in the confirmatory studies. More recently, Dr. Carrington's and several other groups have been able to repeatedly and more concretely demonstrate that several allelic combinations of *KIR3DL1* and *HLA-Bw4* are protective against HIV disease progression[74;216]. In particular, the combination of highly expressed *KIR3DL1* alleles with *HLA-B*57* was demonstrated to have the highest impact on slowing time to AIDS. Remarkably, this same combination has also been observed at an increased frequency in HESN by the Bernard laboratory[57]. Furthermore, this allelic combination has been demonstrated to educate KIR3DL1$^{+}$ NK cells for an increased ability to mediate polyfunctional (i.e., IFN$\gamma^{+}$, TNF$\alpha^{+}$, & CD107a$^{+}$) responses[56]. In chapter 2 of this thesis we will present results on the functional potential NK cells from individuals carrying another KIR/HLA combination associated with slower time to AIDS.

In summary, in uninfected HESN and in HIV infected SP there is a higher frequency of genetic combinations of *KIR3DL1* and *HLA-B* alleles that are epidemiologically associated with protection from infection and/or slow time to AIDS[57;74]. NK cells from individuals carrying these "protective" combinations have higher functional potential than NK cells from individuals having combinations that would have a lesser or no impact on NK education[56]. Therefore, the protection observed in HESN and SP may be related to the educational impact of these *KIR3DL1/HLA-Bw4* combinations on NK cell development, which translates

into a higher frequency of NK cells with more intense and broader functionality upon encountering infected target cells. Such NK cell functionality may be able to reduce viral replication more effectively or more rapidly thus leading to either preventing the establishment of infection or slower disease course. To comprehend how KIR3DL1$^{+}$ NK cells confer protection, it is essential to understand how KIR3DL1$^{+}$ NK cells, and NK cells in general, interact with autologous HIV-infected CD4$^{+}$ T cells. These interactions will be discussed in the next section of this literature review.

## 1.23 Interactions between autologous NK cells and infected CD4$^{+}$ T cells

In general, most studies of the interactions between NK cells and autologous infected CD4$^{+}$ T cells have demonstrated that NK cells have a poor ability to mediate lysis of infected cells[126;127]. Despite the downregulation of HLA-A and HLA-B on infected cells, which should decrease the inhibitory signal received by NK cells bearing inhibitory receptors for these alleles, infected cells upregulate HLA-E, the ligand of the inhibitory NKG2A receptor, and prevent the expression of ligands for the activating NKG2D receptor[108;123;131]. As such, the alteration of ligands for NK cell receptors by HIV occurs in a manner that decreases the likelihood that the autologous infected cell will activate an NK cell.

Despite the reduced ability of NK cells to mediate cytolysis of autologous infected CD4$^{+}$ T cells, several studies have demonstrated that NK cells cultured in

the presence of IL-2 can inhibit the replication of HIV in autologous $CD4^+$ T cells[103;104]. Notwithstanding the questionable validity of the observed functionality of NK cells undergoing prolonged culture in IL-2, these studies have shown that NK cells expressing non-protective KIR, but not those expressing the protective KIR3DL1, degranulate following co-culture with HIV infected autologous $CD4^+$ T cells and thus would be responsible for suppression of viral replication.

As such, data regarding interactions between NK cells and autologous infected $CD4^+$ T cells are unable to explain how allelic combinations of *KIR3DL1* and *HLA-Bw4* mediate protective outcomes in the context of HIV exposure/infection. One alternative possibility is that $KIR3DL1^+$ NK cells require additional signals to overcome the increased inhibitory and decreased activating ligands on infected cells. Studies from the cancer literature suggest that ADCC could provide a mechanism to obtain these additional activating signals[218]. Indeed, Ab-coated tumor cells can stimulate NK cells enough to overcome inhibitory signaling through KIR/HLA interactions.

## 1.24 ADCC and HIV

The idea that $KIR3DL1^+$ NK cells require additional signals through CD16a to be activated by autologous infected $CD4^+$ T cells is not just an attractive explanation for why these NK cells are not activated by infected cells in the

absence of Abs, it is also consistent with a plethora of previous evidence indicting ADCC as a potentially protective response in the context of HIV exposure and infection. Indeed, anti-HIV ADCC has been implicated in the protection from infection observed in vaccinated humans, HESN, as well as rhesus macaques that received passive transfers of the b12 BnAb[55;88;219]. Furthermore, ADCC has also been connected to the slow progression to AIDS observed in SP and some SHIV-infected rhesus macaques[71;220].

As previously mentioned, the association of anti-HIV ADCC with protection from infection was most recently brought to the forefront by the results of the RV144 vaccine trial correlates of protection analysis[88]. The results of this investigation demonstrated that although ADCC did not appear to confer protection from infection in the primary analysis, ADCC responses in individuals with low levels of IgA, which may interfere with the ability of IgG anti-Env Abs to mediate ADCC, were correlated with protection from infection. This observation has created excitement about the possibility of designing an HIV vaccine that harnesses the power of the innate immune system to induce an adaptive immune response. Evidence supporting the potential for such a vaccine has also been provided by studies of HESN and rhesus macaques. Scott-Algara et al. (2003) demonstrated that some IDU HESN carry Abs that can induce NK cell-mediated ADCC of infected $CD4^+$ T cells[55]. However, perhaps the most convincing evidence that ADCC can mediate protection has been put forth by experiments conducted in the laboratory of Dr. Dennis Burton. These experiments involved

passively transferring different variants of the b12 BnAb to rhesus macaques prior to challenge with SHIV[219]. The variants of b12 differed in their capacity to induce ADCC, with one variant being fully ADCC competent and the other lacking the ability to interact with CD16a. Studies prior to this investigation had demonstrated that passive transfer of b12 to rhesus macaques confers protection from SHIV challenge[79;80]. The ability of b12 to neutralize the challenge virus was thought to be responsible for this protection. However, in the more recent study macaques that received the b12 variant that could not induce ADCC were not protected as well as those that received the ADCC-competent Ab[219]. Therefore, the protection capacity of b12 appears to be at least partially due to its ability to induce anti-viral ADCC.

The potential of anti-HIV ADCC to confer protection from HIV is not restricted to preventing infection; several investigators have demonstrated a correlation between ADCC and protection from disease progression. For example, the titer of ADCC- competent Abs from HIV-infected individuals has been associated with $CD4^+$ T-lymphocyte decline within infected individuals[221]. Corroborating this observation is the demonstration that SP carry Abs capable of mediating stronger ADCC than HIV-infected individuals with a typical course of progression[71]. Similar to the data available for humans, studies in SHIV-infected rhesus macaques suggest that anti-viral ADCC may slow disease progression. Indeed, sustained anti-SHIV ADCC is correlated with maintained $CD4^+$ T cell counts in macaques[220].

Cumulatively, these results suggest that targeting ADCC would be a strategy through which the innate immune response could be harnessed to provide protection from HIV infection. Despite the potential of anti-viral ADCC responses to provide protection, the characteristics of protective ADCC responses are largely unknown. Furthermore, it is unknown whether the protection conferred by *KIR3DL1/HLA-Bw4* combinations is linked to the education of these cells to mediate enhanced anti-HIV ADCC. As such, the study of anti-HIV ADCC and the role of education through KIR/HLA combinations, particularly those that confer protection from HIV infection and/or disease progression, is essential for designing vaccines or therapeutics that utilize this immune response. In chapters 3-5 of this thesis I will present results on how *KIR3DL1/HLA-Bw4* genotype combinations that influence NK cell education impact ADCC activity. In the next section of this literature review the assays that are available to study anti-HIV ADCC will be discussed.

## 1.25 Anti-HIV ADCC assays

The assays utilized for measuring anti-HIV ADCC responses can be separated into those that measure the killing of target cells and those that measure NK cell activation through intracellular staining. Both types of assays can provide essential information for evaluating anti-HIV ADCC responses.

## 1.26 ADCC assays that measure target cell killing

Several anti-HIV ADCC assays have been designed to measure the killing of target cells. These include the chromium ($Cr^{51}$) release assay, rapid-fluorescence ADCC (RF-ADCC) assay, and the grantoxilux assay[173;222;223]. These assays can be performed using a variety of target cells, including cell lines or primary infected cells. However, due to the fact that primary infected cells are rarely killed, immortalized cell line target cells are usually used. Normally, an NK cell resistant transformed T-cell line, known as CEM.NKr, is utilized as the ADCC target cell for these assays. These cells, which express surface CD4, can be labeled with HIV gp120 and anti-HIV Abs, and then mixed with NK cell effectors. This cell line is resistant to NK cell lysis prior to Ab coating, which reduces background and allows an effective measurement of anti-HIV ADCC activity.

The assays designed to measure target cell killing differ mostly in the mechanism through which they measure cell lysis. The $Cr^{51}$-release assay involves labeling the CEM.NKr target cells with radioactive $Cr^{51}$ prior to their incubation with effector NK cells[173]. The read out measures the amount of the isotope released by target cells. The RF-ADCC assay involves staining the CEM.NKr cells with a membrane dye, PKH-26, and a viability dye, carboxyfluorescein succinimidyl ester (CFSE), prior to incubation with the effector NK cells[223]. Upon lysis, the target cell will lose the CFSE stain. As such, the degree of cytolysis can be measured as the percentage of $PKH\text{-}26^{+}CFSE^{-}$ target cells. Lastly, the grantoxilux assay involves incubating the CEM.NKr and NK

cells together in the presence of a substrate, which penetrates into the target cells and creates fluorescence only when granzyme B is delivered to the target cell by the NK cell[222].

These three killing assays provide essential information about the level of ADCC occurring upon the combination of NK cells, target cells, and Abs in the presence of HIV antigens. However, these assays do not provide any information about the NK cells mediating the lysis. For a better understanding of the phenotype of the NK cells activated during these incubations, flow cytometry assays incorporating extracellular phenotyping and intracellular staining (ICS) are necessary.

## 1.27 ICS ADCC assays

Similar to anti-HIV ADCC assays that measure target cell killing, several variations of ICS ADCC assays exist. The differences between ICS ADCC assays are mostly the target cells utilized. Targets can include HIV Env-coated T cell lines, or autologous cells coated with HIV proteins or peptides[224;225]. These assays generally involve incubating NK cells with coated target cells and anti-HIV Abs for five hours at 37$^{o}$C in the presence of protein-transport disruptors, such as Brefeldin A and/or Monensin. Abs to CD107a are added at the initiation of the co-culture to detect effector cell degranulation, a surrogate marker for cytolysis.

Afterwards, NK cells are stained for surface markers, and intracellular activation markers, such as IFNγ and TNFα.

The ICS ADCC assay allows the characterization of which NK cells are activated by stimulation through ADCC. As well, the ICS assays provide information about non-cytolytic effector functions mediated upon stimulation through CD16a. Together, the ICS and killing ADCC assays provide powerful tools for assessing NK cell-mediated ADCC responses.

## 1.28 Thesis rationale

Research over the past decade has indicated that allelic combinations of *KIR3DL1* and *HLA-Bw4* that educate NK cells for heightened functional potential confer protection against HIV infection and/or progression to AIDS[56;57;74;216]. Despite these epidemiological observations, NK cells that express KIR3DL1 have been shown to be hypofunctional in the presence of autologous HIV infected $CD4^+$ T-cells[104]. Simultaneous with the accumulation of evidence that KIR/HLA combinations can confer protective outcomes in the context of HIV, several studies have suggested that anti-HIV ADCC is a component of immune responses that protect against HIV infection and disease progression[55;71;88;220]. Given that previous research has demonstrated that KIR/HLA combinations educate NK cells for ADCC against allogeneic cells[178;184], the work described in this thesis will provide evidence supporting the idea that KIR/HLA combinations educate NK

cells for ADCC against autologous target cells and that the enhanced educational capacity of KIR/HLA combinations that are associated with protection from HIV infection and/or progression also confer enhanced ADCC potential.

## 1.29 Thesis objectives

To utilize the ability of NK cells to protect against HIV infection and disease progression, it is essential to understand how NK cells mediate protection. Presently available data suggest that protective KIR/HLA combinations are capable of endowing NK cells with enhanced functional potential through the process of NK cell education[56]. Other data suggest that NK cell mediated ADCC could provide protection against both HIV infection and disease progression[56;57;74;216]. As NK cell education contributes to the potential to mediate CD16a-driven effector functions, it is essential to understand the role of NK cell education in anti-HIV ADCC responses[178;184]. Furthermore, as cytokines can influence NK cell functionality[190-195], it is essential to evaluate if the cytokine environment can alter the ADCC potential of educated and non-educated NK cells. As such, this thesis will present original work that contributes to the general knowledge of NK cell education and its role in anti-HIV ADCC responses. To address this topic and generate a greater understanding of potentially pragmatic anti-HIV ADCC responses, this thesis will evaluate (I) the ability of protective KIR/HLA combinations to confer heightened functional potential upon

NK cells, (II) the ability of NK cell education to endow NK cells with the potential to mediate anti-HIV ADCC against autologous target cells, (III) the potential to alter these responses with exogenous factors, such as cytokines, and (IV) the ability of a protective KIR/HLA combination to confer enhanced anti-HIV ADCC potential.

## Bridge from chapter 1 to 2

Previous research from the Bernard laboratory had demonstrated that the allelic combination of *KIR3DL1*h*y/HLA-B*57*, which has been associated with protection from both HIV infection and disease progression, educates NK cells for enhanced functional potential. This observation led to the hypothesis that protective KIR/HLA combinations could provide protection through enhancing the ability of NK cells to mediate effector functions with anti-viral activity.

The experiments presented in the next chapter were designed to test this hypothesis using NK cells from individuals carrying another protective KIR/HLA combination. The *KIR3DL1*004/HLA-Bw4* combination has been associated with slower progression to AIDS from *KIR3DL1*004/HLA-Bw4* carriers for a functional advantage compared to NK cells from individuals with *KIR3DL1/HLA-B* allotypes not associated with protection. Furthermore, we assessed whether the *KIR3DL1*004/HLA-Bw4* combination was also associated with protection from HIV infection, by determining its frequency in an HESN cohort. Discordance between the ability of KIR/HLA combinations to protect against HIV infection and progression to AIDS could reflect different mechanisms of protection. It should be noted that the term licensing is used to explain NK cell education in this chapter.

# Chapter 2: Mind the gap: Lack of association between KIR3DL1*004/HLA-Bw4-induced natural killer cell function and protection from HIV infection

Mind the gap: Lack of association between KIR3DL1*004/HLA-Bw4 induced NK cell function and protection from human immunodeficiency virus infection.

**Running head:** NK cell licensing and HIV resistance

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**FOOTNOTES**

1) None of the authors has a commercial or other association that might pose a potential conflict of interest related to this manuscript.
2) This work was supported by a grant from the Canadian Institutes for Health Research (CIHR) #HVI 79515 and the Fonds de la Recherche en Santé du Québec (FRSQ) AIDS and Infectious Diseases Network. S Boulet was supported by a Ph.D. scholarship from FRSQ. J-P Routy is the recipient of a Clinician Scientist award from the FRSQ.
3) Portions of this work were presented at the International Symposium on Natural Immunity to HIV, November 15-17$^{th}$, 2009, Winnipeg, Canada.
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6) Matthew S Parsons is supported by a CIHR Vanier scholarship.

## 2.1 Abstract

Several combinations of genes encoding *KIR3DL1* alleles and their *HLA-Bw4* ligands have been linked with favorable outcomes upon exposure to or infection with HIV. Some protective *KIR3DL1/HLA-Bw4* combinations confer elevated NK cell functional potential. The K562 stimulated functionality of NK cells from *KIR3DL1*004/HLA-Bw4* and control genotype carriers was assessed by flow cytometry and found to be higher in *KIR3DL1*004/HLA-Bw4* carriers. However, a comparison of the frequency of this combined genotype among HIV exposed uninfected and HIV-infected subjects revealed no between-group differences. Thus, despite its ability to license NK cells, *KIR3DL1*004/HLA-Bw4* is not associated with a reduced risk of infection.

## 2.2 Introduction:

As observed for other human pathogens, individuals differ in their susceptibility to infection with the human immunodeficiency virus (HIV) and, for those infected, in time to progression to the acquired immune deficiency syndrome (AIDS). Some individuals exhibit HIV resistance, remaining IgG seronegative despite multiple exposures to the virus. A variety of immunologic and genetic factors, differentially expressed in HIV exposed seronegative individuals (HESN) versus control populations, may be important for protection from HIV infection [1;2]. Carriage of certain combined genotypes of natural killer (NK) cell receptors and major histocompatibility complex (MHC) class I (or HLA) ligands appears to protect against HIV infection and slow the rate of HIV disease progression [3-6].

As major effector cells of the innate immune system, NK cells control malignant and virally infected cells and activate dendritic cells (DC), which initiate adaptive immune responses [7;8]. The ability of NK cells to mediate these functions is determined by a process termed licensing, which ensures immunological tolerance to self. Licensing requires the interaction of inhibitory receptors with their ligand to generate functional NK cells that are inhibited by self MHC class I expressed on the surface of normal cells [9-12]. Among the receptors involved in determining NK cell functionality are the killer-cell immunoglobulin-like receptors (KIR). KIR gene products and their HLA ligands may account for observed differences in NK cell functionality, as well as the

observed protective effect of certain KIR/HLA combinations against HIV infection.

Previous work has implicated the *KIR3DL1* locus in protection from HIV infection [3-6]. Different alleles at this locus are activating (i.e., KIR3DS1, henceforth 3DS1) or inhibitory (i.e., KIR3DL1, henceforth 3DL1). 3DL1 alleles are expressed on the cell surface at high (*h) and intermediate/low (*l) levels, or are retained intracellularly (3DL1*004) [13-15]. Co-carriage of the 3DL1 genotype known as *3DL1*h/*y*, which lacks any *l allele, with *HLA-B*57* and homozygosity for *3DS1* are associated with a reduced risk of HIV infection [3;4]. The former KIR/HLA combination also confers elevated NK cell functional potential [16].

There is evidence from epidemiological studies that carriage of *3DL1*004* with *HLA-Bw4* alleles is associated with slower progression to AIDS [6]. The licensing hypothesis predicts that this genotype combination should confer greater NK cell functional potential, as observed with the HESN-associated *3DL1*h/*y/HLA-B*57* combination. We investigated whether NK cells from seronegative individuals carrying *3DL1*004/HLA-Bw4* had higher NK cell functional potential than those from *HLA-Bw6* homozygotes (hmz), who lack the ligand. We also investigated the distribution of *3DL1*004/HLA-Bw4* among HESN and recently infected HIV susceptible individuals.

## 2.3 Materials and Methods

*Study population*

To assess NK cell functional potential we studied 100 HIV seronegative subjects. These included injection drug users (IDU) and seronegative partners of HIV serodiscordant couples. Some (n=49) were classified as HESN based on having at least 5 documented parenteral exposures. Others (n=51) were IDU with insufficient documented HIV exposure for classification as HESN. These subsets were evenly distributed among subjects carrying *3DL1*004/HLA-Bw4* (n=33), *HLA-Bw4* without *3DL1*004* (n=30) and *HLA-Bw6 hmz* (n=37).

In a separate analysis we compared the distribution of carriage of *3DL1*004/HLA-Bw4* among 51 HESN and 261 recently HIV infected *3DL1* hmz enrolled in the Montreal Primary Infection (PI) cohort. Forty six HESN were exposed parenterally with a median (interquartile range) of 14.5 (8, 35) exposures over 3.75 (0.83, 6.12) years; 5 were exposed mucosally for 0.7, 2.2, 6, 15 and 16 years. This analysis focused on *3DL1* hmz to remove any possible confounding effect of carrying the *3DS1* allele at this locus. Informed consent was obtained from all participating subjects and this research conformed to the ethical guidelines of all the author's institutions.

*MHC and KIR typing*

All subjects were typed for *MHC class I* to 4-digit resolution by sequence based typing using kits from Atria Genetics. *KIR3DL/S1* typing was performed as previously described [3;4].

*NK cell activation and staining for phenotype and function*

Frozen peripheral blood mononuclear cells (PBMC) were thawed and resuspended at $10^6$ cells/ml in RPMI 1640 containing 10% fetal bovine serum, 2mM L-glutamine, 50IU/ml penicillin, 50µg/ml streptomycin (Wisent), 5µg/ml brefeldin A (Sigma-Aldrich), 6µg/ml monensin and anti-CD107a antibody (BD Biosciences). One million PBMC were stimulated with (1) HLA-devoid K562 cells at a 5:1 PBMC:K562 ratio, (2) medium alone or (3) PMA (1.25µg/ml); ionomycin (0.25 µg/ml) (Sigma-Aldrich). As previously described, after a 6-hour stimulation cells were stained for viability and cell surface CD3, CD16, CD56 and 3DL1 (using the monoclonal antibody Z27), followed by staining for intracellular Interferon-γ (IFN-γ) and Tumor Necrosis Factor-α (TNF–α) [16]. After washing, samples were fixed with 1% paraformaldehyde (Fisher Scientific) and kept in the dark at $4^oC$ until acquisition.

*Flow cytometry analysis*

After gating on lymphocytes, NK cells were defined as $CD3^-CD56^{+/-}CD16^{+/-}$. Boolean gating was used to determine the tri-functional ($CD107a^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$),

bi-functional (any combination of 2 functions) or mono-functional profile of $3DL1^{+}$ ($Z27^{hi}$), $3DS1^{+}$ ($Z27^{dim}$) and $3DS1^{-}3DL1^{-}$ ($Z27^{-}$) NK cells [17;18]. Between 400,000 and 600,000 total events were acquired with an LSRII flow cytometer (BD) and analyzed using FlowJo version 8.7.1 (TreeStar). Data was corrected for background (unstimulated cells) before statistical analysis.

*Statistics*

Statistical analysis and graphical presentation were performed using GraphPad InStat 3.05 and GraphPad Prism 4.01. Student's T-test or ANOVA with Tukey-Kramer multiple comparisons tests were used for between-group comparisons. The significance of proportional between-group differences in the distribution of KIR/HLA genotypes was tested using the Fisher exact test. P-values $<0.05$ were considered significant.

## 2.4 Results

### Functionality of NK cells from *3DL1*004* carriers

If the 3DL1*004 receptor functionally interacts with HLA-Bw4, the licensing hypothesis predicts that NK cells from *3DL1*004/HLA-Bw4* co-carriers will have higher functionality than *HLA-Bw6* hmz. Because 3DL1*004 is not expressed on the cell surface, NK cells expressing this allele stain $Z27^{-}$. By excluding $Z27^{+}$ NK cells, we excluded the contribution of 3DS1 expressing NK cells and NK cells licensed through other 3DL1-ligand combinations. We compared the K562 stimulated functional potential of $Z27^{-}$ NK cells from subjects who were *3DL1*004/HLA-Bw4* and *HLA-Bw6* hmz for the contribution of each functional response pattern to the total NK cell response. Figure 1A shows a representative example of the gating strategy. Figure 1B shows that the contribution of $Z27^{-}$ tri-functional NK cells to the total K562 stimulated response was significantly greater in NK cells from subjects who were *3DL1*004/HLA-Bw4* (mean ± STD) (3.6% ± 2.7%) versus *HLA-Bw6* hmz (2.2% ± 2.1%) ($p$=0.015, Student's T-test). No significant differences were observed between these 2 study groups for other response patterns, providing a rationale for focusing on tri-functional cells for subsequent analyses. Figure 1C compares the contribution of $Z27^{-}$ K562 stimulated tri-functional NK cells from subjects who were *3DL1*004/HLA-Bw4*, *HLA-Bw6* hmz and who carried *HLA-Bw4* without *3DL1*004*. $Z27^{-}$ NK cells contributed more to total NK cell tri-functionality when from *3DL1*004/HLA-Bw4* carriers than when from either *HLA-Bw6* hmz or carriers of *HLA-Bw4*

without *3DL1*004* (1.8% ± 1.7%) ($p$<0.05 and $p$<0.01 respectively, Tukey-Kramer). Between group comparisons of the frequency of tri-functional $Z27^-$ cells yielded similar conclusions. Together these results show that within the NK subset, which includes cells expressing the intracellular 3DL1*004 receptor, a greater proportion of cells exhibit tri-functionality when from individuals also carrying the HLA-Bw4 ligand compared to those from subjects who express only the ligand or no ligand for 3DL1 alleles.

**The role of the *3DL1*004* allele in protection from HIV infection**

Previous work supports a protective role for carriage of the *3DL1*h/*y/HLA-B*57* genotype against HIV infection and disease progression [3;6]. This KIR/HLA combination also confers higher K562-stimulated functional potential compared to *HLA-Bw6* hmz and carriers of the receptor or ligand alone [16]. We therefore questioned whether the *3DL1*004/HLA-Bw4* genotype was more frequently found in HESN versus PI subjects. Comparison of the distribution of this KIR/HLA genotype, *3DL1*004* alone and *3DL1*004* hmz in 51 HESN and 261 PI subjects who were 3DL1hmz, revealed no proportional between-group differences for any of these genotypes ($p$>0.05, Fisher's exact test) (Table 1).

## 2.5 Discussion

We evaluated the influence of the *3DL1*004/HLA-Bw4* genotype on NK cell functional potential and protection from HIV infection. NK cells from individuals carrying *3DL1*004/HLA-Bw4* had significantly higher functional potential than those from *HLA-Bw6* hmz or carriers of *HLA-Bw4* without *3DL1*004*. Carriage of *3DL1*004/HLA-Bw4*, however, did not protect from HIV infection, as it was similarly distributed among HESN and PI subjects.

For functional studies we focused on NK cells with tri-functional potential. These cells secrete more IFN-γ than bi- or mono-functional cells and may represent NK cells with superior anti-viral activity (unpublished results). The ability of NK cells from *3DL1*004/HLA-Bw4* carriers to exhibit higher levels of K562-stimulated functionality versus *HLA-Bw6* hmz may be one of the factors contributing to slower disease progression in HIV infection. It was not possible to formally attribute increased functionality to the consequences of licensing through 3DL1*004, because there are no antibodies available to label these cells. Therefore, we gated on $Z27^-$ NK cells expressing neither 3DL1 nor 3DS1. Stimulation with K562 activates NK cells with receptors other than 3DL1, which recognize HLA ligands other than Bw4 that would also stain $Z27^-$. This may explain some of the variability in NK cell function observed within each study group. However, assuming there was no significant skewing in the distribution of NK cells expressing receptor/ligand combinations other than *3DL1*004/HLA-Bw4* among the groups studied here, this report provides the first evidence, to our

knowledge, that the 3DL1*004 allele is capable of mediating a licensing interaction with HLA-Bw4, and that a human intracellular inhibitory NK receptor can mediate licensing. How this occurs is not known. However, given that cis interactions between inhibitory receptors and their ligands occur and are important for licensing, it is possible that 3DL1*004 interacts with HLA-Bw4 within an intracellular compartment [19].

Since carriage of *3DL1*h/*y/HLA-B*57* confers protection from both HIV infection and disease progression and licenses NK cells for higher tri-functional potential compared to controls [16], we speculated that increased NK cell functionality plays a role in containing HIV spread soon after transmission to a new host. While the *3DL1*004/HLA-Bw4* genotype combination has been associated with slower progression to AIDS [6] and NK cells from seronegative subjects with this genotype had higher tri-functional potential than those from subjects with control KIR/HLA combinations, carriage of *3DL1*004/HLA-Bw4* was not associated with a reduced risk of HIV infection. Several possibilities may explain why *3DL1*h/*y/HLA-B*57* is associated with protection from infection while *3DL1*004/HLA-Bw4* is not. The percent contribution of tri-functional NK cells from *3DL1*h/*y/HLA-B*57* carriers was higher than it was for *3DL1*004/HLA-Bw4* carriers [16]. In HIV infected subjects the *3DL1*h/*y/HLA-B*57* combination also had a more potent effect than *3DL1*004/HLA-Bw4* genotype on slowing progression to AIDS [6]. Protection from infection may require a higher threshold of NK cell functionality than is achieved by licensing

through 3DL1*004. Alternatively, different licensing interactions may differentially influence distinct effector functions. At an initial threshold, NK cells acquire ability to degranulate; a stronger inhibitory signal is required to achieve cytokine secretion [20]. Therefore, different licensing interactions may promote distinct patterns of effector function and this may explain why only some licensing-competent interactions confer protection from HIV infection. NK cells, as components of the innate immune response, may exert effector functions early enough to prevent the establishment of an infection or later in infection to slow disease progression. The mechanisms underlying NK cell mediated HIV protection are unknown, but of obvious interest. Also unknown is whether the mechanisms responsible for protection are similar to those responsible for slowing disease progression. Further research is needed to address these points.

A limitation of the current study is the use of K562 to stimulate NK cell function. A more physiologically relevant stimulus would be HIV infected cells. Future studies should focus on elucidating the mechanisms used by NK cells mediating anti-HIV activity. A recent assay demonstrating NK cell mediated inhibition of HIV replication provides an ideal tool for such investigations [21]. Another limitation of this study is that we did not assess the impact of co-expression of other 3DL1 alleles with 3DL1*004. Too few subjects carried a second *3DL1* allele that was **l* or **004* hmz to permit statistically relevant comparisons to each other and to those whose second *3DL1* alleles was **h*. However, as we gated on an NK subset that excluded cells expressing *h, *l and

3DS1, it would not be expected that these alleles would differentially influence the functionality of the subset that included those expressing 3DL1*004.

The observation of NK cell licensing through an intracellular receptor is novel. Further studies are required to understand this licensing process and to elucidate the NK cell effector functions important for preventing HIV infection and/or slowing HIV disease progression.

## 2.6 Acknowledgements

The authors wish to acknowledge Mr. Martin Rioux for coordination of the St. Luc Injection Drug User cohort, Mr. Mario Legault for coordination of the Montreal Primary Infection cohort, Mr. Benjamin Tallon, Mr. Saeid Sharafi, Ms. Marianna Kleyman and Ms. Tsoarello Mabanga for technical support.

## 2.7 Figure Captions

**Figure 1.** Z27$^-$ NK cells from *KIR3DL1*004/HLA-Bw4* positive individuals have a distinct functional profile. A, NK cells were gated on from within the CD3$^-$ lymphocyte population. Boolean gating was used to determine the possible combinations of Z27, CD107a, IFN-γ and TNF-α expression on NK cells. B, Demonstration of the percent contribution of seven different possible response patterns to the total K562 stimulated NK cell response for the KIR3DL1/S1 negative subset from individuals carrying *KIR3DL1*004/HLA-Bw4* (n=33) and *HLA-Bw6* hmz (n=37). The response patterns compared are shown below the x-axis. The height of the bars represents the mean and the error bar the standard deviation of the response for that pattern and study population. The significance of between group differences was assessed using Student's T-tests. C, Scatter plot demonstrating the percent contribution of KIR3DL1/S1 negative NK cells to the total K562 stimulated tri-functional response in *KIR3DL1*004/HLA-Bw4*, *HLA-Bw6* hmz and *HLA-Bw4* subjects who were negative for *KIR3DL1*004* (n=30). The line though each study group represents the mean for the group. An ANOVA was used to assess the significance of between-group differences. Significance is indicated by *($p < 0.05$) and ** ($p < 0.01$) over the line linking groups being compared.

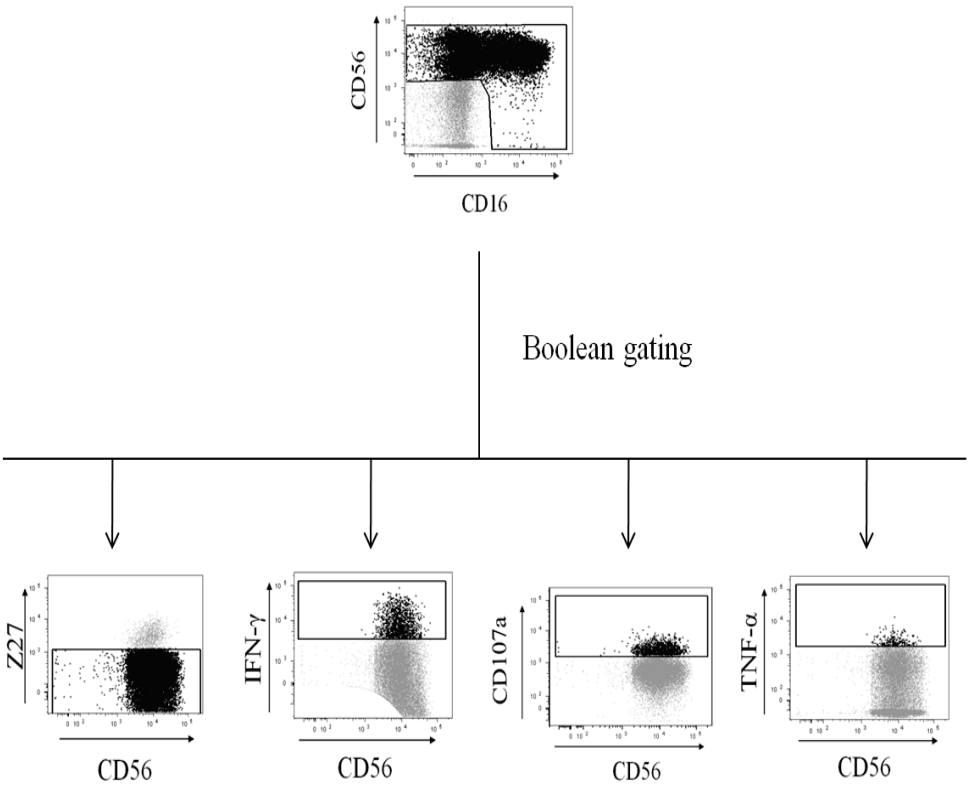
## 2.8 Tables and figures

**Table 1. Frequency of *KIR3DL1*004* in the Primary Infection and HIV-Exposed Seronegative (HESN) Cohorts**

| | No. (%) of individuals by cohort | | |
|---|---|---|---|
| **Genetic Factor** | HESN (n=51) | Primary Infection (n=261) | *p* |
| ****004*** | 22 (43) | 91 (35) | >0.05 |
| ****004 hmz*** | 2 (4) | 10 (4) | >0.05 |
| ****004 +HLA-Bw4*** | 11 (22) | 61 (24) | >0.05 |

Note: hmz, homozygous

**(A)**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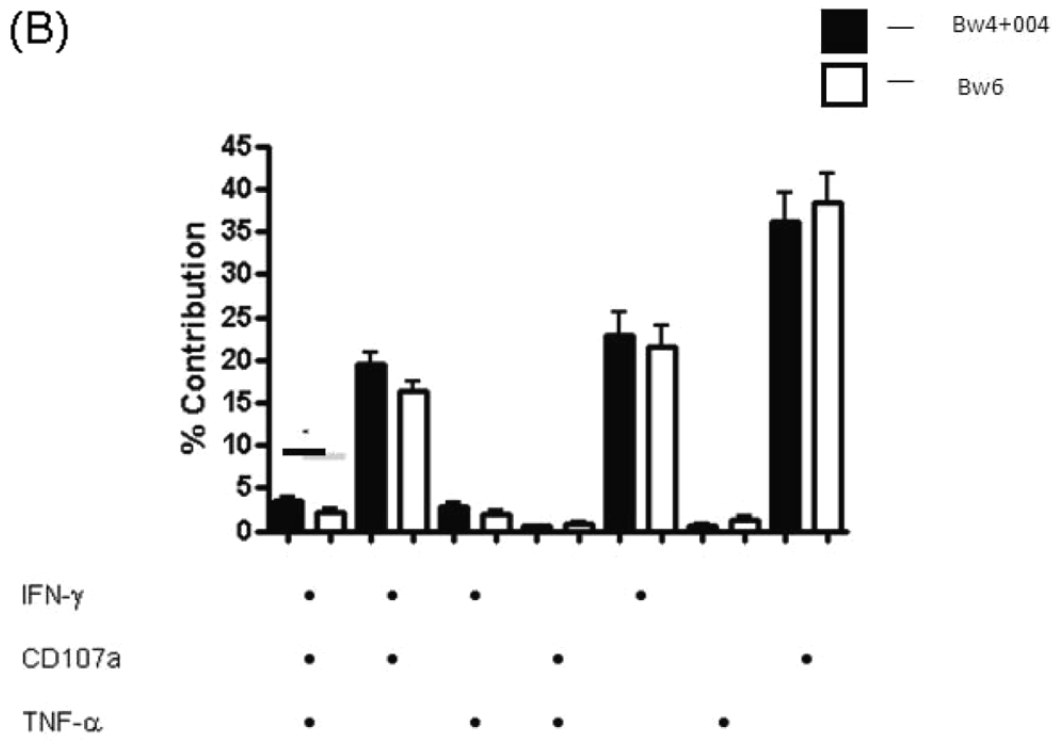
CD56
IFN-γ
CD56
CD107a
CD56
TNF-α
CD56


(B)
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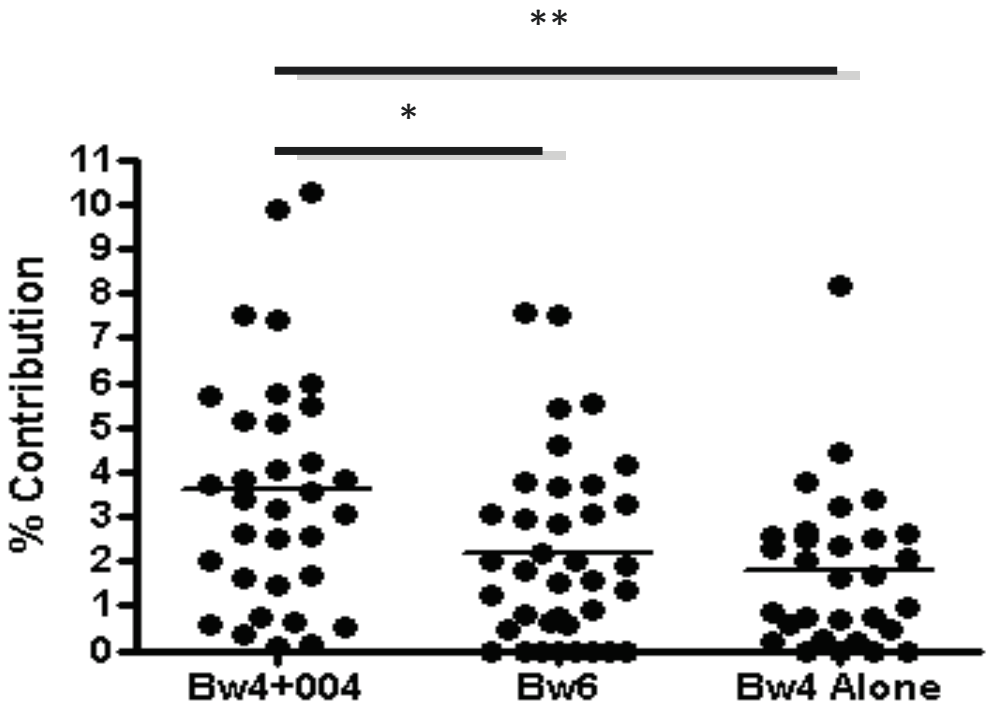
CD107a
TNF-α

**(C)**



## 2.9 References


1 Miyazawa M, Lopalco L, Mazzotta F, et al. The 'immunologic advantage' of HIV-exposed seronegative individuals. AIDS **2009**;23:161-75.

2 Piacentini L, Biasin M, Fenizia C, Clerici M. Genetic correlates of protection against HIV infection: the ally within. J Intern Med **2009**;265:110-24.

3 Boulet S, Kleyman M, Kim JY, et al. A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. AIDS **2008**;22:1487-91.

4 Boulet S, Sharafi S, Simic N, et al. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. AIDS **2008**;22:595-9.

5 Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet **2002**;31:429-34.

6 Martin MP, Qi Y, Gao X, et al. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet **2007**;39:733-40.

7 Sanabria MX, Vargas-Inchaustegui DA, Xin L, Soong L. Role of natural killer cells in modulating dendritic cell responses to Leishmania amazonensis infection. Infect Immun **2008**;76:5100-9.

8 Lanier LL. NK cell recognition. Annu Rev Immunol **2005**;23:225-74.

9 Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature **2005**;436:709-13.

10 Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity **2006**;25:331-42.

11 Kim S, Sunwoo JB, Yang L, et al. HLA alleles determine differences in human natural killer cell responsiveness and potency. Proc Natl Acad Sci U S A **2008**;105:3053-8.

12 Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class I. Immunol Rev **2006**;214:143-54.

13 Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. J Immunol **2003**;171:6640-9.

14 Thomas R, Yamada E, Alter G, et al. Novel KIR3DL1 alleles and their expression levels on NK cells: convergent evolution of KIR3DL1 phenotype variation? J Immunol **2008**;180:6743-50.

15 Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J Exp Med **2006**;203:633-45.

16 Boulet S, Song R, Kamya P, et al. HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. J Immunol **2010**;184:2057-2064.

17 Pascal V, Yamada E, Martin MP, et al. Detection of KIR3DS1 on the cell surface of peripheral blood NK cells facilitates identification of a novel null allele and assessment of KIR3DS1 expression during HIV-1 infection. J Immunol **2007**;179:1625-33.

18 Carr WH, Rosen DB, Arase H, Nixon DF, Michaelsson J, Lanier LL. Cutting Edge: KIR3DS1, a gene implicated in resistance to progression to AIDS, encodes a DAP12-associated receptor expressed on NK cells that triggers NK cell activation. J Immunol **2007**;178:647-51.

19 Chalifour A, Scarpellino L, Back J, et al. A role for cis interaction between the inhibitory Ly49A receptor and MHC class I for natural killer cell education. Immunity **2009**;30: 337-347.

20 Brodin P, Karre K, Hoglund P. NK cell education: Not an on-off switch but a tunable rheostat. Trends Immunol **2009**; 30: 143-149.

21 Alter G, Martin MP, Teigen N, et al. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. J Exp Med **2007**;204:3027-36.

## Bridge from chapter 2 to 3

The data presented in chapter 2 reaffirmed that allelic combinations of *KIR3DL1/HLA-Bw4,* which are associated with protective outcomes in the context of HIV, confer NK cells with enhanced functional potential through the ontological process of NK cell education. Although these observations suggest that protective KIR/HLA combinations may confer protection through enhancement of the ability of KIR3DL1$^{+}$ NK cells to respond to HIV infected-cells, KIR3DL1$^{+}$ NK cells have been shown to be hypofunctional against autologous HIV-infected T cells.

As previous research has demonstrated that NK cells can overcome KIR-induced inhibition to mediate ADCC, we hypothesized that the enhanced activating signalling provided by anti-HIV Abs could allow KIR3DL1$^{+}$ NK cells to respond to autologous anti-HIV ADCC target cells. This hypothesis was evaluated in the experiments described in chapter 3 in both HIV-uninfected and HIV-infected subjects.

# Chapter 3: HIV infection abrogates the functional advantage of natural killer cells educated through KIR3DL1/HLA-Bw4 interactions to mediate anti-HIV antibody-dependent cellular cytotoxicity

# HIV infection abrogates the functional advantage of natural killer cells educated through KIR3DL1/HLA-Bw4 interactions to mediate anti-HIV antibody-dependent cellular cytotoxicity

**Running Title:** NK cell education enhances anti-HIV ADCC

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*None of the authors has a commercial or other association that might pose a potential conflict of interest related to this manuscript.

*This work was supported by National Health and Medical Research Council Grant 510448, Australian Research Council Grant LP0991498, and National Institutes of Health Grant R21AI081541, and by the Australian Centre for HIV and Hepatitis Virology Research, the Royal Australasian College of Physicians, and the Ramaciotti Foundation. MSP is supported by a Vanier Scholarship from the Canadian Institutes for Health Research (CIHR), and is a visiting scholar at the University of Melbourne through a Michael Smith Foreign Study Supplement from the CIHR.

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**3.1 Abstract**

Combinations of *KIR3DL1* and *HLA-Bw4* alleles protect from HIV infection and/or disease progression. These combinations enhance NK cell responsiveness through the ontological process of education. However, educated KIR3DL1$^{+}$ NK cells do not have enhanced degranulation upon direct recognition of autologous HIV-infected cells. Since ADCC is associated with improved HIV infection outcomes and NK cells overcome inhibition through KIR to mediate ADCC, we hypothesized KIR3DL1-educated NK cells mediate anti-HIV ADCC against autologous cells. A whole blood flow cytometry assay was used to evaluate ADCC-induced activation of NK cells. This assay assessed activation (IFNγproduction and/or CD107a expression) of KIR3DL1$^{+}$ and KIR3DL1$^{-}$ NK cells, from HLA-Bw4$^{+}$ and HLA-Bw4$^{-}$ HIV$^{+}$ and HIV$^{-}$ individuals, in response to autologous HIV-specific ADCC targets. KIR3DL1$^{+}$ NK cells were more functional than KIR3DL1$^{-}$ NK cells from HLA-Bw4$^{+}$, but not HLA-Bw4$^{-}$, healthy controls. In HIV-infected individuals, no differences in NK cell functionality were observed between KIR3DL1$^{+}$ and KIR3DL1$^{-}$ NK cells in HLA-Bw4$^{+}$ individuals, consistent with dysfunction of NK cells in the setting of HIV infection. Reflecting the partial normalization of NK cell responsiveness following initiation of anti-retroviral therapy, a significant correlation was observed between the peripheral CD4$^{+}$ T-lymphocyte counts in anti-retroviral therapy treated subjects and the functionality of NK cells. However, peripheral CD4$^{+}$ T-lymphocyte counts were not correlated with an anti-HIV ADCC functional advantage in educated KIR3DL1$^{+}$

NK cells. The abrogation of the functional advantage of educated NK cells may enhance HIV disease progression. Strategies to enhance the potency of NK cell-mediated ADCC may improve HIV therapies and vaccines.

## 3.2 Introduction

A vaccine to protect against infection with the human immunodeficiency virus (HIV) is urgently needed. However, the nature of the most effective immune responses to induce through vaccination is not clear. Attempts to develop antibody (Ab)-based vaccines against HIV have traditionally focused on inducing broadly neutralizing antibodies (BnAbs). Although BnAbs can mediate neutralization of free virions, recent passive transfer studies suggest that their *in vivo* efficacy is in part dependent upon the ability of effector cells of the innate immune system, such as natural killer (NK) cells, to recognize the Ab constant region (Fc) (14). Recognition of the Fc by the NK cell Fc receptor (FcRγIII or CD16) triggers the release of cytokines, chemokines and cytotoxic granules, through a process known as antibody-dependent cellular cytotoxicity (ADCC), which causes the lysis of HIV-infected cells bearing anti-HIV Abs. Several other investigations support a role for ADCC in protection from HIV infection and disease progression, demonstrating the activity at higher levels in HIV exposed seronegative individuals, elite controllers and Rhesus macaques protected through vaccination (9, 19, 29). Moreover, the recent RV144 Thai vaccine trial, which provided partial protection from HIV infection, induced anti-HIV ADCC-competent Abs (35). Coinciding with the increasing evidence for ADCC as a protective anti-HIV immune response, it is also well established that NK cell responsiveness decreases with progressive HIV infection (13, 27), ultimately decreasing the ability of NK cells to mediate ADCC. While HIV infection induces altered NK cell

responsiveness, experiments on NK cells from HIV-uninfected subjects have revealed remarkable variability in the ability of NK cells to mediate anti-HIV ADCC responses (11). A greater understanding of the role of NK cell ontogeny in determining the ADCC functional potential of NK cells could establish ADCC as a correlate of protection against HIV and enhance our knowledge of how to best manipulate these responses to achieve more successful vaccination strategies.

The concept that NK cells are protective against HIV infection and disease progression is supported by a large body of epidemiological evidence that links co-carriage of certain allelic combinations of killer cell immunoglobulin-like receptors (KIR) and their major histocompatibility complex (MHC) class I (or HLA-I) alleles with protection from both infection and/or progression to AIDS (5, 22). In particular, alleles from the *KIR3DL1* (henceforth *3DL1*) locus, which recognize HLA-B molecules carrying the HLA-Bw4 public epitope, have been associated with protection. As these protective *3DL1/HLA-Bw4* combinations have also been demonstrated to confer higher responsiveness upon NK cells expressing the 3DL1 receptor (6, 26), it has generally been assumed that these receptor/ligand combinations mediate protection through direct lysis of HIV-infected cells. Such direct lysis, however, has recently been demonstrated to be unlikely, as $3DL1^{+}$ NK cells do not degranulate upon exposure to autologous infected cells (1). As Ab coated target cells provide additional activating signals, which have been demonstrated to overcome simultaneous inhibitory NK cell receptor signals (20),

we hypothesized that an alternative mechanism of protection is that 3DL1$^{+}$ NK cells demonstrate enhanced anti-HIV ADCC.

The ability of NK cells to mediate effector functions is determined by the cumulative signal generated via the ligation of activating and inhibitory NK cell receptors during the interaction of NK cells with putative target cells. NK cells obtaining similar signals during this interaction, however, will not necessarily mediate equal responses. The strength of NK cell responses is determined by the ligation of activating and inhibitory receptors during NK cell development (15). This ontological process, known as NK cell "education" (or licensing), involves the interaction of activating and inhibitory NK cell receptors with their HLA-I ligands, which can be recognized on the NK cell (*cis*) or on autologous cells (*trans*). In general, NK cell education predicts that NK cells carrying an inhibitory receptor without the HLA-I ligand will be hypofunctional, NK cells co-carrying inhibitory receptors and their HLA-I ligands will mediate higher functionality and NK cells co-carrying activating receptors and their HLA-I ligands will demonstrate decreased functionality. This process, however, does not appear to be a simple "on-off" procedure, as the total input from all inhibitory and activating receptors contributes to the education of NK cells. Recent research demonstrates that stronger cumulative inhibitory signals during NK cell education prepare NK cells to mediate stronger effector functions against appropriate target cells (10). As such, NK cells appear to be regulated by two separate processes involving ligation of inhibitory and activating receptors. The first of these processes

determines NK cell functional potential, while the second determines whether the NK cell will respond to potential target cells.

The establishment of NK cell education as the regulatory mechanism for determining the functional potential of NK cells offers a putative explanation for how certain allelic combinations of *3DL1* and *HLA-Bw4* protect from HIV infection and progression to AIDS. As NK cells educated through interactions between 3DL1 and HLA-Bw4 have higher functional potential, it seemed probable that $3DL1^+$ NK cells could more efficiently recognize and destroy autologous HIV-infected cells, which downregulate HLA-B alleles (12). Previous research, however, has demonstrated $3DL1^+$ NK cells fail to degranulate in response to autologous HIV-infected cells (1), making direct NK cell killing of HIV infected cells an unlikely mechanism of the protective *3DL1/HLA-Bw4* effect. Activating signals through CD16 can, however, overcome inhibitory signals through KIR to facilitate NK cell activation (20). As such, we hypothesized that NK cells educated through 3DL1 mediate potent anti-HIV ADCC against autologous $HLA\text{-}Bw4^+$ target cells. A prediction of this hypothesis is that $3DL1^+$ NK cells are a more functional ADCC-mediating NK cell subset in individuals also carrying the HLA-Bw4 ligand. We used an intracellular cytokine staining (ICS) ADCC assay to evaluate whether this was indeed the case. Whole blood, from $HLA\text{-}Bw4^+$ and $HLA\text{-}Bw4^-$ subjects, was cultured with HIV envelope antigens and anti-HIV ADCC-competent Abs. NK cells from both HIV-negative and HIV-positive donors

responding to ADCC antibodies were analyzed for 3DL1 expression, cytokine production and degranulation.

## 3.3 Materials and Methods

### Study population

Whole blood was collected into vacutainers containing sodium heparin anti-coagulant from 19 HIV-uninfected healthy control individuals and 27 HIV-infected individuals recruited through the Melbourne Sexual Health Clinic. All participants were screened for 3DL1 expression with PE-conjugated DX9 monoclonal Ab (BD Biosciences), which detects inhibitory 3DL1 alleles that are expressed on the NK cell surface (32). Expression of 3DL1 was detected in 27/27 HIV-infected subjects and 17/19 healthy control subjects. As 3DL1-mediated education could not be evaluated in the 2 $DX9^-$ subjects, they were excluded from all subsequent analyses. *HLA-B* typing was performed by the Victorian Transplant and Immunogenetics Service (Parkville, Australia), using sequence-based typing. Supplementary table 1 (Appendix) provides the *HLA-B* typings of the HIV-uninfected healthy controls and HIV-infected participants. All subjects provided informed consent for participating in this study and all participating study sites approved this study.

### Anti-HIV ADCC ICS assay

A whole blood ICS assay was used to assess NK cell activation by ADCC Abs (30). Briefly, 200µl of sodium-heparinized HIV-infected whole blood, or 150µl

of healthy control whole blood plus 50µl of ADCC-competent HIV-infected plasma, was incubated at 37$^{o}$C for 5 hours with 1µg/ml of HIV Env peptide pool, Brefeldin A (5µg/ml) (Sigma) and Monensin (6µg/ml) (Sigma). ADCC responses were assessed using a peptide pool containing 15-mers that overlapped by 11 amino acids. This peptide pool spanned the HIV-1 consensus subtype B Env protein (NIH AIDS Reagent Repository). Control incubations of whole blood with just peptides or just HIV-infected plasma were also set up to confirm that any observed functionality was ADCC. After the incubation, cells were surface stained with Per-CP-conjugated anti-CD3, FITC-conjugated anti-CD2, PE-conjugated anti-KIR3DL1, PE-Cy7-conjugated anti-CD56 and APC-conjugated anti-CD107a (All from BD Biosciences). Next, whole blood was treated with lysing solution (BD Biosciences) to remove red blood cells, and the remaining white blood cells were treated with permeabilization solution (BD Biosciences) and stained with Alexa700-conjugated anti-IFNγ antibody (BD Biosciences). Flow cytometry data was collected using a FACS Canto II Flow cytometer (BD Biosciences), and was analyzed using Flow Jo Version 9.2 software (Tree Star).

The role of antibody-coated target cells and immune complexes in stimulating NK cells in this assay was evaluated by assessing the activation of enriched NK cells, which were exposed to HIV Env peptides and ADCC-competent plasma under the same conditions as the whole blood assay. NK cells were enriched by negative selection using the Easysep human NK cell enrichment kit

(Stemcell Technologies). After using the kit as suggested by the manufacture, populations of cells consisting of greater than 90% NK cells were obtained.

**Statistical analysis**

Data analyses were performed using GraphPad Prism Version 4.0 software. Wilcoxon matched pairs tests were used to compare within group differences in the functionalities of $3DL1^+$ and $3DL1^-$ NK cell subsets in HLA-$Bw4^+$ and HLA-$Bw4^-$ groups. Between group differences, in the functionality of $3DL1^+$ NK cells between HLA-$Bw4^+$ and HLA-$Bw4^-$ groups, were assessed using Mann-Whitney tests. Spearman correlation coefficients were used to assess correlations between clinical and functional data.

## 3.4 Results

### HIV-specific ADCC activity of NK cells

Although NK cells from healthy controls and HIV-infected individuals can mediate efficient ADCC against autologous target cells in the presence of HIV Env antigens and anti-HIV Abs, it is unknown which NK cells are most potently activated by these ADCC Abs. While educated $3DL1^+$ NK cells mediate enhanced natural cytotoxicity and ADCC against non-self target cells (18, 28), it is unknown if these NK cells can mediate ADCC against autologous target cells that would induce an inhibitory signal through the interaction between 3DL1 and its HLA-Bw4 ligand. To evaluate whether 3DL1-educated NK cells mediate anti-HIV ADCC, we employed a whole blood ADCC ICS assay to examine the ADCC mediated by NK cells from healthy controls and HIV-infected individuals. This assay assesses the activation of $CD56^+CD2^+$ NK cells as measured by IFNγ synthesis and CD107a expression (Fig. 1). That the NK cell activation observed is ADCC and is induced by the recognition of anti-HIV antibody-coated target cells is demonstrated by the necessity of both HIV Env peptides and ADCC-competent plasma for NK cell activation (Fig. 1B). Target cells are necessary to present these peptide antibody combinations, as enriched NK cells alone are not activated in the presence of peptides and ADCC-competent plasma (Fig. 1C). Furthermore, this activation induces immense CD16 downregulation, which is a common characteristic of stimulation through CD16 (7). Corroborating previous data (11), NK cells from both healthy controls and HIV-infected individuals mediated efficient ADCC

against autologous target cells. Despite the well-known inhibitory nature of the 3DL1/HLA-Bw4 interaction (21), $3DL1^+$ NK cells from $HLA\text{-}Bw4^+$ individuals were observed to mediate efficient anti-HIV ADCC against $HLA\text{-}Bw4^+$ autologous target cells. Interestingly, when we compared the $3DL1^+$ and $3DL1^-$ NK cells from an $HLA\text{-}Bw4^+$ healthy control, we observed that the $3DL1^+$ NK cells were the more responsive subset.

**Impact of NK cell education on anti-HIV ADCC functionality in healthy controls**

NK cells educated through 3DL1/HLA-Bw4 interactions have been previously demonstrated to exhibit higher cytokine production and degranulation against non-self target cells than $3DL1^-$ NK cells from $HLA\text{-}Bw4^+$ individuals (6). Our initial results (Fig. 1A) suggested that educated $3DL1^+$ NK cells mediate robust anti-HIV ADCC against $HLA\text{-}Bw4^+$ autologous target cells. We therefore recruited a total of 11 $HLA\text{-}Bw4^+$ healthy control subjects to investigate more thoroughly whether $3DL1^+$ NK cells were more functional than $3DL1^-$ NK cells from within $HLA\text{-}Bw4^+$ individuals. As a control, we also recruited 6 HLA-Bw6 homozygous subjects, who do not carry HLA-Bw4 alleles and would not be expected to demonstrate NK cell education through 3DL1. The whole blood anti-HIV ADCC ICS assay demonstrated $3DL1^+$ NK cells to be a more functional NK cell subset than $3DL1^-$ NK cells in HLA-Bw4 carriers. In the HLA-Bw4 carriers significantly higher mean percentages of the $3DL1^+$ than $3DL1^-$ NK cell subset

produced IFNγ (20+/-2.9 vs. 14.2+/-2.3, $p<0.01$) and/or expressed the CD107a degranulation marker (34.8+/-3.2 vs. 25.9+/-2.9, $p<0.01$, Fig. 2A). In HLA-Bw6 homozygous individuals no difference was observed in IFNγ production (10.1+/-2.3 vs. 8.5+/-2.1, ns) or CD107a expression (22.7+/-4.4 vs. 20.3+/-4.1, ns) between the $3DL1^+$ and $3DL1^-$ NK cells (Fig. 2A). These results demonstrate that NK cell education through 3DL1/HLA-Bw4 interactions enhances the anti-HIV ADCC functionality of $3DL1^+$ NK cells against $HLA\text{-}Bw4^+$ autologous target cells.

Previous research has demonstrated that polyfunctional cell-based immune responses are associated with better control of viral infections. Indeed, this has been demonstrated in the context of HIV infection for both cytotoxic T-lymphocytes and NK cells from individuals with protective KIR/HLA allelic combinations (6, 26, 31). We investigated whether the functional advantage of $3DL1^+$ NK cells, as compared to $3DL1^-$ NK cells, from HLA-Bw4 carriers was observed for IFNγ and CD107a monofunctionality and bifunctionality. The functional advantage of the $3DL1^+$ NK cell subset was largely restricted to bifunctional responses ($CD107a^+$ $IFN\gamma^+$). While $3DL1^+$ and $3DL1^-$ NK cells demonstrated similar monofunctional CD107a ($CD107a^+$ $IFN\gamma^-$) and IFN-γ ($CD107a^-$ $IFN\gamma^+$) responses, a significantly higher percentage of $3DL1^+$ NK cells mediated bifunctional responses than $3DL1^-$ NK cells (Table 1). It should be noted that this difference was still significant after the Bonferroni correction was applied. A similar comparison of monofunctional and bifunctional $3DL1^+$ and $3DL1^-$ NK cell responses in HLA-Bw6 homozygotes revealed no significant

differences in any of the functional permutations (Table 1). These data demonstrate that NK cell education enhances the anti-HIV ADCC function of 3DL1$^{+}$ NK cells, and that this enhanced function is driven primarily by an increased ability of 3DL1-educated NK cells to mediate multiple functions simultaneously.

NK cell education through 3DL1 and HLA-Bw4 interactions has been demonstrated to induce higher responsiveness against non-self target cells in 3DL1$^{+}$ NK cells than 3DL1$^{-}$ NK cells from HLA-Bw4 subjects, and drives higher responsiveness in 3DL1$^{+}$ NK cells from HLA-Bw4$^{+}$ subjects than HLA-Bw6 homozygotes (6, 18, 28). Given that our results demonstrated higher bifunctional responses in 3DL1$^{+}$ NK cells than 3DL1$^{-}$ NK cells in HLA-Bw4$^{+}$ individuals, but not in HLA-Bw6 homozygotes, we hypothesized that 3DL1$^{+}$ NK cells would be more responsive to autologous ADCC target cells in HLA-Bw4$^{+}$ individuals than in HLA-Bw6 homozygotes. Indeed, HLA-Bw4$^{+}$ individuals did have overall higher frequencies of 3DL1$^{+}$ NK cells capable of mediating bifunctional anti-HIV ADCC compared to HLA-Bw6 homozygotes (18.0+/-2.8 vs. 8.9+/-2.3, $p<0.05$) (Fig. 2B). Cumulatively, these results demonstrate that NK cell education enhances the ability of NK cells to mediate anti-HIV ADCC against autologous target cells.

**The effect of HIV infection on the impact of NK cell education on the relative anti-HIV ADCC functionalities of 3DL1$^+$ and 3DL1$^-$ NK cells**

Numerous studies demonstrate that HIV infection has a detrimental effect on NK cell functional potential (13, 23, 33). Given that NK cell education determines the functional potential of NK cells, it might be expected that HIV-infected individuals would experience a decrease in the functionality of NK cells educated through 3DL1 and HLA-Bw4 interactions. As such, we recruited 27 HIV-infected subjects to evaluate ADCC activity in NK cell subsets. This unselected heterogenous cohort included consecutive subjects attending an outpatient clinic and included viremeic and aviremic patients at different stages of disease progression and treatment status (Supplementary Table 1 - Appendix). We used a whole blood anti-HIV ADCC ICS assay to evaluate whether HIV$^+$ HLA-Bw4$^+$ individuals maintain a functional advantage in their 3DL1$^+$ NK cells. HIV$^+$ HLA-Bw6 homozygous individuals were also evaluated as a control group. No difference in bifunctional anti-HIV ADCC responses was observed between the 3DL1$^+$ and 3DL1$^-$ NK cell subsets from either HLA-Bw4$^+$ (8.8+/-2.3 vs. 9.2+/-1.9, ns) or HLA-Bw6 homozygous (10.2+/-2.9 vs. 12.6+/-4.4, ns) groups (Fig. 3A). It should also be known that no differences were observed in monofunctional IFNγ or monofunctional CD107a responses between the 3DL1$^+$ and 3DL1$^-$ NK cell subsets from HLA-Bw4$^+$ (1.03+/-0.33 vs. 1.16+/-0.39, ns; 6.42+/-1.29 vs. 7.23+/-1.15, ns) or HLA-Bw6 homozygous (0.05+/-0.05 vs. 0.21+/-0.11, ns; 8.54+/-1.95 vs. 8.54+/-1.63, ns) groups (data not shown). Although some HLA-Bw4$^+$ individuals were

found to have higher bifunctionality in their 3DL1$^{+}$ NK cell subset than in their 3DL1$^{-}$ NK cells, the population as a whole demonstrates no functional advantage in their 3DL1$^{+}$ NK cells. Coinciding with this observation, no difference was observed in the frequency of bifunctional 3DL1$^{+}$ NK cells in HLA-Bw4 carriers and HLA-Bw6 homozygous individuals (8.8+/-2.3 vs. 10.2+/-2.9, ns) (Fig. 3B). These results suggest that HIV-infection has a negative impact on the ADCC functional advantage of NK cells educated through the interaction of 3DL1 and HLA-Bw4.

**Clinical correlates of post-HAART NK cell functionality**

NK cell responsiveness decreases with progressive HIV infection and subsequent immunodeficiency (13, 23, 33). We studied the relationship between 3DL1 expression and ADCC activity of NK cells from HIV-infected HLA-Bw4 carriers. Our results demonstrate a loss of the functional advantage of educated 3DL1$^{+}$ NK cells in both viremic and aviremic HIV$^{+}$ individuals (Fig. 3 and data not shown). As the majority of the HLA-Bw4$^{+}$ HIV-infected subjects recruited were on effective HAART and had undetectable viral loads (Supplementary Table 1 - Appendix), subsequent analyses of the impact of disease progression on the function of NK cells focused on the latest peripheral CD4$^{+}$ T-lymphocyte count. We hypothesized that the relative normalization of NK cell functionality observed after immune reconstitution following the initiation of HAART would result in a reestablishment of the functional advantage within the educated NK

cell subset. We therefore expected to see a correlation between treatment efficacy, as measured by counts of peripheral $CD4^+$ T-lymphocytes, and the bifunctionality of total NK cells. We also expected to observe a correlation between the counts of peripheral $CD4^+$ T-lymphocytes and the NK cell education ratio in HLA-$Bw4^+$ subjects (i.e., bifunctionality observed in $3DL1^+$ NK cells:bifunctionality observed in $3DL1^-$ NK cells). As such, we calculated the percentage of bifunctional total NK cells and the NK cell education ratio for the HLA-$Bw4^+$ treated and aviremic $HIV^+$ individuals and correlated these values with the counts of peripheral $CD4^+$ T-lymphocytes. A significant correlation was observed between the percentage of bifunctional total NK cells and the count of peripheral $CD4^+$ T-lymphocytes (R=0.57, $p<0.05$) (Fig. 4A). However, no significant correlation was observed between the NK cell education ratio and the $CD4^+$ T-lymphocyte counts within these patients (R=0.36, $p>0.05$) (Fig. 4B). It should be noted that a similar pattern was observed when we correlated the increase in $CD4^+$ T-lymphocyte counts with the percentage of bifunctional total NK cells (R=0.52, $p<0.05$) and the NK cell education ratio (R=0.26, $p>0.05$) (data not shown). These data demonstrate that the post-HAART counts of peripheral $CD4^+$ T-lymphocytes are associated with a partial normalization of NK cell functionality. However, the partial reconstitution of immune competence on HAART is not associated with a full restoration of an anti-HIV ADCC functional advantage for educated $3DL1^+$ NK cells over $3DL1^-$ NK cells.

## 3.5 Discussion

Several epidemiological studies demonstrate an association between co-carriage of several *3DL1* and *HLA-Bw4* alleles and protection from HIV infection and/or disease progression (5, 22). Although these *3DL1/HLA-Bw4* combinations increase the responsiveness of $3DL1^+$ NK cells against HLA-devoid target cells (6, 26), $3DL1^+$ NK cells do not degranulate upon direct exposure to autologous HIV infected cells (1). As such, the mechanism of *3DL1/HLA-Bw4* conferred protection remains undefined. In this study, we evaluated the role of 3DL1-driven NK cell education in anti-HIV ADCC activity to autologous target cells. A simple and robust whole blood ICS assay was employed to assess the activation of NK cell subsets from both HIV-uninfected healthy controls and a cohort of HIV-infected patients. This experimental system involves the combination of whole blood with HIV Env peptides and plasma from an HIV-infected source. This results in presentation of HIV peptides, in an as of yet non-elucidated mechanism, by target cells, which become coated with anti-HIV Abs and activate NK cells for ADCC (Stratov et al. Manuscript in preparation). As these targets are nucleated cells that express surface HLA-I, this assay serves to evaluate the ability of NK cells to mediate anti-HIV ADCC in a self-HLA-I environment. Our results demonstrate that educated NK cells from healthy controls mediate responses against autologous target cells, which express HLA-Bw4 ligands that have a known inhibitory effect upon binding inhibitory KIRs (21). Not only were educated NK cells capable of responding to autologous ADCC targets, they

represented a more responsive subset. This study also linked functional alterations in NK cells from HIV-infected individuals to the abrogation of the functional benefit of educated NK cells. Further, we demonstrated that although successful HAART partially restores the function of total NK cells, treated aviremic individuals, regardless of peripheral $CD4^+$ T-lymphocyte counts, still exhibit a loss of the functional advantage of educated $3DL1^+$ NK cells for mediating anti-HIV ADCC.

This study provides the first evidence that educated human NK cells are a more functional NK cell subset than non-educated NK cells against autologous ADCC target cells. Although this observation coincides with previous research demonstrating educated NK cells are more responsive to Ab-coated murine P815 cells and Ab-coated heterologous EBV-transformed human B-lymphocytes (2, 28), the results contrast from previous evidence of non-responsive educated NK cells in the context of infections. For example, a recent investigation of the role of educated NK cells in the context of murine cytomegalovirus demonstrated that non-educated NK cells were the major effector cells in infected mice (25). Corroborating this observation is the demonstration, in humans, that $3DL1^+$ NK cells do not mediate Ab-independent degranulation against autologous HIV-infected target cells (1). Since previous research has demonstrated that Ab-dependent NK cell responses can overcome inhibitory signals through inhibitory KIR to mediate functionality (20), we investigated if educated NK cells represented a more functional subset in the context of Ab-dependent responses

against autologous target cells. Indeed, our results demonstrated that Ab-dependent responses can overcome inhibitory signals, and that educated NK cells preferentially mediate Ab-dependent responses against autologous target cells. This observation is particularly intriguing, as the ADCC measured in our assay was mediated against target cells that did not have altered HLA-B expression. Although previous work has suggested that autologous HIV-infected target cells need to downregulate HLA-B to be susceptible to NK cell-mediated functions (3), our results suggests that with optimal stimulation NK cells can be triggered to mediate effector functions against autologous target cells with normal HLA-B expression.

Our results also provide the first evidence linking the NK cell dysfunction observed in HIV infection with an abrogation of the functional advantage of educated NK cells mediating ADCC effector functions. Although higher bifunctional responses were observed in the educated $3DL1^+$ NK cell subset of HIV-uninfected HLA-$Bw4^+$ healthy controls than in their $3DL1^-$ subset, no significant difference in bifunctional responses was observed between these NK cell subsets in HLA-$Bw4^+$ HIV-infected individuals. Coinciding with this observation, it was also noted that while HIV-uninfected HLA-$Bw4^+$ healthy controls demonstrate a higher proportion of bifunctional $3DL1^+$ NK cells than HIV-uninfected HLA-Bw6 homozygous healthy controls, no significant difference was observed between bifunctional $3DL1^+$ responses in HIV-infected HLA-$Bw4^+$ and HLA-Bw6 homozygous individuals. Several NK cell abnormalities have been

noted in HIV infection, including a decrease in total functionality and NK cell-mediated destruction of uninfected $CD4^+$ T-lymphocytes (13, 23, 33, 34). The abrogated function of educated NK cells observed in this study may reflect an overall reduction in NK cell functionality. Although it is unknown how HIV infection decreases the function of educated $3DL1^+$ NK cells, it is possible that recently described escape peptides that abrogate the 3DL1/HLA-Bw4 interaction could interfere with NK cell education (8). Alternatively, the alteration of educated $3DL1^+$ NK cell functionality may reflect an infection-induced increase in the function of non-educated NK cells that would be more likely to mediate the destruction of uninfected autologous $CD4^+$ T-lymphocytes. Lastly, alterations to other NK cell receptors that influence NK cell education could contribute the dysfunction observed in educated $3DL1^+$ NK cells. However, this situation is unlikely, as the dysfunction in educated $3DL1^+$ NK cells was observed in both treated and untreated HIV-infected subjects, and successful treatment of HIV infection has been reported to normalize the expression levels of most receptors that exhibit altered expression due to viremia (13). Indeed, we investigated expression of one such receptor, NKG2A, on total NK cells and $3DL1^+$ NK cells from HIV-uninfected and HAART treated HIV-infected subjects, and observed no differences between the groups (data not shown). Future studies using serial samples from HIV-infected individuals, prior to and following initiation of HAART, will help further clarify these observations.

HIV infection abrogated the functional advantage of educated NK cells. A correlation between the effectiveness of immune reconstitution following anti-retroviral treatment, measured as $CD4^+$ T-lymphocyte counts, with the bifunctionality of total NK cells demonstrated that more effective control of HIV disease was associated with higher NK cell functionality. However, the lack of correlation between treatment efficacy and the NK cell education ratio is interesting, as it may provide a partial explanation for the higher incidence of non-AIDS defining malignancies (NADMs) in HAART-treated HIV-infected individuals than in the general population (24). Future studies using larger sample sizes and following subjects over time will help elucidate the factors determining why some HIV-infected individuals exhibit normal NK cell education ratios, and the potential role of these ratios on susceptibility to NADMs.

Although the present study demonstrates that NK cells educated through 3DL1/HLA-Bw4 interactions can mediate potentially beneficial anti-HIV ADCC responses against autologous target cells, it is conceivable that the similar activity of 3DL1-educated NK cells in the presence of autoantibodies or uninfected cells carrying viral proteins could mediate immunopathology. As HIV infected individuals often carry anti-lymphocyte Abs (4) and potentially demonstrate soluble gp120 binding to uninfected $CD4^+$ T-lymphocytes (16), future research needs to evaluate whether 3DL1-educated NK cells are functional in these potentially pathological situations. It is essential to establish which educated NK cell-mediated effector functions are truly beneficial and

which are potentially pathogenic before such effector functions can be targeted as therapies and/or vaccines to HIV-infected individuals or individuals at risk.

Demonstrating educated NK cells mediate anti-HIV ADCC against autologous target cells is essential to understanding the NK cell education process and its potential implications for vaccines and therapies. While this study evaluated the anti-HIV ADCC activity of NK cells educated through the epidemiologically interesting 3DL1/HLA-Bw4 interactions (22), future research should investigate the impact of other HLA/KIR combinations and the cumulative HLA/KIR phenotype of NK cells on the ability of NK cells to mediate autologous anti-HIV ADCC. Interestingly, Kamya *et al* recently showed that while *HLA-Bw4/KIR3DL1* combinations contribute to the ability of NK cells to be activated by HLA-devoid K562 cells, combinations of *HLA-C/KIR2DL1/2/3* did not contribute to the ability of NK cells to be activated by this stimulation (17). In contrast, both *HLA-Bw4/KIR3DL1* and *HLA-C/KIR2DL1/2/3* combinations contributed to the ability of NK cells from healthy controls to be activated by K562 stimulation. These results illustrate the importance of future research investigating other HLA/KIR interactions that may be important in the context of NK cell-mediated ADCC. Furthermore, it will be necessary to elucidate the effector functions mediated by educated NK cells that are important for viral control. Such a detailed understanding of the impact of NK cell education on anti-HIV ADCC is essential for understanding NK cell anti-viral responses, and for the design of more effective ADCC-inducing HIV vaccines.

### 3.6 Acknowledgements

We wish to acknowledge nurses J Silvers and H Kent and Drs M Chen, R Moore, J Sasadeusz, C Fairley, T Schmidt, C Bradshaw, T Read, from the Melbourne Sexual Health Centre, and Christos M Tsoukas, from the McGill University Health Center, for their assistance in collecting clinical samples. We are also grateful to all of the study's participants.

### 3.7 Figure captions

**Figure 1. NK cell-mediated Anti-HIV ADCC responses.** (A) $CD3^-$ lymphocytes were gated on. Flow cytometry plots illustrate the gating strategy used to identify the anti-HIV ADCC responses of $3DL1^+$ and $3DL1^-$ NK cells. The gating strategy is illustrated for an HIV negative donor and an HIV infected donor. (B) Dot plots illustrate the requirement for HIV Env peptide and plasma-derived anti-HIV antibodies for NK cell activation in the whole blood assay. (C) Dot plots depict a representative example of 3 experiments assessing the ability of NK cells within a whole blood, HIV Env peptide, and HIV-infected plasma culture to express CD107a and produce IFNγ as compared to enriched NK cells incubated with HIV Env peptides and HIV-infected plasma.

**Figure 2. Anti-HIV ADCC responsiveness of $3DL1^+$ and $3DL1^-$ NK cells from HLA-$Bw4^+$ and HLA-Bw6 homozygous HIV-uninfected healthy controls.** (A) The graphs on the left illustrate the relative abilities of $3DL1^+$ and $3DL1^-$ NK cell subsets from HLA-$Bw4^+$ individuals to produce IFNγ and express CD107a. Graphs on the right illustrate the relative abilities of $3DL1^+$ and $3DL1^-$ NK cell subsets from HLA-Bw6 homozygous individuals to produce IFNγ and express CD107a. The significance of within-group differences of $3DL1^+$ and $3DL1^-$ NK cell subset responses were assessed with Wilcoxon matched pairs tests. (B) The scatter plot depicts the relative anti-HIV ADCC bifunctional (i.e., $CD107a^+IFN\gamma^+$) activity of the

3DL1$^{+}$ NK cell subset in HLA-Bw4$^{+}$ and HLA-Bw6 homozygous individuals. The significance of the between-group difference of responses from 3DL1$^{+}$ NK cells originating from HLA-Bw4$^{+}$ and HLA-Bw6 homozygous individuals was assessed with a Mann-Whitney test.

**Figure 3. Anti-HIV ADCC responsiveness of 3DL1$^{+}$ and 3DL1$^{-}$ NK cells from HLA-Bw4$^{+}$ and HLA-Bw6 homozygous HIV-infected individuals.** (A) The graph on the left illustrates the relative abilities of 3DL1$^{+}$ and 3DL1$^{-}$ NK cell subsets from HLA-Bw4$^{+}$ individuals to mediate bifunctional anti-HIV ADCC responses (IFN$\gamma^{+}$CD107a$^{+}$). The graph on the right illustrates the relative abilities of 3DL1$^{+}$ and 3DL1$^{-}$ NK cell subsets from HLA-Bw4$^{+}$ individuals to mediate bifunctional anti-HIV ADCC responses (IFN$\gamma^{+}$CD107a$^{+}$). The significance of within-group differences in responses of 3DL1$^{+}$ and 3DL1$^{-}$ NK cell subsets were assessed with Wilcoxon matched pairs tests. (B) The scatter plot depicts the relative anti-HIV ADCC bifunctional (i.e., CD107a$^{+}$IFN$\gamma^{+}$) activity of the 3DL1$^{+}$ NK cell subset from HLA-Bw4$^{+}$ and HLA-Bw6 homozygous individuals. The significance of the between-group difference in responses of 3DL1$^{+}$ NK cell subsets from HLA-Bw4$^{+}$ and HLA-Bw6 homozygous individuals was assessed with a Mann-Whitney test.

**Figure 4. The relationship between HAART efficacy and the functionalities of total and 3DL1-educated NK cells of HIV-infected HLA-Bw4$^{+}$ individuals. (A)** The graph shows the relationship between the post-HAART counts of peripheral CD4$^{+}$ T-lymphocytes and the percentage of bifunctional total NK cells in post-HAART aviremic HLA-Bw4$^{+}$ individuals. (B) The graph shows the relationship between the post-HAART counts of peripheral CD4$^{+}$ T-lymphocytes and the ratio of bifunctionality between 3DL1$^{+}$ and 3DL1$^{-}$ NK cells in post-HAART aviremic HLA-Bw4$^{+}$ individuals. The strength of the relationships were evaluated with Spearman correlation coefficients.

### 3.8 Tables and figures

Table 1. Impact of NK cell education on anti-HIV ADCC functional profile.

| Healthy control group (Functional profile) | 3DL1$^{+}$ (Mean$^{*}$+/-SE) | 3DL1$^{-}$ (Mean+/-SE) | $P^{\dagger}$ |
|---|---|---|---|
| HLA-Bw4 (IFNγ$^{+}$CD107a$^{-}$) | 2.2 +/- 0.5 | 1.7 +/- 0.3 | 0.23 |
| HLA-Bw4 (IFNγ$^{-}$CD107a$^{+}$) | 16.8 +/- 2.7 | 13.4 +/- 1.8 | 0.07 |
| HLA-Bw4 (IFNγ$^{+}$CD107a$^{+}$) | 18.0 +/- 2.8 | 12.5 +/- 2.3 | 0.01 |
| HLA-Bw6 (IFNγ$^{+}$CD107a$^{-}$) | 1.2 +/- 0.3 | 1.3 +/- 0.3 | 0.84 |
| HLA-Bw6 (IFNγ$^{-}$CD107a$^{+}$) | 13.8 +/- 2.8 | 13.1 +/- 2.8 | 0.31 |
| HLA-Bw6 (IFNγ$^{+}$CD107a$^{+}$) | 8.9 +/- 2.3 | 7.2 +/- 1.9 | 0.22 |

$^{*}$ Mean percentage of NK cell subsets expressing effector molecules is shown.
$^{\dagger}$ p values obtained from Wilcoxon matched pairs tests.

**Figure 1**

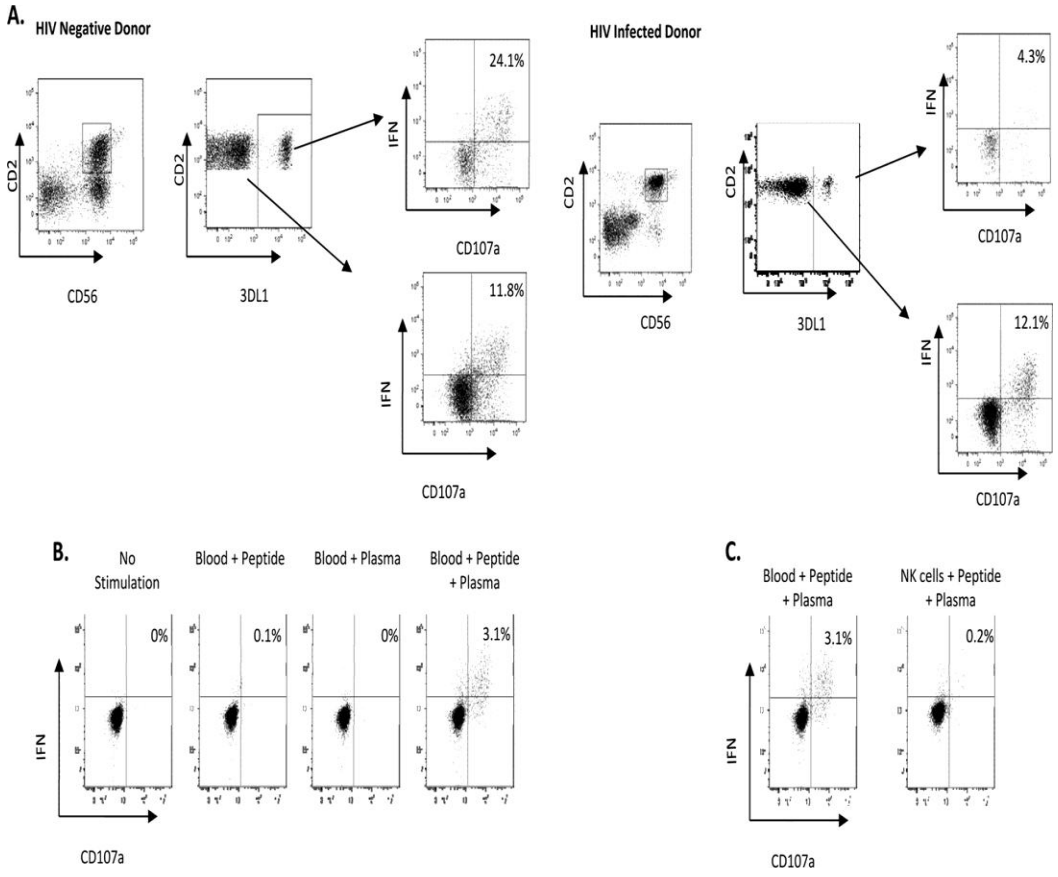

A.
HIV Negative Donor
CD2
CD56
CD2
3DL1
24.1%
IFN
CD107a
11.8%
IFN
CD107a
HIV Infected Donor
CD2
CD56
CD2
3DL1
4.3%
IFN
CD107a
12.1%
IFN
CD107a
B.
No Stimulation
Blood + Peptide
Blood + Plasma
Blood + Peptide + Plasma
0%
0.1%
0%
3.1%
IFN
CD107a
C.
Blood + Peptide + Plasma
NK cells + Peptide + Plasma
3.1%
0.2%
IFN
CD107a

**Figure 2**

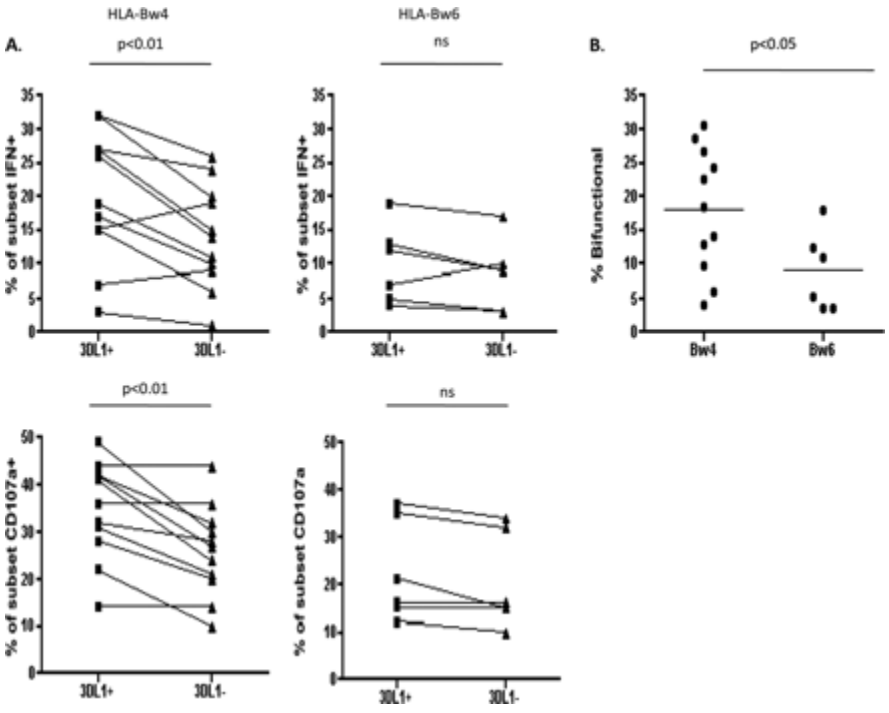

A.
HLA-Bw4
p<0.01
HLA-Bw6
ns
B.
p<0.05
% of subset IFN+
% Bifunctional
3DL1+
3DL1-
Bw4
Bw6
p<0.01
ns
% of subset CD107a+
% of subset CD107a

**Figure 3**

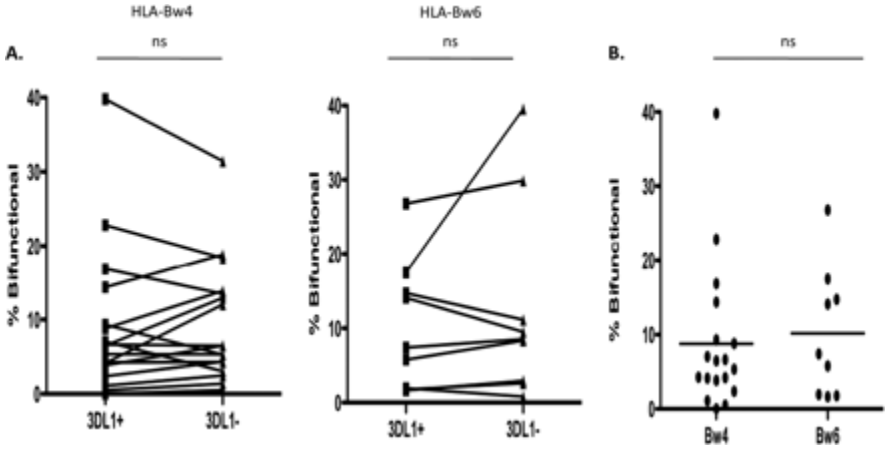
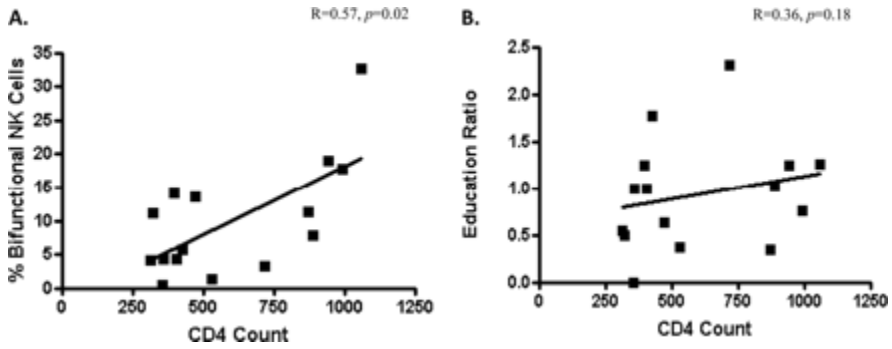

A.
HLA-Bw4
ns
% Bifunctional
40
30
20
10
0
3DL1+
3DL1-
HLA-Bw6
ns
% Bifunctional
40
30
20
10
0
3DL1+
3DL1-
B.
ns
% Bifunctional
40
30
20
10
0
Bw4
Bw6

**Figure 4**


A.
R=0.57, p=0.02
% Bifunctional NK Cells
CD4 Count
B.
R=0.36, p=0.18
Education Ratio
CD4 Count

## 3.9 References


1. **Alter, G., M. P. Martin, N. Teigen, W. H. Carr, T. J. Suscovich, A. Schneidewind, H. Streeck, M. Waring, A. Meier, C. Brander, J. D. Lifson, T. M. Allen, M. Carrington, and M. Altfeld.** 2007. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. J Exp Med **204:**3027-3036.

2. **Anfossi, N., P. Andre, S. Guia, C. S. Falk, S. Roetynck, C. A. Stewart, V. Breso, C. Frassati, D. Reviron, D. Middleton, F. Romagne, S. Ugolini, and E. Vivier.** 2006. Human NK cell education by inhibitory receptors for MHC class I. Immunity **25:**331-342.

3. **Bonaparte, M. I., and E. Barker.** 2004. Killing of human immunodeficiency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules. Blood **104:**2087-2094.

4. **Bonner, B. C., and T. A. Poulton.** 1991. Cytofluorometric analysis of anti-lymphocyte antibodies in AIDS. FEMS Microbiol Immunol **4:**33-40.

5. **Boulet, S., M. Kleyman, J. Y. Kim, P. Kamya, S. Sharafi, N. Simic, J. Bruneau, J. P. Routy, C. M. Tsoukas, and N. F. Bernard.** 2008. A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. AIDS **22:**1487-1491.

6. **Boulet, S., R. Song, P. Kamya, J. Bruneau, N. H. Shoukry, C. M. Tsoukas, and N. F. Bernard.** 2010. HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. J Immunol **184:**2057-2064.

7. **Bowles, J. A., and G. J. Weiner.** 2005. CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells. J Immunol Methods **304:**88-99.

8. **Brackenridge, S., E. J. Evans, M. Toebes, N. Goonetilleke, M. K. Liu, K. di Gleria, T. N. Schumacher, S. J. Davis, A. J. McMichael, and G. M. Gillespie.** 2011. An Early HIV Mutation within an HLA-B*57-Restricted T

Cell Epitope Abrogates Binding to the Killer Inhibitory Receptor 3DL1. J Virol **85:**5415-5422.

9. **Brocca-Cofano, E., K. McKinnon, T. Demberg, D. Venzon, R. Hidajat, P. Xiao, M. Daltabuit-Test, L. J. Patterson, and M. Robert-Guroff.** 2011. Vaccine-elicited SIV and HIV envelope-specific IgA and IgG memory B cells in rhesus macaque peripheral blood correlate with functional antibody responses and reduced viremia. Vaccine **29:**3310-3319.

10. **Brodin, P., T. Lakshmikanth, S. Johansson, K. Karre, and P. Hoglund.** 2009. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood **113:**2434-2441.

11. **Chung, A. W., E. Rollman, R. J. Center, S. J. Kent, and I. Stratov.** 2009. Rapid degranulation of NK cells following activation by HIV-specific antibodies. J Immunol **182:**1202-1210.

12. **Cohen, G. B., R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, and D. Baltimore.** 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity **10:**661-671.

13. **Fauci, A. S., D. Mavilio, and S. Kottilil.** 2005. NK cells in HIV infection: paradigm for protection or targets for ambush. Nat Rev Immunol **5:**835-843.

14. **Hessell, A. J., L. Hangartner, M. Hunter, C. E. Havenith, F. J. Beurskens, J. M. Bakker, C. M. Lanigan, G. Landucci, D. N. Forthal, P. W. Parren, P. A. Marx, and D. R. Burton.** 2007. Fc receptor but not complement binding is important in antibody protection against HIV. Nature **449:**101-104.

15. **Hoglund, P., and P. Brodin.** 2010. Current perspectives of natural killer cell education by MHC class I molecules. Nat Rev Immunol **10:**724-734.

16. **Iannello, A., O. Debbeche, S. Samarani, and A. Ahmad.** 2008. Antiviral NK cell responses in HIV infection: II. viral strategies for evasion and lessons for immunotherapy and vaccination. J Leukoc Biol **84:**27-49.

17. **Kamya, P., Tallon, B., Melendez-Pena, C., Parsons, M. S., Migueles, S. A., Connors, M., Miconiatis, S., Song, R., Boulet, S., Bruneau, J., Tremblay, C. L., Bernard, N. F.** 2012. Inhibitory Killer Immunoglobulin-like receptors

to self HLA-B and HLA-C ligands contribute differentially to Natural Killer cell functional potential in HIV infected slow progressors. Clinical Immunology In Press.

18. **Kim, S., J. B. Sunwoo, L. Yang, T. Choi, Y. J. Song, A. R. French, A. Vlahiotis, J. F. Piccirillo, M. Cella, M. Colonna, T. Mohanakumar, K. C. Hsu, B. Dupont, and W. M. Yokoyama.** 2008. HLA alleles determine differences in human natural killer cell responsiveness and potency. Proc Natl Acad Sci U S A **105:**3053-3058.

19. **Lambotte, O., G. Ferrari, C. Moog, N. L. Yates, H. X. Liao, R. J. Parks, C. B. Hicks, K. Owzar, G. D. Tomaras, D. C. Montefiori, B. F. Haynes, and J. F. Delfraissy.** 2009. Heterogeneous neutralizing antibody and antibody-dependent cell cytotoxicity responses in HIV-1 elite controllers. AIDS **23:**897-906.

20. **Lang, P., M. Pfeiffer, R. Handgretinger, M. Schumm, B. Demirdelen, S. Stanojevic, T. Klingebiel, U. Kohl, S. Kuci, and D. Niethammer.** 2002. Clinical scale isolation of T cell-depleted CD56+ donor lymphocytes in children. Bone Marrow Transplant **29:**497-502.

21. **Lanier, L. L., J. E. Gumperz, P. Parham, I. Melero, M. Lopez-Botet, and J. H. Phillips.** 1995. The NKB1 and HP-3E4 NK cells receptors are structurally distinct glycoproteins and independently recognize polymorphic HLA-B and HLA-C molecules. J Immunol **154:**3320-3327.

22. **Martin, M. P., Y. Qi, X. Gao, E. Yamada, J. N. Martin, F. Pereyra, S. Colombo, E. E. Brown, W. L. Shupert, J. Phair, J. J. Goedert, S. Buchbinder, G. D. Kirk, A. Telenti, M. Connors, S. J. O'Brien, B. D. Walker, P. Parham, S. G. Deeks, D. W. McVicar, and M. Carrington.** 2007. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet **39:**733-740.

23. **Mavilio, D., J. Benjamin, M. Daucher, G. Lombardo, S. Kottilil, M. A. Planta, E. Marcenaro, C. Bottino, L. Moretta, A. Moretta, and A. S. Fauci.** 2003. Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. Proc Natl Acad Sci U S A **100:**15011-15016.

24. **Nguyen, M. L., K. J. Farrell, and C. J. Gunthel.** 2010. Non-AIDS-Defining Malignancies in Patients with HIV in the HAART Era. Curr Infect Dis Rep **12:**46-55.

25. **Orr, M. T., W. J. Murphy, and L. L. Lanier.** 2010. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. Nat Immunol **11:**321-327.

26. **Parsons, M. S., S. Boulet, R. Song, J. Bruneau, N. H. Shoukry, J. P. Routy, C. M. Tsoukas, and N. F. Bernard.** 2010. Mind the gap: lack of association between KIR3DL1*004/HLA-Bw4-induced natural killer cell function and protection from HIV infection. J Infect Dis **202 Suppl 3:**S356-360.

27. **Parsons, M. S., and M. Grant.** 2009. Natural killer cell receptors in human immunodeficiency virus infection: pathways to protection or doors to disappointment? Curr HIV Res **7:**487-496.

28. **Parsons, M. S., K. Zipperlen, M. Gallant, and M. Grant.** 2010. Killer cell immunoglobulin-like receptor 3DL1 licenses CD16-mediated effector functions of natural killer cells. J Leukoc Biol **88:**905-912.

29. **Scott-Algara, D., L. X. Truong, P. Versmisse, A. David, T. T. Luong, N. V. Nguyen, I. Theodorou, F. Barre-Sinoussi, and G. Pancino.** 2003. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. J Immunol **171:**5663-5667.

30. **Stratov, I., A. Chung, and S. J. Kent.** 2008. Robust NK cell-mediated human immunodeficiency virus (HIV)-specific antibody-dependent responses in HIV-infected subjects. J Virol **82:**5450-5459.

31. **Thobakgale, C. F., H. Streeck, N. Mkhwanazi, Z. Mncube, L. Maphumulo, F. Chonco, A. Prendergast, G. Tudor-Williams, B. D. Walker, P. J. Goulder, M. Altfeld, and T. Ndung'u.** 2011. CD8(+) T Cell Polyfunctionality Profiles in Progressive and Nonprogressive Pediatric HIV Type 1 Infection. AIDS Res Hum Retroviruses.

32. **Trundley, A., H. Frebel, D. Jones, C. Chang, and J. Trowsdale.** 2007. Allelic expression patterns of KIR3DS1 and 3DL1 using the Z27 and DX9 antibodies. Eur J Immunol **37:**780-787.

33. **Ullum, H., P. C. Gotzsche, J. Victor, E. Dickmeiss, P. Skinhoj, and B. K. Pedersen.** 1995. Defective natural immunity: an early manifestation of human immunodeficiency virus infection. J Exp Med **182:**789-799.

34. **Vieillard, V., J. L. Strominger, and P. Debre.** 2005. NK cytotoxicity against CD4+ T cells during HIV-1 infection: a gp41 peptide induces the expression of an NKp44 ligand. Proc Natl Acad Sci U S A **102:**10981-10986.

35. **Wren, L., and S. J. Kent.** 2011. HIV Vaccine efficacy trial: Glimmers of hope and the potential role of antibody-dependent cellular cytotoxicity. Hum Vaccin **7**.

## Bridge from chapter 3 to 4

The data presented in chapter 3 demonstrated that NK cell education through KIR3DL1/HLA-Bw4 interactions confers NK cells from healthy controls with an ADCC functional advantage, but that this functional advantage is abrogated during HIV infection. As HIV infection is associated with alterations in the levels of numerous soluble plasma factors, including cytokines, we next assessed whether plasma factors and purified cytokines could influence the ability of NK cells from HIV-uninfected controls to mediate anti-HIV ADCC. The experiments presented in this chapter also assessed the effect of cytokine treatment on the anti-HIV ADCC activity of KIR3DL1$^{+}$ and KIR3DL1$^{-}$ NK cells from HLA-Bw4 carriers

# Chapter 4: Influence of cytokines on HIV-specific antibody-dependent cellular cytotoxicity activation profile of natural killer cells

# Influence of cytokines on HIV-specific antibody-dependent cellular cytotoxicity activation profile of natural killer cells

**Running Head:** Cytokines and NK cell-mediated ADCC

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## 4.1 Abstract

There is growing interest in HIV-specific antibody-dependent cellular cytotoxicity (ADCC) as an effective adaptive immune response to prevent or control HIV infection. ADCC relies on innate immune effector cells, particularly NK cells, to mediate control of virus-infected cells. The activation of NK cells (i.e., expression of cytokines and/or degranulation) by ADCC antibodies in serum is likely subject to the influence of other factors that are also present. We observed that the HIV-specific ADCC antibodies, within serum samples from a panel of HIV-infected individuals induced divergent activation profiles of NK cells from the same donor. Some serum samples primarily induced NK cell cytokine expression (i.e., IFN$\gamma$), some primarily initiated NK cell expression of a degranulation marker (CD107a) and others initiated a similar magnitude of responses across both effector functions. We therefore evaluated a number of HIV-relevant soluble factors for their influence on the activation of NK cells by HIV-specific ADCC antibodies. Key findings were that the cytokines IL-15 and IL-10 consistently enhanced the ability of NK cells to respond to HIV-specific ADCC antibodies. Furthermore, IL-15 was demonstrated to potently activate "educated" KIR3DL1$^+$ NK cells from individuals carrying its HLA-Bw4 ligand. The cytokine was also demonstrated to activate "uneducated" KIR3DL1$^+$ NK cells from HLA-Bw6 homozygotes, but to a lesser extent. Our results show that cytokines influence the ability of NK cells to respond to ADCC antibodies *in vitro*. Manipulating the immunological

environment to enhance the potency of NK cell-mediated HIV-specific ADCC effector functions could be a promising immunotherapy or vaccine strategy.

## 4.2 Introduction

The development of a safe and effective HIV vaccine is urgently needed. Traditional HIV vaccine constructs have focused on the induction of broadly neutralizing antibodies (BnAbs) and cytotoxic T-lymphocytes (CTL) [1]. However, several lines of evidence suggest that non-neutralizing HIV-specific antibodies could play an important role in preventing or controlling HIV infection. These antibodies can bind to infected cells and recruit innate immune effector cells, such as natural killer (NK) cells, to lyse infected cells through antibody-dependent cellular cytotoxicity (ADCC). A recent HIV vaccine efficacy trial based on a recombinant Canarypox virus prime and envelope protein boost showed partial protection from HIV infection, despite inducing only narrow neutralizing antibody responses and very modest CTL responses [2]. High levels of HIV-specific ADCC-competent antibodies were induced by this regimen, suggesting such responses may play a role in protective immunity [3,4]. This idea is supported by elegant passive transfer experiments in Rhesus macaques, which demonstrate decreased effectiveness of BnAbs no longer capable of eliciting antibody constant region (Fc)-dependent ADCC responses [5]. Furthermore, ADCC responses have been associated with protective outcomes against immunodeficiency viruses [6,7], in particular this has been observed in highly exposed seronegative intravenous drug users, HIV-infected elite controllers and vaccinated Rhesus macaques [8–10].

The potential effectiveness of infection-induced HIV-specific ADCC responses suggests that attempts to improve or modulate these responses could provide a therapeutic benefit or assist in protective immunity. Understanding the best mechanisms to present ADCC epitopes for immune recognition, or to improve the potency with which they activate NK cells, may be important for improving ADCC-based therapeutic or preventative strategies. Recent research has highlighted the importance of antibody specificity and the glycosylation of antibody Fc regions for driving efficient ADCC responses [11,12]. It has also been demonstrated that NK cells are more likely to mediate ADCC if NK cell function was conferred through the interaction of inhibitory killer cell immunoglobulin-like receptors (KIR) and their major histocompatibility complex (MHC) class I (or HLA-I) ligands during the process of NK cell education [13–15]. Furthermore, soluble factors within plasma, such as cytokines, have been demonstrated to effect NK cell responsiveness [16] and have been associated with the rate of disease progression [17], potentially due to a synergistic effect on NK cell mediated anti-HIV ADCC.

Alterations in production and plasma/sera levels of cytokines during HIV infection are extensive. Investigators have reported increases in the ability of peripheral blood mononuclear cells (PBMC) to produce IL-4 [18] and IL-10 [19], while increased levels of soluble IL-10 [20], IL-7 [21], GM-CSF [22], and TNF α [23] have been observed in the plasma of infected individuals. Furthermore, reductions in the production of IL-12 and IL-15 have been reported in HIV-

infected individuals [24,25]. The presence of other soluble plasma factors has also been noted to be enhanced during HIV infection. Most notably, LPS levels are increased in HIV-infected individuals [26], and have been correlated with the level of immune activation and disease progression [27]. The influence of cytokines on NK cell-mediated HIV-specific ADCC has not been thoroughly studied. Such studies could ultimately lead to understanding how best to influence the immune environment to obtain optimal ADCC levels during therapeutic and/or prophylactic interventions.

NK cells are exquisitely sensitive to exogenous cytokines, which can increase or decrease multiple effector functions [16]. Most anti-HIV ADCC assays utilize whole serum or plasma that contains not only the antibody of interest but also variable levels of a suite of biologically activate cytokines. Indeed, chronic viral infections, such as HIV, substantially perturb plasma cytokine levels, which could plausibly influence the effectiveness of ADCC responses *in vivo*. Upon activation NK cells mediate a variety of effector functions, including the release of cytokines and chemokines, and degranulation to kill neighboring virus-infected cells. We hypothesized that exposure of NK cells to different exogenous cytokines prior to activation would differentially alter the effector functions mediated by activated NK cells. Furthermore, as educated NK cells are conferred with higher functional potential [28], we hypothesized that NK cells educated through KIR3DL1/HLA-Bw4 interactions would be more susceptible to the effects of exogenous cytokines than KIR3DL1$^{+}$ NK cells from HLA-Bw6 homozygous

individuals. We used a recently developed flow-based ADCC assay that measures NK cell activation (IFNγ synthesis and CD107a degranulation marker expression) to evaluate these hypotheses [29,30]. Whole blood from HIV-uninfected healthy controls was incubated with HIV antigens and ADCC-competent plasma, serum or purified IgG from HIV-infected individuals. These incubations were done in the presence and absence of exogenous cytokines. NK cells responding to ADCC antibodies were evaluated for IFNγ production and expression of the CD107a degranulation marker.

## 4.3 Methods and Materials

### *Study population*

We studied ADCC induced NK cell activation responses elicited by sera samples from 32 HIV-infected subjects not on antiretroviral therapy recruited through the Kirby Institute (Sydney, Australia). Table 1 provides the demographics and clinical characteristics of these patients. Plasma and sera samples from an additional 19 HIV-infected individuals recruited from the Melbourne Sexual Health Centre and one HIV-infected individual from La Clinique l'Actuel, Montreal, Quebec, Canada were utilized to study envelope-specific ADCC responses. HIV-uninfected healthy laboratory volunteers provided whole blood, for assessment of NK cell function. All subjects provided informed consent for participating in this study and human research and ethics committees from all participating study sites approved this study.

### *HLA typing*

Kits from Atria Genetics was used to conduct sequence-based typing of *HLA-B* alleles. Otherwise, *HLA-B* typing was performed by the Victorian Transplant and Immunogenetics Service (Parkville, Australia), using sequence-based typing.

### *Anti-HIV ADCC NK cell activation assay*

As previously described [12], a whole blood assay was used to assess NK cell activation by ADCC Abs. Briefly, 150µl of HIV-uninfected healthy control whole blood plus 50µl of ADCC-competent HIV-infected plasma/serum (stored at -

20°C), or purified IgG, was incubated at 37°C for 5 hours with 1μg/ml of HIV Env peptide pool or whole gp140 protein, Brefeldin A (5μg/ml, Sigma) and Monensin (6μg/ml, Sigma). ADCC responses were assessed using either a peptide pool containing 15-mers that overlapped by 11 amino acids or whole gp140 protein. The peptide pool spanned the HIV-1 consensus subtype B Env protein (kindly supplied by the NIH AIDS Reagent Repository). As previous described, the gp140 protein was obtained from purification of the supernatant of HeLa or 293T cells transfected with the gp140 gene from the subtype B AD8 isolate [31]. After incubation, cells were surface stained with Per-CP-conjugated anti-CD3, FITC-conjugated anti-CD2, PE-conjugated anti-KIR3DL1, PE-Cy7-conjugated anti-CD56 and APC-conjugated anti-CD107a (All from BD Biosciences). Next, whole blood was treated with lysing solution (BD Biosciences) to remove red blood cells, and the remaining white blood cells were treated with permeabilization solution (BD Biosciences) and stained with Alexa700-conjugated anti-IFNγ antibody (BD Biosciences). Flow cytometry data was collected using a FACS Canto II Flow cytometer (BD Biosciences), and was analyzed using Flow Jo Version 9.2 software (Tree Star). We have previously shown this assay is not dependent on immune complexes activating NK cells [30]. The assay works efficiently using either overlapping peptides or whole Env protein as the HIV antigen.

***IgG purification and depletion from sera***

To assess the impact of soluble serum/plasma factors on the skewing of NK cell activation profiles, we purified total IgG from serum over a protein G spin column (Thermo Fisher Scientific) to use in the ADCC ICS assay [12]. Sera were bound to protein G columns for 4 hours with end over end agitation before elution of IgG as per the manufacturer's instruction. Purified IgG samples were then dialyzed in 2 ml mini dialysis tubes (GE Healthcare) before they were concentrated in 30kDa Amicon ultra centrifugal filter devices (Millipore). IgG was also depleted from sera using protein G spin columns (Thermo Fisher Scientific). IgG-depleted sera was then combined with IgG from a single source and used in the ADCC ICS assay described above, to assess the effect of soluble sera factors on a known ADCC-mediated NK cell activation response.

***Anti-Env IgG ELISA***

100 ng of HIV-$1_{AD8}$ Env gp140 purified from media conditioned by a stable gp140-expressing cell line (31) was absorbed onto the bottom of ELISA plate wells in coating buffer (20 mM Tris pH 8.8, 100 mM NaCl) overnight at 4˚C. Wells were then blocked with blocking buffer (5% skim milk powder in PBS/0.1% Tween 20) for 1 hour. Patient samples were added as half log dilution series in block buffer and incubated for 4 hours at room temperature followed by washing with PBS/0.1% Tween 20. HRP conjugated antibody against human IgG in blocking buffer was then added and incubated for 1 hour. After washing ELISAs were

developed using standard techniques. Background was measured by titration of HIV negative human sera. Wells were considered positive when OD was at least 5-fold higher than background. Endpoint titers (most dilute samples giving a positive reading) were averaged over two assays.

***Cytokines***

*In vitro* supplementation with the following cytokines and growth factors, for the five hour duration of the anti-HIV ADCC assay, was studied to assess their influence on NK cell activation profiles: IL-10 (50ng/ml) (BD Biosciences), IL-15 (5ng/ml) (R&D Systems), IL-4 (50Units/ml) (BD Biosciences), GM-CSF (1μg/ml) (BD Biosciences), IL-12 (100ng/ml) (R&D Systems), IL-7 (50ng/ml) (BD Biosciences), TNFα (200ng/ml) (eBioscience) and LPS (1μg/ml) (Sigma).

***Statistical analyses***

Data analyses were performed using GraphPad Prism Version 4.0 software. Data sets were tested for normal distribution using the Kolmogorov-Smirnov test. Parametric data was analyzed using T-tests or paired T-tests. Non-parametric data was compared using Mann-Whitney tests, Wilcoxon Matched Pairs tests, or Spearman correlations

## 4.4 Results

### Skewed ADCC-induced NK cell activation profiles mediated by sera from HIV-infected individuals

NK cells exhibit a number of functions when activated by the Fc portion of ADCC antibodies, including the expression of cytokines and the degranulation and lysis of target cells. We hypothesized that ADCC-induced NK cell effector functions may be differentially regulated depending on the cytokine milieu of the plasma. To evaluate this hypothesis we simultaneously evaluated sera samples obtained from a cohort of 32 antiretroviral therapy naive HIV-infected subjects for their ability to activate fresh NK cells in blood obtained from a single healthy donor in the presence of Env peptide or protein antigens. We utilized a previously described flow cytometric assay of antibody-mediated NK cell activation [12,15,29,30,32–34]. This simple assay studies the ability of donor NK cells within whole blood to be activated by antibodies within $HIV^+$ sera samples recognizing HIV peptides or Env proteins. The NK cell activation in this assay occurs only when both peptides/proteins and antibodies are present (Fig 1a). Furthermore, this assay is not dependent on NK cell recognition of immune complexes.

We observed marked differences in the ability of NK cells from the same donor to express IFN$\gamma$, CD107a or both effector molecules in response to the same HIV Env antigens using different $HIV^+$ sera samples - examples are shown in

Fig 1b. We quantified this difference by assessing whether there was markedly greater IFNγ expression, greater CD107a expression or similar expression of both molecules from NK cells. Quantification was performed by using a 1.5% differential to determine if responses were skewed towards a particular response profile. As such, responses that exhibited equal to or higher than 1.5% NK cell expression of one effector molecule than the other were considered skewed for that response, while responses with less than 1.5% differences were considered equal. For stimulations involving Env peptides CD107a was the predominant effector molecule expressed in 22% of samples, whereas in a minority (6% of samples) IFNγ expression predominated (Fig 1c). Similarly, for stimulations involving GP140 protein CD107a was the predominant effector molecule expressed for 35% of samples, whereas in a minority (6% of samples) IFNγ expression predominated (Fig 1c). It should be noted that plasma samples that induce skewed responses appear to induce similarly skewed responses regardless of the source of donor NK cells. We assessed anti-HIV ADCC using a single plasma source on 20 HIV-uninfected controls. We found that the same source of plasma consistently induced a CD107a-skewed NK cell functional profile; the mean ratio between percent of NK cells expressing CD107a and percent of NK cells expressing IFNγ was 3.0 (range 1.2-16.9) across the 20 subjects.

Previous research has suggested that individual NK cells within an organism obtain diverse functional potentials through ontological processes,

such as NK cell education. As such, we questioned if the differential responses induced by different serum samples were reflective of the activation of different arrays of functionally competent NK cells. To answer this question we compared the total percentage of activated NK cells present in individuals that mediated "skewed" and "even" ADCC responses against HIV gp140. This analysis demonstrated that skewed responses were associated with higher percentages of activated NK cells (7.9+/-0.8 vs. 3.4+/-0.6, $p<0.01$, Mann-Whitney test) (Fig 1d). It should be noted that a similar observation was made for ADCC responses against HIV Env peptides (Data not shown).

Next, we evaluated if the differences in magnitude and functional profiles of NK cell-mediated ADCC responses to different plasmas could be explained by different levels of anti-HIV IgG present in the sera, or if it was related to other soluble sera factors. As such, an anti-Env IgG ELISA was utilized to assess the levels of anti-Env IgG in 31/32 sera samples (Due to limited sample availability). Figure 1E illustrates that no correlation was observed between the level of sera anti-Env IgG and the magnitude the anti-HIV ADCC response ($r=0.2$, $p=0.2$, Spearman correlation). Figure 1F illustrates that no difference in sera anti-Env IgG was observed between sera mediating "skewed" responses and those mediating "even" responses ($p>0.05$, Mann-Whitney test). These observations suggest that plasma factors in additional to Env-specific IgG levels are influencing the magnitude and functional profile of NK cell-mediated anti-HIV ADCC responses.

Previous research has demonstrated that soluble plasma factors, such as cytokines, can alter the immune responsiveness of NK cells [16]. To assess if plasma-derived factors were responsible for these skewed ADCC responses, we next purified IgG from the sera of 10 HIV-infected subjects with predominantly CD107a expression profiles and assessed the Env-specific ADCC-induced activation profiles of NK cells activated with purified IgG in comparison to whole sera. Four representative samples from these experiments are shown in figure 2A. Despite having no effect on the total percentage of responding NK cells ($p$>0.05, Wilcoxon Matched Pairs Test) (Fig 2B), IgG purification was consistently observed to alter the response towards a more IFNγ$^{+}$ response profile (Fig 2C). This is illustrated by the shift from plasma induced responses that consisted of 94% of activated NK cells expressing CD107a and 36% of activated NK cells producing IFNγ to pure IgG induced responses that consisted of 80% of activated NK cells expressing CD107a and 63% of activated NK cells producing IFNγ. There was significantly increased synthesis of IFNγ in ADCC mediated by purified IgG compared to that mediated by sera (63+/-3.3 vs. 36 +/-5.1, $p$=0.0001, paired T-test) and significantly decreased expression of CD107a in ADCC mediated by purified IgG compared to that mediated by sera (80+/-2.9 vs. 94+/-1.0, $p$=0.001, paired T-test). These results suggest that soluble factors, other than IgG, within sera, are affecting the cytokine expression/degranulation profile of NK cells following ADCC activation.

To confirm that the alteration of the NK cell responses towards higher IFNγ and lower CD107a expression following IgG purification was due to soluble factors present within the plasma, we next performed a set of experiments that involved separately adding IgG-depleted sera from 10 different HIV-infected donors that exhibited CD107a dominant responses to the purified IgG from a single donor. Four representative examples of these experiments are depicted in figure 2D. Similar to the results suggesting that ADCC responses induced by whole plasma are characterized by higher CD107a expression and lower IFNγ synthesis than ADCC responses mediated by purified IgG, the addition of IgG-depleted plasma from different donors to a common source of purified IgG increased the responding NK cells that expressed CD107a and decreased the responding NK cells that produced IFNγ (Fig 2E). Cumulatively, these results demonstrate that soluble factors other than IgG, which are present within the plasma/sera of HIV-infected individuals, influence the functional profile of NK cells mediating anti-HIV ADCC.

**Influence of cytokines on HIV-specific ADCC activation of NK cells**

As our initial results suggested that soluble plasma factors have an impact on NK cell-mediated effector functions, we selected a series of soluble factors to assess their influence on the anti-HIV ADCC induced activation profiles of NK cells. As discussed in the introduction, these factors were chosen due to their previously observed relevance to HIV infection [18–27]. These factors were added into a standard culture of healthy donor whole blood, Env antigens and plasma from an

HIV-infected donor with known anti-Env ADCC antibodies. This experiment was repeated with whole blood from four HIV-uninfected donors. We observed consistent alterations in the profile of NK cell activation in the presence of several cytokines (Figure 3). GM-CSF and IL-4 both exhibited an inhibitory effect on overall Env-specific ADCC-induced NK cell activation, decreasing responses in all donors studied. IL-12, TNF-α and IL-7 had little overall effect on ADCC-mediated NK cell activation in the donors. However, both IL-10 and IL-15 had a markedly positive effect on ADCC-induced NK cell activation, increasing ADCC responses in all 4 donors. These results suggest that cytokines can alter the immunological microenvironment within which anti-HIV ADCC responses occur, increasing or decreasing the potency of these anti-viral responses.

**Effects of IL-10 and IL-15 on anti-HIV ADCC induced NK cell activation**

As IL-10 and IL-15 both enhanced NK cell-mediated anti-HIV ADCC effector functions, we performed a detailed assessment of the influences of these cytokines on NK cell-mediated anti-HIV ADCC. Since we observed an impact of these cytokines on the ability of NK cells to both produce IFNγ and express CD107a, we first performed titrations to elucidate the concentrations at which these cytokines are active (Figure 4A). These titrations also served to confirm the consistency of the effect of IL-10 and IL-15 across NK cells from several donors. While IL-10 required relatively high levels (i.e., 25-50ng/ml) to enhance anti-HIV ADCC induced NK cell IFNγ production and degranulation, IL-15

maximally increased both NK cell effector functions at low concentrations (i.e., 5ng/ml). These results demonstrate that cytokines can influence the effector functions of NK cells at concentrations that could be achievable through therapeutic mechanisms.

Control of viral infections has been associated with simultaneous mediation of several cell-based effector functions [35–37]. Indeed, natural control of HIV-infection has been associated with polyfunctional CTL and NK cell responses. As polyfunctional, rather than monofunctional, NK cell-mediated responses would be preferable to obtain by prophylactic and/or therapeutic interventions, we next assessed the impact of exogenous cytokines on the ability of NK cells to mediate more than one effector function simultaneously. Since different plasmas contain different arrays of biologically active cytokines that could influence the effects of exogenous cytokines, we assessed if the effects of adding IL-10 and IL-15 were consistent across a series of HIV-infected plasmas. Regardless of the plasma used in the whole blood ICS assay, we observed a consistent increase in the frequency of cells capable of mediating bifunctional ($IFN\gamma^{+}CD107a^{+}$) responses when IL-10 (2.5+/-0.8 vs. 3.9+/-1.2, $p<0.01$, paired T-test) and IL-15 (2.5+/-0.8 vs. 4.9+/-1.5, $p<0.05$, paired T-test) were added (Figure 4B). Indeed, exogenous IL-10 (19.7+/-3.2 vs. 24.8+/-3.4, $p<0.01$, paired T-test) and IL-15 (19.7+/-3.2 vs. 32.0+/-3.9, $p<0.01$, paired T-test) increased the contribution that bifunctional responses made to the total NK cell response (Figure 4B). The observation that exogenous cytokines have consistent effects

across a series of HIV-infected plasmas suggests that therapeutic use of exogenous cytokines would have a similar effect across many HIV-infected individuals. Overall, these results suggest that polyfunctional cellular responses, which have been associated with natural protection from HIV disease progression [1,35–37], are achievable through anti-HIV ADCC antibodies, especially in the presence of exogenous cytokines.

**Importance of NK cell education for effects of exogenous cytokines on NK cell-mediated anti-HIV ADCC**

The ability of NK cells to mediate effector functions is controlled by a developmental process known as education [28]. In short, this process ensures self-tolerance of NK cells by only conferring functional potential upon NK cells that express inhibitory receptors for self-ligands. Recent research suggests that the stronger the inhibitory signal delivered to NK cells during this process, the stronger and wider array of effector functions mediated by the NK cell [38]. Indeed, polyfunctional NK cell responses occur more frequently in NK cells that are educated by KIR3DL1/HLA-Bw4 combinations that are protective against HIV infection and/or disease progression [36,37]. Interestingly, KIR3DL1$^{+}$ NK cells from HLA-Bw4 carriers have also been demonstrated to mediate higher bifunctional anti-HIV ADCC responses than the same NK cell subset from HLA-Bw6 homozygotes [15]. As we observed a greater effect of exogenous cytokines on bifunctional NK cells, we next assessed if NK cells educated through KIR3DL1/HLA-Bw4 interactions are more sensitive than KIR3DL1$^{+}$ NK cells from

HLA-Bw6 homozygotes to exogenous cytokine treatment. Figure 5a illustrates the gating strategy we employed to distinguish KIR3DL1$^{+/-}$ NK cells. Since exogenous IL-15 induced the largest increase in bifunctional ADCC responses, we assessed the role of educated NK cells in exogenous cytokine enhanced functionality using IL-15. We assessed groups of 9 HLA-Bw4$^{+}$ individuals and 5 HLA-Bw6 homozygous donors and found that exogenous IL-15 treatment significantly increased the bifunctional activity of KIR3DL1$^{+}$ NK cells from HLA-Bw4 carriers (4.5+/-1.1 vs. 13.3+/-3.2, $p$<0.01, paired T-test), but not from HLA-Bw6 homozygotes (1.98+/-0.8 vs. 6.4+/-2.8, $p$>0.05, paired T-test) (Figure 5B). Although increases in bifunctional activity were consistently observed in HLA-Bw6 homozygotes, the reason these changes did not reach significance could reflect the lower responsiveness of non-educated NK cells to cytokine stimulation [14,39].

## 4.5 Discussion

Several studies have linked NK cell-mediated anti-HIV ADCC to protection against HIV infection and/or disease progression [6–10]. Although several assays exist to evaluate anti-HIV ADCC, the readouts of these experimental systems are influenced by several factors that could confound associations of ADCC with HIV disease progression. Most assays measuring anti-HIV ADCC analyze plasma or sera from HIV-infected individuals. As HIV infection is associated with immune activation and perturbations of plasma cytokine levels [16], these samples often contain a suite of biologically active factors that can skew the ADCC measurement. Indeed, using a NK cell activation anti-HIV ADCC assay we observed that, depending on the source of serum used, NK cells from a common donor mediated a series of responses either skewed towards cytokine production, degranulation or equally spread across both effector functions. These skewed responses were dependent upon soluble plasma/sera factors and not solely due to intrinsic qualities of anti-HIV antibodies, as purification of IgG was associated with alteration of NK cell effector functions. Exogenous addition of IL-10 or IL-15 markedly increased NK cell-mediated anti-HIV ADCC effector functions. The effects of IL-15 were observed most potently on NK cells educated through co-expression of KIR3DL1 and HLA-Bw4, which have previously been associated with protection against HIV infection and/or disease progression [40,41]. Of the molecules tested, IL-4 and GM-CSF had a negative influence on NK cell activation, and IL-4 has been implicated in HIV pathogenesis [18].

Surprisingly, no effect was observed for IL-12 on anti-HIV ADCC. This could reflect differences in the methodology utilized in the current manuscript compared to other investigations. For example, the current investigation added IL-12 to the NK cells for a brief five hour period. Other investigators generally add IL-12 to the NK cells for an overnight period prior to NK cell functional assays [42].

The effects of cytokines on NK cell-mediated anti-HIV ADCC effector functions are potently observed within the KIR3DL1 educated NK cell subset mediating both IFN$\gamma$ production and degranulation as assessed by CD107a expression. As educated NK cells mediating polyfunctionality have been associated with protection from HIV infection and/or disease progression [36,37], the ability of cytokines to alter the functionality of these NK cells may partially explain the association of certain cytokine perturbations with protection from HIV infection or advancement of HIV disease. Indeed, the functional observations of this study suggest this possibility. We observed that IL-10 and IL-15 enhanced NK cell functionality. Previous research has demonstrated that both IL-10 and IL-15 can enhance NK cell functions, including ADCC [14,43–48]. The current investigation, however, has built upon these previous studies by investigating the role for IL-15 in a novel assay, as well as the ability of this cytokine to improve upon the functions of “educated” and “uneducated” NK cells. Interestingly, IL-15 has been demonstrated to be increased in the breast milk that is fed to exposed uninfected infants [49]. Meanwhile, exposed-uninfected individuals and slow progressing individuals have been demonstrated

to carry IL-10 promoter polymorphisms that result in high levels of plasma IL-10 [50,51]. Although IL-10 and IL-15 could act through multiple mechanisms to assist in control of HIV, we speculate that an important mechanism could be their ability to enhance NK cell responsiveness to ADCC antibodies. Future studies should evaluate the association of overlap between NK cell education and plasma cytokine levels with protection from HIV infection.

The observation that IL-10 and IL-15 can enhance anti-HIV NK cell activity suggests that these cytokines may be potential resources for prophylactic and/or therapeutic interventions. Although the concentrations of exogenous cytokines that are required to observe increases in NK cell functionality are above what is observed *in vivo* [52], it is worth noting that long-term changes in *in vivo* concentrations of cytokines could still be mediating the skewed responses mediated by several plasma samples. Previous research has demonstrated that the effects of cytokines are synergistic [53]. Furthermore, neutralization of plasma cytokines has been demonstrated to rescue NK cells from the effects of inhibitory endogenous cytokines, such as TGF-β [54]. Lastly, although the high concentrations of exogenous cytokines required to observe NK cell activation may not reflect *in vivo* concentrations, the concentrations may still be safely utilized for preventative or therapeutic interventions. The levels of IL-15 required to activate NK cells to mediate anti-HIV ADCC are particularly intriguing, as it may be feasible to therapeutically administer similar levels of IL-15 to HIV-infected individuals [55]. Although, the levels of exogenous IL-10 required to stimulate

increased anti-HIV ADCC are much higher, they too could likely be supplied safely from an exogenous source [56]. The fact that IL-10 enhances NK cell-mediated anti-HIV ADCC, however, makes it a particularly attractive target. Although some previous research has associated IL-10 with poor viral control, this is mostly due the detrimental effects of IL-10 on CTL [19]. Future research should investigate IL-10 as a component of an antibody containing anti-HIV microbicide that utilizes the ADCC effector function of NK cells. Our results, in combination with previous demonstrations of high-producing IL-10 promoter polymorphisms in exposed uninfected individuals [50], suggest that IL-10 may be particularly well-suited to protect against HIV infection.

Demonstrating that exogenous cytokines can enhance NK cell-mediated anti-HIV ADCC responses that have been associated with protection from HIV infection and/or disease progression is an important step towards understanding how to design better ADCC-based therapeutics or vaccines. Our data suggests vaccines co-expressing IL-15 [57] could result in strongly enhanced ADCC potency. While this study investigated the effect of exogenous cytokines on a small number of NK cell effector functions, future research should elucidate the effector functions involved in viral suppression/control and evaluate the effects of exogenous cytokine stimulation on these functions. Additionally, analysis of monoclonal ADCC antibodies could also evaluate the influence of Fc region glycosylation patterns on NK cell activation. With a broader understanding of the factors determining the potency of anti-HIV ADCC responses, such as NK cell

education, antibody specificity and soluble plasma factors, enhanced ADCC-based therapies and vaccines can be more rationally designed.

**4.6 Acknowledgements**

We are grateful to A. Chung, M. Navis, J. Silvers and H. Kent for helpful assistance, the physicians caring for the $HIV^+$ subjects recruited (T Read, M Chen, C Fairley, T Schmidt, C Bradshaw, R Moore, R McFarlane, D Baker, M McMurchie, D Cooper, S Pett, A Carr, R Finlayson, D Smith, TM Soo, M Kelly, J Patten, B Anderson, S Marlton, D Smith, M Bloch, N Doong, N Roth), and to all the subjects recruited to donate blood samples for this study.

## 4.7 Figure captions

**Figure 1. Differential NK cell activation patterns by HIV-specific ADCC.** The ability of NK cells to respond to anti-HIV ADCC antibodies was assessed using a flow-based assay. (A) Stimulated cells were stained with fluorochrome conjugated antibodies against CD3, CD2, CD56, CD107a and IFNγ. After collection on a FACS II Canto, lymphocytes were gated upon and NK cells were identified as $CD3^{-}CD2^{+}CD56^{+}$. Cells within the NK cell population were assessed for IFNγ production and CD107a expression prior to and following activation. (B) Zebra plots depict examples of the diverse anti-HIV ADCC responses obtained when NK cells from a common donor are stimulated with sera from different HIV-infected individuals in the presence of Env peptides (top) or gp140 protein (bottom). The numbers in the quadrants represent the percentages of responding NK cells that are mediating $IFN\gamma^{+}CD107a^{-}$, $IFN\gamma^{-}CD107a^{+}$ and $IFN\gamma^{+}CD107a^{+}$ responses. (C) The pie chart on the left illustrates the frequency with which IFNγ dominant, CD107a dominant and even response profiles were observed, when sera samples from 32 HIV-infected individuals were used to stimulate NK cells from a common donor in the presence of gp140 protein. The pie chart on the right depicts the same analysis for stimulation with Env peptides. (D) The box and whiskers plot depicts the assessment of the relationship between the ability of different sera to induce diverse anti-HIV ADCC functional profiles against HIV gp140 and the magnitude of the total ADCC response. The total percent of NK cell activation was compared between sera that induced “even” or “skewed” ADCC responses,

using a Mann-Whitney test. (E) The scatter plot illustrates the relationship between the levels of sera anti-HIV gp140 IgG and the percent of total NK cells, from a common donor, activated by the sera in the whole blood ADCC assay in the presence of gp140. This correlation was assessed with the Spearman correlation. (F) The scatter plot depicts the assessment of the impact of the level of sera-associated anti-HIV gp140 IgG on the functional profile induced by different sera, evaluated by a Mann-Whitney test.

**Figure 2. Effect of IgG purification on NK cell-mediated ADCC.** The impact of non-IgG soluble sera factors was assessed by activating NK cells for ADCC functionality in the presence of whole sera, purified IgG, or common purified IgG in the presence of IgG-depleted sera from a series of donors. (A) Zebra plots depict the anti-HIV ADCC mediated by NK cells when incubated with whole sera or IgG purified from the same sera. The values depicted represent the percentages of total NK cells mediating $CD107a^{+}IFN\gamma^{-}$, $CD107a^{-}IFN\gamma^{+}$, or $CD107a^{+}IFN\gamma^{+}$ functional profiles. (B) The scatter plot depicts a comparison of the percentage of total NK cells mediating ADCC-induced effector functions after stimulation of NK cells from a common donor with sera or IgG purified from sera. This difference was assessed with a Wilcoxon Matched Pairs test. (C) The graph on the top illustrates the percent of responding NK cells expressing CD107a after stimulation with sera or IgG purified from the same sera. The graph on the bottom illustrates the percent of responding NK cells producing IFNγ after stimulation with sera or IgG purified from the same sera. Differences were

assessed with paired T-tests. (D) Zebra plots depict the response of NK cells from a common donor to anti-HIV ADCC when incubated with purified IgG from a common donor, sera from a series of donors, or purified IgG from a common donor combined with IgG-depleted sera from the series of donors. The values depicted represent the percentages of responding NK cells mediating $CD107a^{+}IFN\gamma^{-}$, $CD107a^{-}IFN\gamma^{+}$, or $CD107a^{+}IFN\gamma^{+}$ functional profiles. (E) The graph illustrates the alteration of the NK cell-mediated ADCC functional profile induce by the common purified IgG after the addition of IgG-depleted sera from a series of donors.

**Figure 3. Effect of exogenous cytokines on ADCC-induced NK cell activation.** A suite of 7 cytokines and LPS was added separately to a mix of $HIV^{+}$ plasma co-incubated with healthy donor blood and HIV-1 Env antigens. Gated $CD3^{-}$ $CD2^{+}CD56^{+}$ NK cells were studied for CD107a and IFNγ expression. These experiments were repeated using whole blood from four separate donors (A) Zebra plots depict the anti-HIV ADCC mediated by NK cells when incubated with cytokines in the absence of anti-HIV antibodies and Env antigens, incubated with anti-HIV antibodies and Env antigens with no cytokines added, or with the addition of cytokines. The values shown represent the percentages of total NK cells expressing both IFNγ and CD107a. (B) The effect of all cytokines and LPS on ADCC-driven NK cell activation is shown, expressed as a ratio of the response with cytokine added to the response without cytokine. The effect of cytokine

addition on both total $CD107a^+$ and total $IFN\gamma^+$ responses is shown. Error bars represent variation between different whole blood donors.

**Figure 4. Consistent effects of IL-10 and IL-15 across numerous donors.** (A) Titration curves depict the effects of adding IL-10 or IL-15 to donor NK cells from three donors, which were all stimulated with the same $HIV^+$ plasma in the presence of HIV-1 Env antigen. (B) NK cells from a common donor were stimulated with plasma from 9 HIV-infected individuals in the presence of HIV-1 Env antigen, with or without exogenous cytokines. Graphs on the top demonstrate the consistency of the effect of exogenous IL-10 and IL-15 on bifunctional ($IFN\gamma^+CD107a^+$) response profiles, regardless of the source of the plasma. Graphs on the bottom depict the consistency of the effect of exogenous IL-10 (50ng/ml) and IL-15 (5ng/ml) on the percentage contribution bifunctional responses make to the total NK cell response.

**Figure 5. Role of NK cell education in exogenous cytokine stimulation.** Whole blood from nine HLA-Bw4 carriers and five HLA-Bw6 homozygous healthy controls was incubated with ADCC-competent plasma in the presence of HIV-1 Env antigen, with or without exogenous IL-15. (A) $CD3^-$ $CD56^+$ NK cells were gated upon and assessed for KIR3DL1 expression. (B) $KIR3DL1^+$ NK cells from HLA-Bw4 carriers and HLA-Bw6 homozygotes were assessed for their ability to mediate bifunctional (i.e., $CD107a^+IFN\gamma^+$) anti-HIV ADCC responses in the absence and presence of IL-15. The graph represents the influence of IL-15 on the bifunctionality of NK cells from the $KIR3DL1^+$ subset in both groups. The

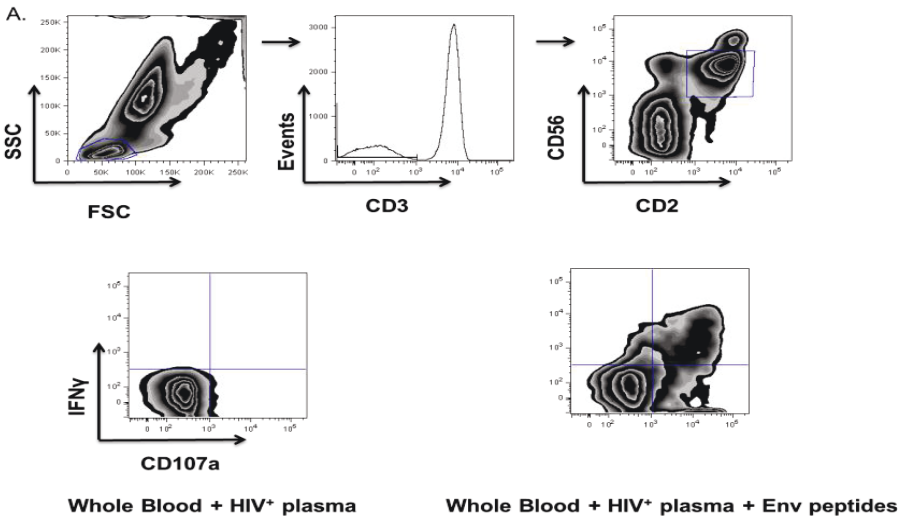
impact of IL-15 on the bifunctionality of these NK cells was assessed using paired T-tests.

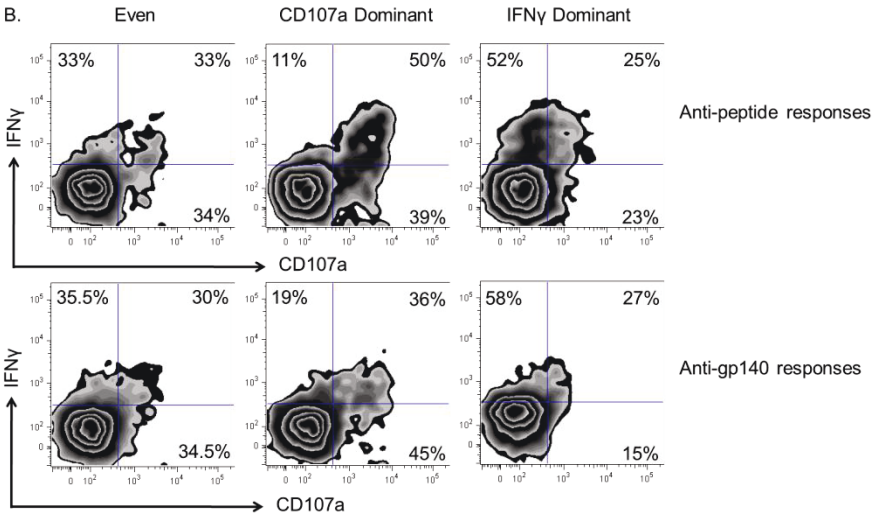
### 4.8 Tables and figures

Table 1. Clinical characteristics of HIV-infected donors.

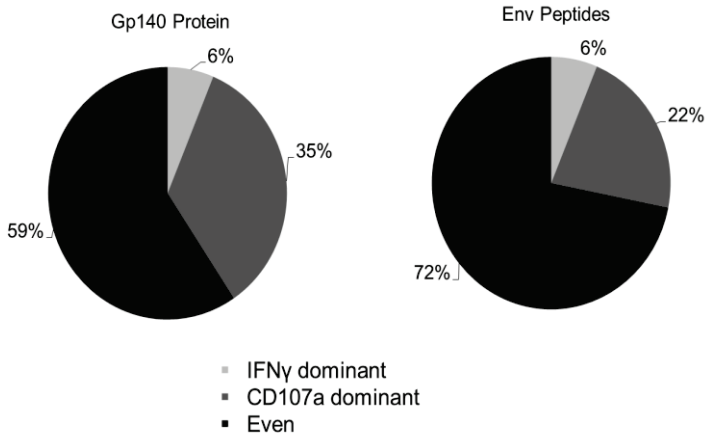
| | **Mean (range)** |
|---|---|
| **Number** | 32 |
| **Age** | 41 (28-65) |
| **Female/Male** | 1/31 |
| **$CD4^+$ cell count (cells/µl)** | 746.5 (504-1310) |
| **Viral load (copies/ml)** | 290 (<50-203100) |

**Figure 1**

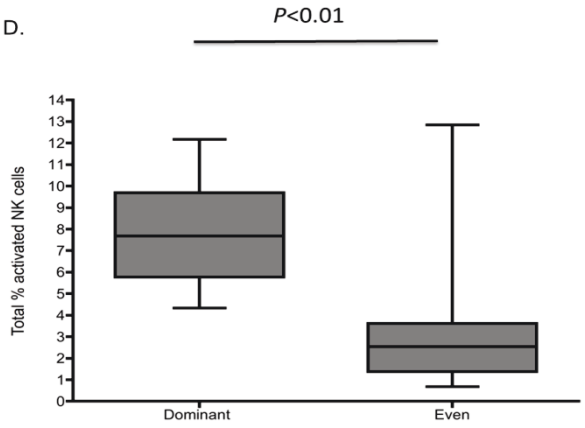

A.
SSC
FSC
Events
CD3
CD56
CD2
IFNγ
CD107a
Whole Blood + HIV+ plasma
Whole Blood + HIV+ plasma + Env peptides


B.
Even
CD107a Dominant
IFNγ Dominant
33%
33%
34%
11%
50%
39%
52%
25%
23%
IFNγ
CD107a
Anti-peptide responses
35.5%
30%
34.5%
19%
36%
45%
58%
27%
15%
IFNγ
CD107a
Anti-gp140 responses

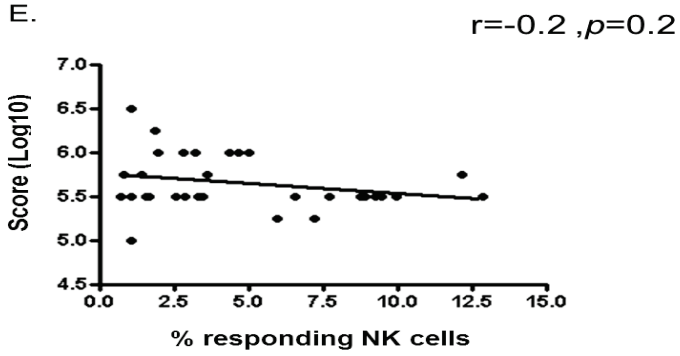
C.


Gp140 Protein
6%
35%
59%
Env Peptides
6%
22%
72%
IFNγ dominant
CD107a dominant
Even

D.


P<0.01
Total % activated NK cells
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Dominant
Even

E.


r=-0.2 ,p=0.2
Score (Log10)
7.0
6.5
6.0
5.5
5.0
4.5
0.0
2.5
5.0
7.5
10.0
12.5
15.0
% responding NK cells

F.

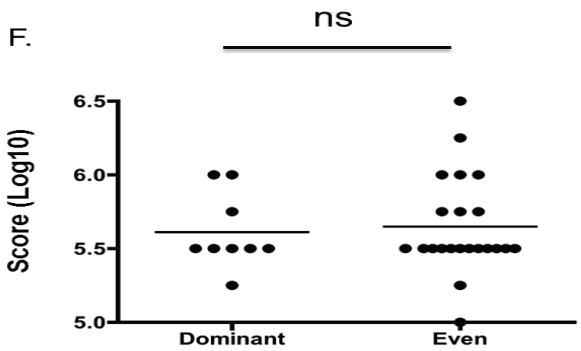


**Figure 2**

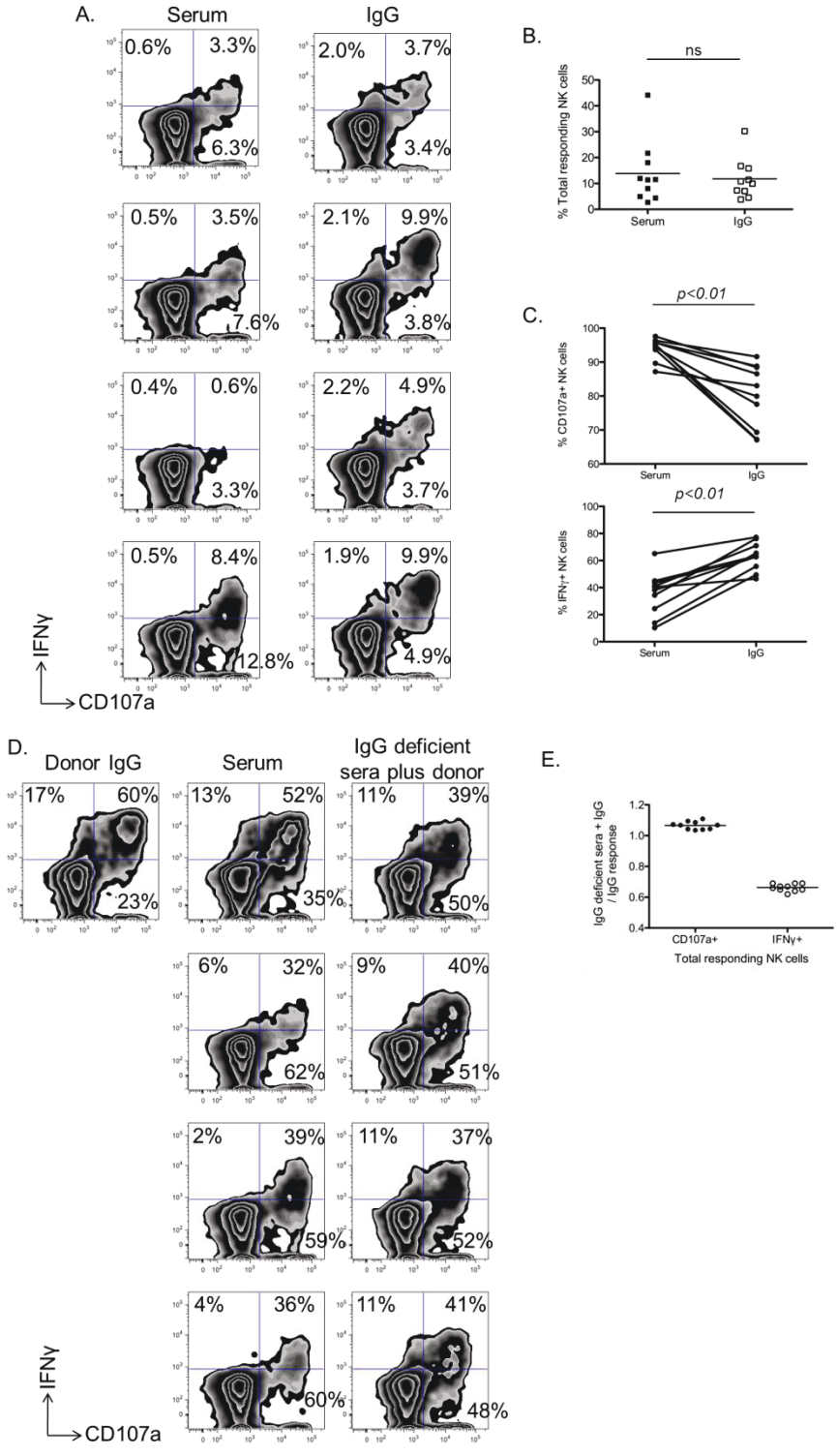
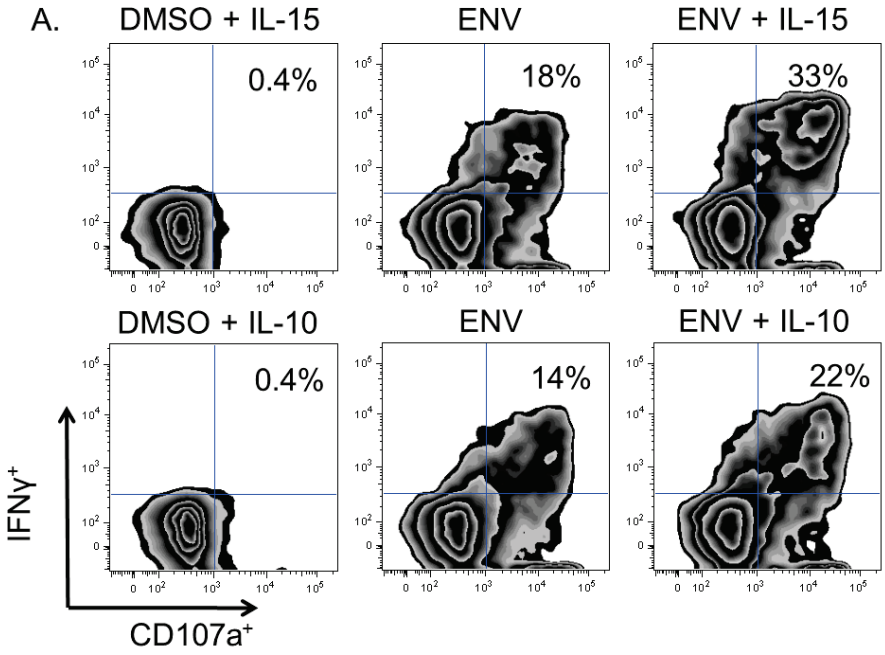

A.
Serum
IgG
0.6%
3.3%
6.3%
2.0%
3.7%
3.4%
0.5%
3.5%
7.6%
2.1%
9.9%
3.8%
0.4%
0.6%
3.3%
2.2%
4.9%
3.7%
0.5%
8.4%
12.8%
1.9%
9.9%
4.9%
IFNγ
CD107a
B.
ns
% Total responding NK cells
Serum
IgG
C.
p<0.01
% CD107a+ NK cells
p<0.01
% IFNγ+ NK cells
D.
Donor IgG
Serum
IgG deficient sera plus donor
17%
60%
23%
13%
52%
35%
11%
39%
50%
6%
32%
62%
9%
40%
51%
2%
39%
59%
11%
37%
52%
4%
36%
60%
11%
41%
48%
E.
IgG deficient sera + IgG / IgG response
CD107a+
IFNγ+
Total responding NK cells

**Figure 3**


A.
DMSO + IL-15
ENV
ENV + IL-15
0.4%
18%
33%
DMSO + IL-10
ENV
ENV + IL-10
0.4%
14%
22%
IFNγ+
CD107a+

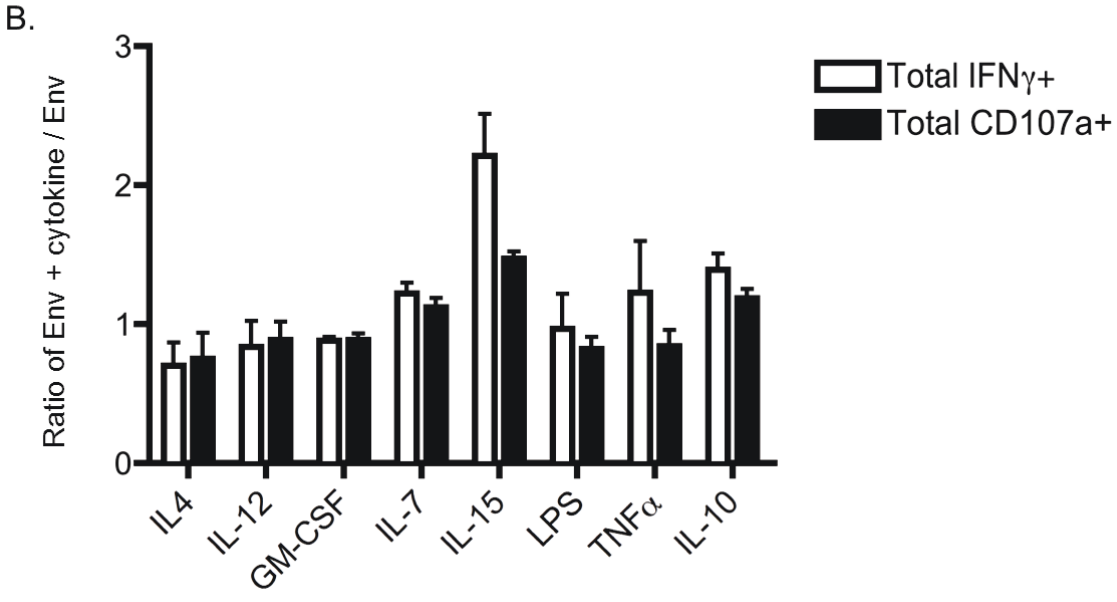
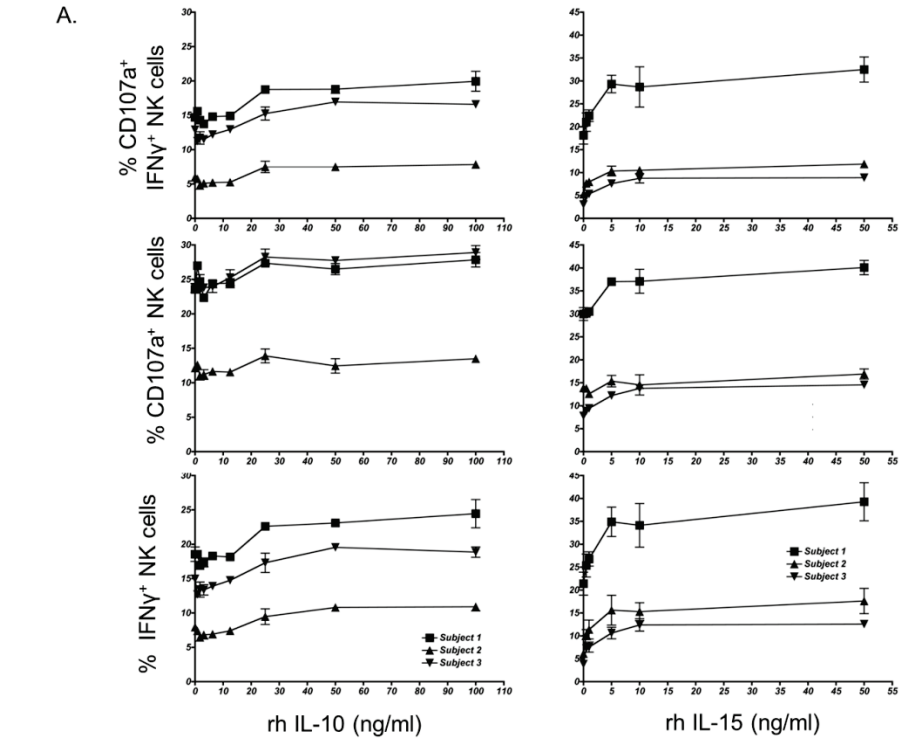

B.
Ratio of Env + cytokine / Env
Total IFNγ+
Total CD107a+
IL4
IL-12
GM-CSF
IL-7
IL-15
LPS
TNFα
IL-10

**Figure 4**


A.
% CD107a+ IFNγ+ NK cells
% CD107a+ NK cells
% IFNγ+ NK cells
Subject 1
Subject 2
Subject 3
rh IL-10 (ng/ml)
rh IL-15 (ng/ml)

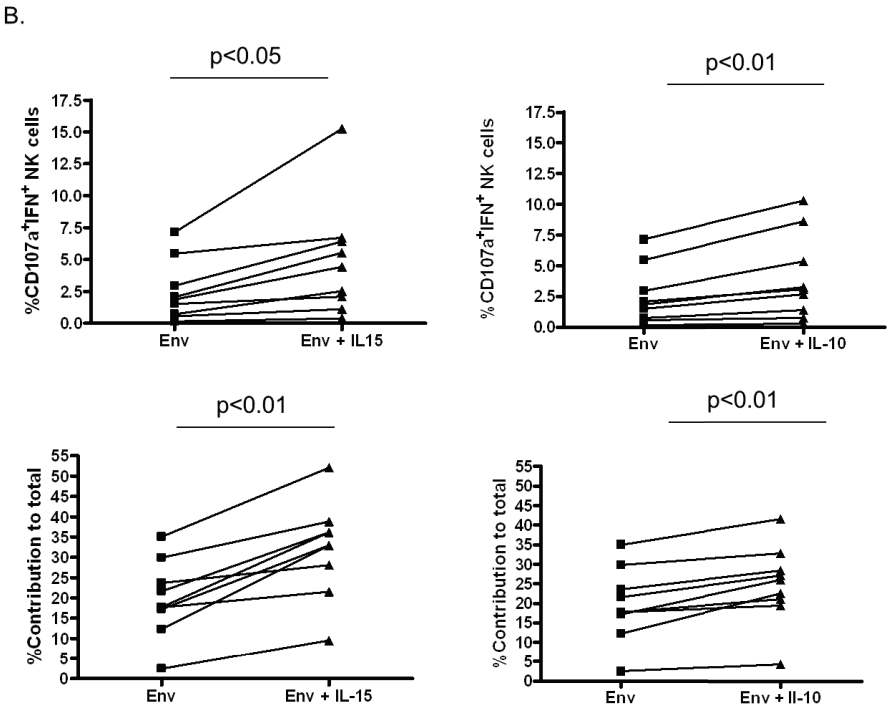
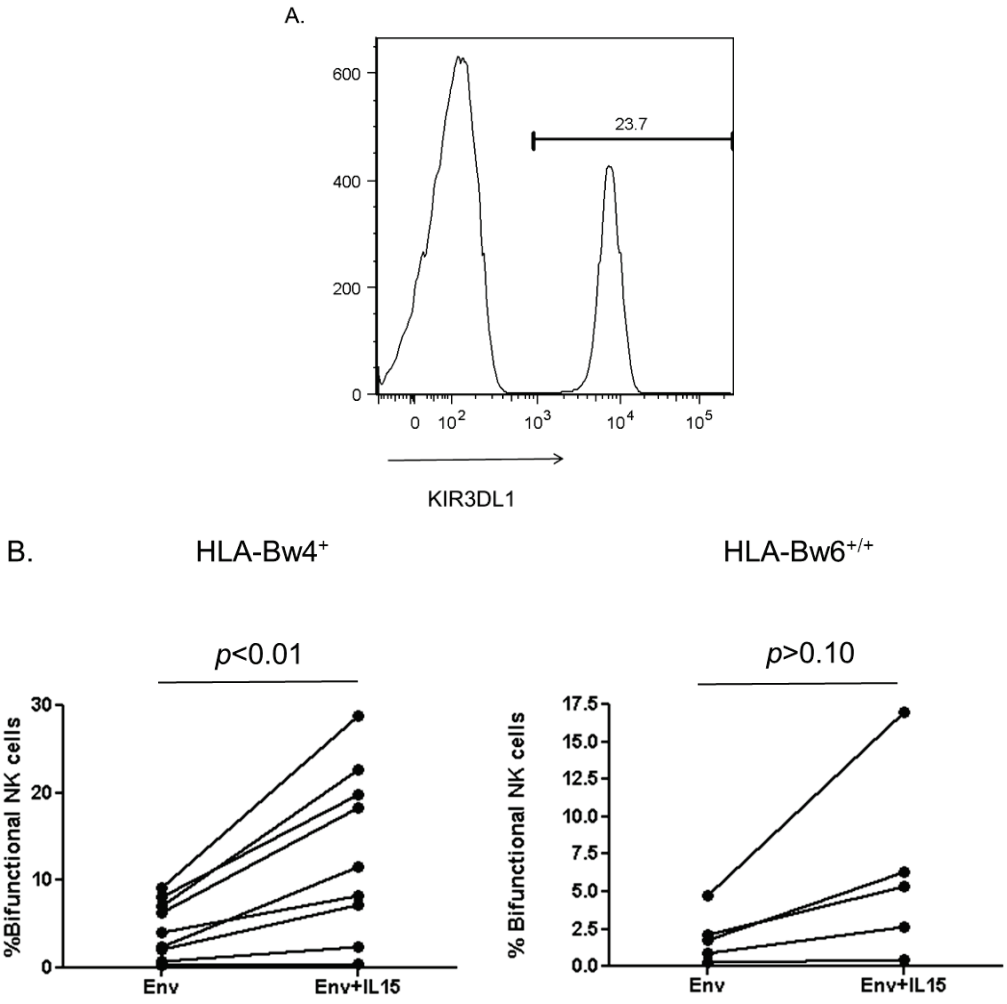

B.
p<0.05
%CD107a+IFN+ NK cells
Env
Env + IL15
p<0.01
%CD107a+IFN+ NK cells
Env
Env + IL-10
p<0.01
%Contribution to total
Env
Env + IL-15
p<0.01
%Contribution to total
Env
Env + Il-10

**Figure 5**


A.
600
400
200
0
23.7
0
$10^2$
$10^3$
$10^4$
$10^5$
KIR3DL1
B.
HLA-Bw4+
HLA-Bw6+/+
p<0.01
p>0.10
%Bifunctional NK cells
% Bifunctional NK cells
30
20
10
0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Env
Env+IL15
Env
Env+IL15

## 4.9 Reference


1. Walker BD, Burton DR (2008) Toward an AIDS vaccine. Science 320: 760-764.

2. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, et al. (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 361: 2209-2220.

3. Wren L, Kent SJ (2011) HIV Vaccine efficacy trial: glimmers of hope and the potential role of antibody-dependent cellular cytotoxicity. Hum Vaccin 7: 466-473.

4. Karnasuta C, Paris RM, Cox JH, Nitayaphan S, Pitisuttithum P, et al. (2005) Antibody-dependent cell-mediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDSVAX B/E prime-boost HIV-1 vaccine trial in Thailand. Vaccine 23: 2522-2529.

5. Hessell AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, et al. (2007) Fc receptor but not complement binding is important in antibody protection against HIV. Nature 449: 101-104.

6. Sun Y, Asmal M, Lane S, Permar SR, Schmidt SD, Mascola JR, et al. (2011) Antibody-dependent cell-mediated cytotoxicity in simian immunodeficiency virus-infected rhesus monkeys. J Virol 85: 6906-6912.

7. Baum LL, Cassutt KJ, Knigge K, Khattri R, Margolick J, et al. (1996) HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. J Immunol 157: 2168-2173.

8. Scott-Algara D, Truong LX, Versmisse P, David A, Luong TT, et al. (2003) Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. J Immunol 171: 5663-5667.

9. Lambotte O, Ferrari G, Moog C, Yates NL, Liao HX, et al. (2009) Heterogeneous neutralizing antibody and antibody-dependent cell cytotoxicity responses in HIV-1 elite controllers. AIDS 23: 897-906.

10. Brocca-Cofano E, McKinnon K, Demberg T, Venzon D, Hidajat R, et al. (2011) Vaccine-elicited SIV and HIV envelope-specific IgA and IgG memory B cells in rhesus macaque peripheral blood correlate with functional antibody responses and reduced viremia. Vaccine 29: 3310-3319.

11. Patel D, Guo X, Ng S, Melchior M, Balderes P, et al. (2010) IgG isotype, glycosylation, and EGFR expression determine the induction of antibody-dependent cellular cytotoxicity in vitro by cetuximab. Hum Antibodies 19: 89-99.

12. Chung AW, Isitman G, Navis M, Kramski M, Center RJ, et al. (2011) Immune escape from HIV-specific antibody-dependent cellular cytotoxicity (ADCC) pressure. Proc Natl Acad Sci U S A 108: 7505-7510.

13. Parsons MS, Zipperlen K, Gallant M, Grant M (2010) Killer cell immunoglobulin-like receptor 3DL1 licenses CD16-mediated effector functions of natural killer cells. J Leukoc Biol 88: 905-912.

14. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, et al. (2006) Human NK cell education by inhibitory receptors for MHC class I. Immunity 25: 331-342.

15. Parsons MS, Wren L, Isitman G, Navis M, Stratov I, et al.(2012) HIV infection abrogates the functional advantage of natural killer cells educated through KIR3DL1/HLA-Bw4 interactions to mediate anti-HIV antibody-dependent cellular cytotoxicity. J Virol .

16. Iannello A, Debbeche O, Samarani S, Ahmad A (2008) Antiviral NK cell responses in HIV infection: II. viral strategies for evasion and lessons for immunotherapy and vaccination. J Leukoc Biol 84: 27-49.

17. Roberts L, Passmore JA, Williamson C, Little F, Bebell LM, et al. (2010) Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression. AIDS 24: 819-831.

18. Navikas V, Link J, Wahren B, Persson C, Link H (1994) Increased levels of interferon-gamma (IFN-gamma), IL-4 and transforming growth factor-beta (TGF-beta) mRNA expressing blood mononuclear cells in human HIV infection. Clin Exp Immunol 96: 59-63.

19. Brockman MA, Kwon DS, Tighe DP, Pavlik DF, Rosato PC, et al. (2009) IL-10 is up-regulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells. Blood 114: 346-356.

20. Srikanth P, Castillo RC, Sridharan G, John TJ, Zachariah A, et al. (2000) Increase in plasma IL-10 levels and rapid loss of CD4+ T cells among HIV-infected individuals in south India. Int J STD AIDS 11: 49-51.

21. Napolitano LA, Grant RM, Deeks SG, Schmidt D, De Rosa SC, et al. (2001) Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat Med 7: 73-79.

22. Hober D, Ajana F, Petit MC, Sartiaux C, Boniface M, et al. (1993) Granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha in patients with human immunodeficiency virus (HIV) type 1 infection. Microbiol Immunol 37: 785-792.

23. Rautonen J, Rautonen N, Martin NL, Philip R, Wara DW (1991) Serum interleukin-6 concentrations are elevated and associated with elevated tumor necrosis factor-alpha and immunoglobulin G and A concentrations in children with HIV infection. AIDS 5: 1319-1325.

24. D'Ettorre G, Forcina G, Lichtner M, Mengoni F, D'Agostino C, et al. (2002) Interleukin-15 in HIV infection: immunological and virological interactions in antiretroviral-naive and -treated patients. AIDS 16: 181-188.

25. Marshall JD, Chehimi J, Gri G, Kostman JR, Montaner LJ, et al. (1999) The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. Blood 94: 1003-1011.

26. Gregson JN, Steel A, Bower M, Gazzard BG, Gotch FM, et al. (2009) Elevated plasma lipopolysaccharide is not sufficient to drive natural killer cell activation in HIV-1-infected individuals. AIDS 23: 29-34.

27. Douek D (2007) HIV disease progression: immune activation, microbes, and a leaky gut. Top HIV Med 15: 114-117.

28. Hoglund P, Brodin P (2010) Current perspectives of natural killer cell education by MHC class I molecules. Nat Rev Immunol 10: 724-734.

29. Stratov I, Chung A, Kent SJ (2008) Robust NK cell-mediated human immunodeficiency virus (HIV)-specific antibody-dependent responses in HIV-infected subjects. J Virol 82: 5450-5459.

30. Chung AW, Rollman E, Center RJ, Kent SJ, Stratov I (2009) Rapid degranulation of NK cells following activation by HIV-specific antibodies. J Immunol 182: 1202-1210.

31. Center RJ, Wheatley AK, Campbell SM, Gaeguta AJ, Peut V, et al. (2009) Induction of HIV-1 subtype B and AE-specific neutralizing antibodies in mice and macaques with DNA prime and recombinant gp140 protein boost regimens. Vaccine 27: 6605-6612.

32. Isitman G, Chung AW, Navis M, Kent SJ, Stratov I (2011) Pol as a target for antibody dependent cellular cytotoxicity responses in HIV-1 infection. Virology 412: 110-116.

33. Chung AW, Navis M, Isitman G, Wren L, Silvers J, et al. (2011) Activation of NK cells by ADCC antibodies and HIV disease progression. J Acquir Immune Defic Syndr 58: 127-131.

34. Johansson SE, Rollman E, Chung AW, Center RJ, Hejdeman B, et al. (2011) NK cell function and antibodies mediating ADCC in HIV-1-infected viremic and controller patients. Viral Immunol 24: 359-368.

35. Thobakgale CF, Streeck H, Mkhwanazi N, Mncube Z, Maphumulo L, et al. (2011) Short Communication: CD8(+) T Cell Polyfunctionality Profiles in Progressive and Nonprogressive Pediatric HIV Type 1 Infection. AIDS Res Hum Retroviruses 27: 1005-1012.

36. Boulet S, Song R, Kamya P, Bruneau J, Shoukry NH, et al. (2010) HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. J Immunol 184: 2057-2064.

37. Parsons MS, Boulet S, Song R, Bruneau J, Shoukry NH, et al. (2010) Mind the gap: lack of association between KIR3DL1*004/HLA-Bw4-induced natural killer cell function and protection from HIV infection. J Infect Dis 202 Suppl 3: S356-S360.

38. Brodin P, Lakshmikanth T, Johansson S, Karre K, Hoglund P (2009) The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood 113: 2434-2441.

39. Juelke K, Killig M, Thiel A, Dong J, Romagnani C (2009) Education of hyporesponsive NK cells by cytokines. Eur J Immunol 39: 2548-2555.

40. Boulet S, Kleyman M, Kim JY, Kamya P, Sharafi S, et al. (2008) A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. AIDS 22: 1487-1491.

41. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, et al. (2007) Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet 39: 733-740.

42. Roda JM, Joshi T, Butchar JP, McAlees JW, Lehman A, et al. (2007) The activation of natural killer cell effector functions by cetuximab-coated, epidermal growth factor receptor positive tumor cells is enhanced by cytokines. Clin Cancer Res 13: 6419-6428.

43. Lin SJ, Roberts RL, Ank BJ, Nguyen QH, Thomas EK, et al. (1998) Effect of interleukin (IL)-12 and IL-15 on activated natural killer (ANK) and antibody-dependent cellular cytotoxicity (ADCC) in HIV infection. J Clin Immunol 18: 335-345.

44. Moga E, Canto E, Vidal S, Juarez C, Sierra J, et al. (2011) Interleukin-15 enhances rituximab-dependent cytotoxicity against chronic lymphocytic leukemia cells and overcomes transforming growth factor beta-mediated immunosuppression. Exp Hematol 39: 1064-1071.

45. Loubeau M, Ahmad A, Toma E, Menezes J (1997) Enhancement of natural killer and antibody-dependent cytolytic activities of the peripheral blood mononuclear cells of HIV-infected patients by recombinant IL-15. J Acquir Immune Defic Syndr Hum Retrovirol 16: 137-145.

46. Fauriat C, Ivarsson MA, Ljunggren HG, Malmberg KJ, Michaelsson J (2010) Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. Blood 115: 1166-1174.

47. Cai G, Kastelein RA, Hunter CA (1999) IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. Eur J Immunol 29: 2658-2665.

48. Mocellin S, Panelli M, Wang E, Rossi CR, Pilati P, et al. (2004) IL-10 stimulatory effects on human NK cells explored by gene profile analysis. Genes Immun 5: 621-630.

49. Walter J, Ghosh MK, Kuhn L, Semrau K, Sinkala M, et al. (2009) High concentrations of interleukin 15 in breast milk are associated with protection against postnatal HIV transmission. J Infect Dis 200: 1498-1502.

50. Chatterjee A, Rathore A, Sivarama P, Yamamoto N, Dhole TN (2009) Genetic association of IL-10 gene promoter polymorphism and HIV-1 infection in North Indians. J Clin Immunol 29: 71-77.

51. Oleksyk TK, Shrestha S, Truelove AL, Goedert JJ, Donfield SM, et al. (2009) Extended IL10 haplotypes and their association with HIV progression to AIDS. Genes Immun 10: 309-322.

52. Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, et al. (2009) Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. J Virol 83: 3719-3733.

53. DeBlaker-Hohe DF, Yamauchi A, Yu CR, Horvath-Arcidiacono JA, Bloom ET (1995) IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK cells. Cell Immunol 165: 33-43.

54. Peppa D, Micco L, Javaid A, Kennedy PT, Schurich A, et al. (2010) Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog 6: e1001227.

55. Mueller YM, Do DH, Altork SR, Artlett CM, Gracely EJ, et al. (2008) IL-15 treatment during acute simian immunodeficiency virus (SIV) infection increases viral set point and accelerates disease progression despite the induction of stronger SIV-specific CD8+ T cell responses. J Immunol 180: 350-360.

56. Rosenblum IY, Johnson RC, Schmahai TJ (2002) Preclinical safety evaluation of recombinant human interleukin-10. Regul Toxicol Pharmacol 35: 56-71.

57. Boyer JD, Robinson TM, Kutzler MA, Vansant G, Hokey DA, et al. (2007) Protection against simian/human immunodeficiency virus (SHIV) 89.6P in macaques after coimmunization with SHIV antigen and IL-15 plasmid. Proc Natl Acad Sci U S A 104: 18648-18653.

## Bridge from chapter 4 to 5

In chapters 3 and 4 we demonstrate that KIR3DL1$^{+}$ NK cells from HLA-Bw4 carriers have an educational advantage to mediate anti-HIV ADCC and that cytokine treatment increases the ADCC functional potential of educated KIR3DL1$^{+}$ NK cells to a greater extent than it does uneducated KIR3DL1$^{+}$ NK cells. If KIR/HLA allotype combinations associated with protective outcomes against HIV are mediating protection through ADCC, it should be expected that protective combinations confer enhanced ADCC functional potential as compared to non-protective combinations. As such, we evaluated NK cells from individuals carrying the most protective combination of *KIR3DL1*h*y/HLA-B*57* for ADCC potential, as compared to individuals with other *KIR3DL1/HLA-B* combinations.

# Chapter 5: The AIDS-protective allelic combination of HLA-B*57 and highly expressed variants of KIR3DL1 confers natural killer cells with robust antibody-dependent cellular cytotoxicity functional potential

# The AIDS-protective allelic combination of HLA-B*57 and highly expressed variants of KIR3DL1 confers natural killer cells with robust antibody-dependent cellular cytotoxicity functional potential

**Running head:** NK cell education and ADCC

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[*]None of the authors has a commercial or other association that might pose a potential conflict of interest related to this manuscript.

[*]This work was supported by a grant from the Canadian Institutes for Health Research (CIHR) #HVI 79515 and the Fonds de la Recherche en Santé du Québec (FRSQ) AIDS and Infectious Diseases Network. M.S.P is supported by a CIHR Vanier Scholarship.

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## 5.1 Abstract

Highly-expressed variants of KIR3DL1 in combination with HLA-B57 (**h/*y+B*57*) associate with protection from HIV infection and slower disease progression. Although KIR3DL1$^{+}$ NK cells do not degranulate against autologous HIV-infected cells, stimulation via ADCC represents a mechanism to achieve additional activating signals. We observed higher ADCC by NK cells from **h/*y+B*57* carriers than individuals with other *KIR3DL1/HLA-Bw4* combinations or *KIR3DL1* without its ligand. These results reaffirm the importance of NK cell education in ADCC and HIV vaccine design.

## 5.2 Manuscript

Natural killer (NK) cells have been implicated in several protective outcomes upon exposure and/or infection with the human immunodeficiency virus (HIV). Particularly, allelic variants of the killer cell immunoglobulin-like receptor 3DL1 (*KIR3DL1*) have been associated with protection against HIV infection and/or slower progression to the acquired immune deficiency syndrome (AIDS), when carried in combination with major histocompatibility complex class I (MHC-I or HLA-I) alleles that express the HLA-Bw4 public epitope [1, 2]. Alleles from both the *HLA-A* and *HLA-B* loci can exhibit the HLA-Bw4 epitope, which serves as a ligand for KIR3DL1, to the mutual exclusion of HLA-Bw6 [3]. Although the mechanism by which *KIR3DL1/HLA-Bw4* carriage confers protection has not yet been elucidated, it has been hypothesized that the potency of NK educational signals received through inhibitory receptors such as KIR3DL1 following HLA-Bw4 ligation is directly related with the strength of cell functional potential [4]. Therefore, NK cells from co-carriers of *KIR3DL1/HLA-Bw4* may be better able to mediate cytolysis and/or secrete cytokines upon encountering autologous HIV-infected cells.

Throughout their lifecycle, NK cells undergo a continuous developmental process known as NK cell education during which activating and inhibitory receptors expressed on NK cells interact with their ligands on cells within a normal self-environment [4]. Resulting signals from these interactions tune the NK cell functional potential in a manner that prevents autoaggressive immune

responses. Thus, signals induced by the interaction of activating and inhibitory receptors to self-ligands tune NK cell functional potential down or up, respectively [5-7]. This tuning process decreases the chance that NK cells will become activated upon encountering healthy autologous cells, and increases the likelihood that NK cells will recognize virus-infected or transformed cells that have downregulated classical HLA-I ligands for inhibitory receptors [8].

As predicted by the NK cell education model, KIR3DL1$^{+}$ NK cells from individuals co-carrying HLA-Bw4 are more likely to respond to activating signals than are the same cells from individuals that do not carry HLA-Bw4 (i.e., HLA-Bw6 homozygotes) [9, 10]. Interestingly, allelic combinations of *KIR3DL1* and *HLA-Bw4* that confer protection in the context of HIV have been demonstrated to mediate more potent educational interactions, conferring KIR3DL1$^{+}$ NK cells with an enhanced ability to mediate polyfunctional responses [11]. Despite these observations, KIR3DL1$^{+}$ NK cells mediate only weak degranulation when stimulated with autologous HIV-infected CD4$^{+}$ T-cells [12]. These results suggest that if protective NK cell-mediated responses are the result of NK cell activation by infected cells, then additional activating signals are required to activate KIR3DL1$^{+}$ NK cells.

Additional stimulatory signals can be provided to NK cells through ligation of the activating Immunoglobulin G (IgG) constant region receptor (FC$\gamma$RIIIa or CD16a) by anti-viral IgG antibodies (Abs) bound to infected cells, resulting in the

Ab-dependent cell-mediated cytotoxicity (ADCC) of the infected cell. We have recently demonstrated that the ability of KIR3DL1$^{+}$ NK cells to respond to healthy autologous cells coated with HIV antigens and anti-HIV Abs is determined by NK cell education in HIV-uninfected individuals [13]. It should be noted that this study did not assess the function of NK cells from individuals with protective allelic combinations of *KIR3DL1* and *HLA-Bw4*.

If ADCC contributes to the protection mediated by allelic combinations of *KIR3DL1* and *HLA-Bw4*, NK cells from individuals carrying these protective combinations should mediate enhanced anti-HIV ADCC compared to individuals carrying other allelic combinations of *KIR3DL1* and *HLA-Bw4*. To evaluate this hypothesis, we assessed the ability of NK cells obtained from 39 HIV-uninfected individuals to mediate anti-HIV ADCC. As previously described [11], these individuals were allotyped for *KIR3DL1* and *HLA-A* and *HLA-B*. Eleven of these individuals carried *KIR3DL1* alleles for high expression variants in combination with *HLA-B*57* (**h/*y+ B*57*), a combination associated with protection from HIV infection and progression to AIDS [1, 2]. Another 20 individuals carried other combinations of *KIR3DL1* and *HLA-Bw4*, while 8 were *HLA-Bw6* homozygotes (hmz) who were *KIR3DL1* hmz.

The ability of NK cells from these individuals to mediate ADCC was assessed using the recently described GranToxiLux assay [14]. Briefly, the CD4$^{+}$ CEM.NKr cell line was coated with HIV gp120 and stained with target (TFL4) and viability (NFL1) dyes. Target cells were incubated for 1 hour at 37$^{O}$C at a 30:1

effector-to-target cell ratio with peripheral blood mononuclear cells (PBMC) from each study participant in the presence of anti-HIV ADCC competent plasma or seronegative plasma as a control, and a substrate that is cleaved to emit fluorescence upon granzyme B delivery to target cells. After incubation, cells were washed and analyzed on a FACSCanto II flow cytometer.

The gating strategy used to analyze data generated from the GranToxiLux assay is shown in figure 1A. Results demonstrate that the cytolysis observed is dependent upon the presence of anti-HIV Abs as it is observed in the presence of plasma from HIV-infected, but not seronegative subjects (Figure 1B). The responses observed appear to be determined by NK cell education, as ADCC was higher in the 31 individuals carrying *KIR3DL1* and *HLA-Bw4* compared to the 8 *HLA-Bw6* homozygotes (7.8+/-1.5 vs. 3.3+/-1.7, $p$=0.08, Mann-Whitney test) (Figure 1C). However, when the *HLA-Bw4* carriers were split into those carrying the protective **h/*y+ B*57* combination versus other *KIR3DL1/HLA-Bw4* combinations, the educational capacity of the protective allelic combination superseded that of other *KIR3DL1/HLA-Bw4* combinations. Indeed, a Kruskal-Wallis test followed by Dunn's multiple comparison tests demonstrated that individuals carrying the protective **h/*y+B*57* (13.4+/-2.6) combination mediated significantly higher ADCC than individuals with other *KIR3DL1/HLA-Bw4* (4.7+/-1.4, $p$<0.05) combinations or *HLA-Bw6* HMZ (3.3+/-1.7, $p$<0.01) (Figure 1D).

Signals propagated through inhibitory KIR2DL receptors that recognize self HLA-C1/2 can also educate NK cells for ADCC [15]. As previously described [16], we typed all 39 subjects for *HLA-C* and *KIR2DL1/2/3* to confirm that the educational advantage in **h/*y+B*57* carriers was due to education through KIR3DL1 and not because these individuals had a higher number of other educationally competent KIR/HLA combinations. No differences were observed between the three groups in terms of *HLA-C/KIR2DL1/2/3* combinations (Data not shown). These data support the conclusion that increased ADCC in individuals carrying **h/*y+B*57* is due to the heightened educational capacity of this receptor-ligand pair.

Collectively, these data reaffirm previous observations that NK cell education confers NK cells with ADCC functional potential [9, 13, 15]. Similar to other NK cell-mediated effector functions, the education of NK cells to mediate ADCC by the protective **h/*y+B*57* combination is more potent than that observed for other *KIR3DL1* and *HLA-Bw4* combinations [11]. This observation may have consequences for attempts to harvest the functional potential of NK cells through vaccines inducing ADCC-competent Abs, as functionally similar Abs will mediate drastically divergent ADCC in different individuals.

## 5.3 Figure captions

**Figure 1.** (A) The dot plots depict the gating strategy used. First target and effector cells were gated on. Target cells were identified as being $TFL4^{+}$ and those that were dead prior to incubation with peripheral blood mononuclear cell (PBMC) effectors were excluded on the basis of their being $NFL1^{+}$. The read out for this assay was the percent of live target cells that received a lethal hit of Granzyme B during incubation with PBMC in the absence or presence of plasma from an HIV-infected individual. (B) The percentage of Granzyme B positive target cells is shown for 1 individual following a 1 hr incubation of PBMC effectors and gp120 coated CEM.NKr targets at a 30:1 ratio in the absence of plasma (left panel), presence of plasma from an HIV negative subject (middle panel), or plasma from an HIV positive subject (right panel). The y-axis for these plots is the side scatter and the x-axis the mean fluorescent intensity of the GranToxiLux readout. (C) The scatter plot depicts the background subtracted anti-HIV ADCC responses mediated by PBMC from *KIR3DL1* homozygous *HLA-Bw4* carriers compared to that mediated by *KIR3DL1* homozygotes (hmz) *HLA-Bw6* hmz. Between group differences were assessed with a Mann-Whitney test. (D) The scatter plot depicts the ADCC responses of *KIR3DL1* hmz that co-carry the **h/*y+B*57* genotype (*B*57*), other combinations of *KIR3DL1* and *HLA-Bw4* (*Bw4*), or *KIR3DL1* who are *HLA-Bw6* hmz (*Bw6*). The significance of between-group differences were assessed using a Kruskal-Wallis test and Dunn's multiple

comparisons tests. The p-values shown over the lines linking 2 groups refer to the significance of comparisons between these 2 groups.

## 5.4 Figures

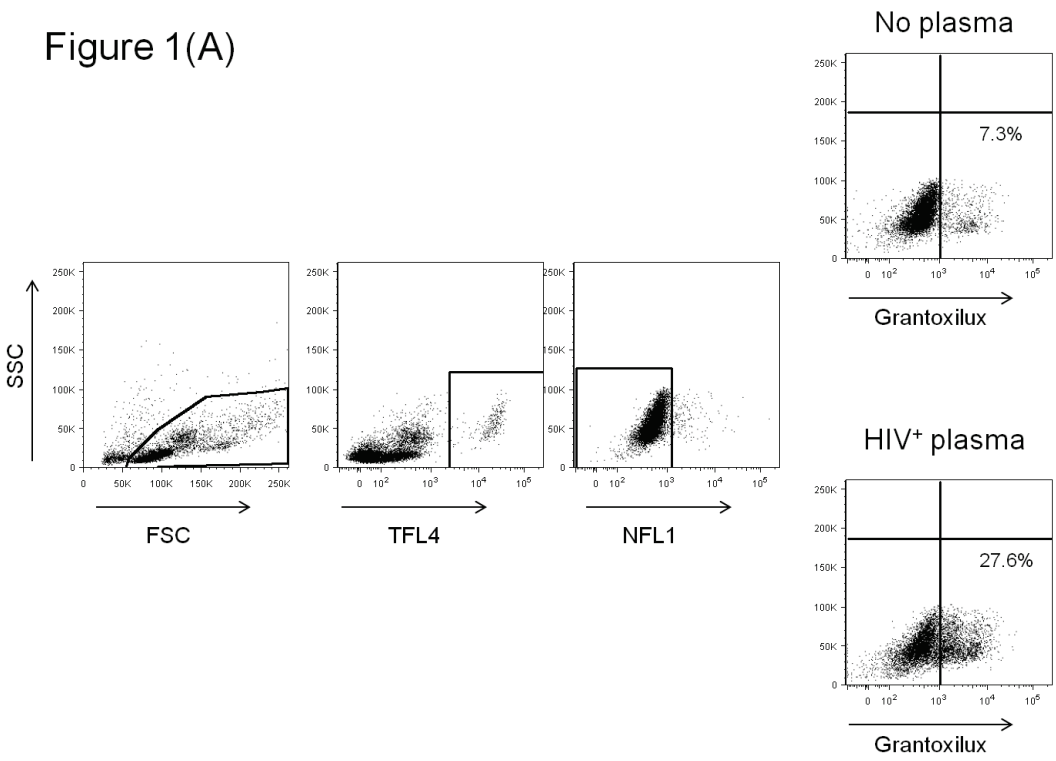
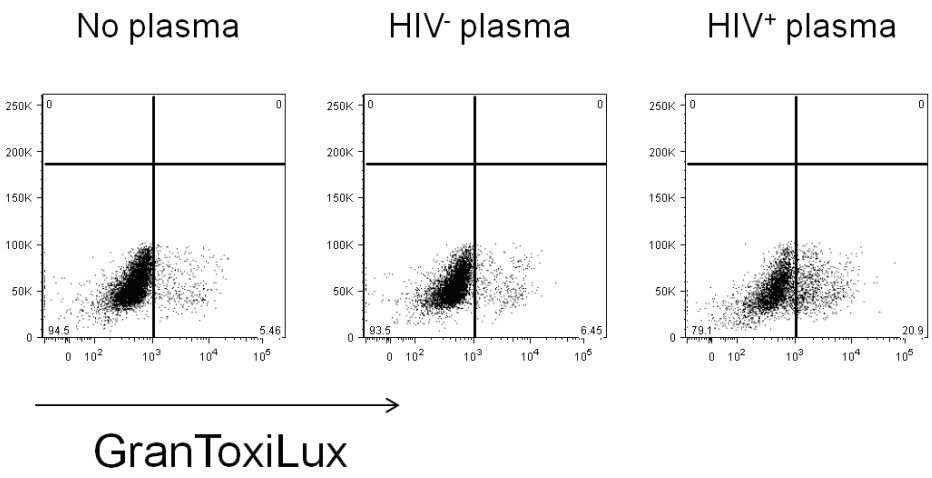
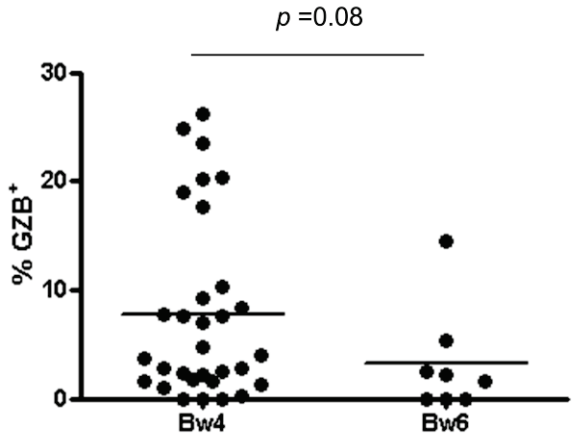


Figure 1(A)
No plasma
7.3%
Grantoxilux
HIV+ plasma
27.6%
Grantoxilux
SSC
FSC
TFL4
NFL1

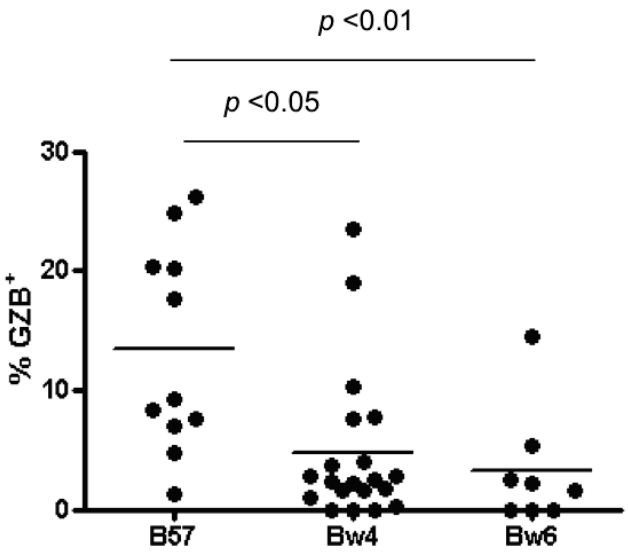
(B)


No plasma
HIV- plasma
HIV+ plasma
250K
200K
150K
100K
50K
0
94.5
5.46
93.5
6.45
79.1
20.9
GranToxiLux

(C)



(D)



## 5.5 References


1. Boulet S, Kleyman M, Kim JY, Kamya P, Sharafi S, Simic N*, et al.* A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. *AIDS* 2008; **22(12)**:1487-1491.

2. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, Pereyra F*, et al.* Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet* 2007; **39(6)**:733-740.

3. Wan AM, Ennis P, Parham P, Holmes N. The primary structure of HLA-A32 suggests a region involved in formation of the Bw4/Bw6 epitopes. *J Immunol* 1986; **137(11)**:3671-3674.

4. Hoglund P, Brodin P. Current perspectives of natural killer cell education by MHC class I molecules. *Nat Rev Immunol* 2010; **10(10)**:724-734.

5. Brodin P, Karre K, Hoglund P. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol* 2009; **30(4)**:143-149.

6. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 2010; **115(11)**:2167-2176.

7. Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* 2009; **182(8)**:4572-4580.

8. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* 1998; **391(6665)**:397-401.

9. Parsons MS, Zipperlen K, Gallant M, Grant M. Killer cell immunoglobulin-like receptor 3DL1 licenses CD16-mediated effector functions of natural killer cells. *J Leukoc Biol* 2010; **88(5)**:905-912.

10. Kim S, Sunwoo JB, Yang L, Choi T, Song YJ, French AR*, et al.* HLA alleles determine differences in human natural killer cell responsiveness and potency. *Proc Natl Acad Sci U S A* 2008; **105(8)**:3053-3058.

11. Boulet S, Song R, Kamya P, Bruneau J, Shoukry NH, Tsoukas CM*, et al.* HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. *J Immunol* 2010; **184(4)**:2057-2064.

12. Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A*, et al.* Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* 2007; **204(12)**:3027-3036.

13. Parsons MS, Wren L, Isitman G, Navis M, Stratov I, Bernard NF*, et al.* HIV infection abrogates the functional advantage of natural killer cells educated through KIR3DL1/HLA-Bw4 interactions to mediate anti-HIV antibody-dependent cellular cytotoxicity. *J Virol* 2012; **86(8)**:4488-4495.

14. Pollara J, Hart L, Brewer F, Pickeral J, Packard BZ, Hoxie JA*, et al.* High-throughput quantitative analysis of HIV-1 and SIV-specific ADCC-mediating antibody responses. *Cytometry A* 2011; **79(8)**:603-612.

15. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA*, et al.* Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 2006; **25(2)**:331-342.

16. Kamya P, Tallon B, Melendez-Pena C, Parsons MS, Migueles SA, Connors M*, et al.* Inhibitory Killer Immunoglobulin-like Receptors to self HLA-B and HLA-C ligands contribute differentially to Natural Killer cell functional potential in HIV infected slow progressors. *Clin Immunol* 2012; **143(3)**:246-255.

# Chapter 6: Summary of original scholarship

**Summary of Original Scholarship**

From chapter 2

1. The results presented in chapter 2 are the first to show evidence that human NK cells are educated through interactions between KIR3DL1*004, an allele that is not detected on the surface of NK cells by Abs recognizing other KIR3DL1 alleles, and HLA-Bw4.
2. These results imply that either KIR3DL1*004 cycles to the cell surface long enough to support NK cell licensing through interactions with its Bw4 ligand or that NK cell licensing can somehow occur through interactions with this molecule intracellularly.
3. While others have shown that the combination of KIR3DL1*004 and HLA-Bw4 is associated with slower time to AIDS in those infected, we are the first to demonstrate that this combination is not associated with protection from infection in HESN.

From chapter 3

1. Results presented in chapter 3 demonstrate for the first time that in HIV uninfected subjects NK cell education is a determinant of the ADCC functional potential of NK cells activated by autologous anti-HIV ADCC target cells.

2. Results in this chapter also provide the first evidence that the education of NK cells for ADCC function is abrogated in HIV-infected individuals.

From chapter 4

1. Data shown in chapter 4 provide the first demonstration that soluble factors in sera from HIV-infected individuals can skew anti-HIV ADCC responses towards a degranulation-biased response.

2. This information is the first to demonstrate that IL-10 enhances NK cell-mediated anti-HIV ADCC.

3. In chapter 4 we also show for the first time that the functional advantage of educated NK cells against autologous anti-HIV ADCC targets is maintained in the presence of IL-15.

From chapter 5

1. Included in chapter 5 are results that provide the first demonstration that the AIDS-protective **h*y+B*57* combination confers NK cells with enhanced ADCC functional potential.

Together these observations provide additional support for the importance of NK cell education as a determinant of diverse NK cell-mediated effector functions. Indeed, the research described in this thesis demonstrates that the NK cell education model can at least partially explain: (i) the ability of NK cells to respond to HLA-devoid target cells (chapter 2), (ii) the ability of NK cells to mediate anti-HIV ADCC against autologous target cells (chapter 3), (iii) the loss of NK cell functionality observed in the context of HIV infection (chapter 3), (iv) the differential ability of NK cells from different individuals to mediate ADCC after cytokine stimulation (chapter 4), and (v) the enhanced ADCC functional potential observed in individuals carrying the AIDS-protective **h*y+B*57* combination (chapter 5).

Aside from demonstrating the impact of NK cell education on multiple aspects of NK cell functions, these data also provide a basis for understanding how NK cell-mediated effector functions confer protection against HIV infection or progression to AIDS . As such, the data presented in this thesis also provide a foundation for the rational design and immune monitoring of HIV vaccines. As well, the work described in this thesis contributed to improving our understanding of the potential protective immune responses induced by the recent RV144 trial.

# Chapter 7: Discussion

## 7.1: Building a paradigm: Does education conferred NK cell functionality influence protective outcomes in the context of HIV?

As previously discussed, epidemiological studies have demonstrated that co-carriage of several allelic combinations of *KIR3DL1* and *HLA-Bw4* ligands is associated with protection from both HIV infection and time to AIDS[57;74]. Of relevance to this thesis Martin et al. (2007) reported that the *KIR3DL1*004/HLA-Bw4* combination had a significant effect on slowing time to AIDS, as compared to homozygosity for *HLA-Bw6*[74]. HLA-Bw6 does not interact with any KIR3DL1 receptor and therefore cannot participate in NK cell education through KIR3DL1. Previous work from our lab showed that another protective *KIR3DL1/HLA-Bw4* combination, **h*y+B*57*, was associated with educating NK cells for potent polyfunctional potential[56]. Consistent with the literature regarding polyfunctional anti-HIV $CD8^+$ T-lymphocyte responses, polyfunctional NK cells exhibited higher levels of each function than corresponding monofunctional NK cells[226]. Cumulatively, polyfunctional $CD8^+$ T cells and NK cells have been associated with protective outcomes in the context of HIV infection[56;65]. In chapter 2 we sought to determine whether there was a link between the influence of carriage of *KIR3DL1*004/HLA-Bw4* on the time to progression to AIDS and NK cell education mediated by this receptor ligand pair. We sought to address this by determining whether NK cells from individuals with this KIR/HLA genotype combination had superior polyfunctional potential compared to NK

cells from subjects carrying other *KIR3DL1/HLA-Bw4* combinations, or carrying *KIR3DL1* in the absence of *HLA-Bw4* (i.e. *Bw6* hmz).

We found that NK cells from individuals with the *KIR3DL1*004/HLA-Bw4* combination had superior polyfunctional potential to those from subjects who were *HLA-Bw6* hmz or who carried different *KIR3DL1/HLA-Bw4* combinations. This result is consistent with the hypothesis that the KIR3DL1*004/HLA-Bw4 combination functions in NK cell education, that NK cell education is a determinant of NK function and that potent NK functional potential is associated with slow time to AIDS in those infected. The observation that NK cells from *KIR3DL1*004/HLA-Bw4* carriers have superior functional potential to carriers of the receptor alone or ligand alone raises an interesting question. How does the inhibitory KIR3DL1*004 receptor participate in education since it is not expressed on the cell surface? One explanation may be provided by Taner et al. (2011), who demonstrated the KIR3DL1*004 allele is transiently expressed on the cell surface[227]. Indeed, the surface expression of KIR3DL1*004 can be visualized, if the cells are cultured at sub-physiological temperatures that slow cellular protein turnover. This observation may be useful for future investigations addressing the ability of KIR3DL1*004 to participate in NK cell education. First, it could be investigated whether NK cells mediate responses against HLA-devoid targets at sub-physiological temperatures. If so, it may be possible to assess whether KIR3DL1*004-expressing NK cells interact with HLA-Bw4 expressing cells. Furthermore, this cell culture system could be used to

determine whether KIR3DL1*004-expressing NK cells from HLA-Bw4 carriers mediate higher responses in this environment than the same cells from HLA-Bw6 hmz.

The observation that *KIR3DL1*004/HLA-Bw4* co-carriage confers NK cells with heightened functional potential is consistent with similar observations made regarding NK functional profiles for other KIR/HLA combinations that protect against HIV infection and/or disease progression[56]. Cumulatively, these observations are consistent with the paradigm that NK cells are protective against HIV infection and disease progression due to their ability to mediate potent anti-viral effector functions.

## 7.2: Current views on how NK cells protect against HIV infection and progression to AIDS

Coinciding with the observation that protective receptor/ligand pairs confer enhanced functional potential, it has been generally assumed that NK cells mediate protection against HIV through cytolysis of HIV infected targets and/or the secretion of pro-inflammatory cytokines and chemokines that impact viral replication[198]. This is supported by the fact that many studies have demonstrated the *ex vivo* functional behavior of NK cells to be an important indicator of HIV disease status. For example, *ex vivo* analyses of NK cells from progressors with high viral loads and/or low CD4 T-cell counts exhibit decreased ADCC potential

and lysis of HLA-devoid tumor cells[199;228]. These alterations in functionality are often associated with phenotypic changes within the NK cell population, which is reflected by a decrease in highly functional $CD16^{+}CD56^{dim}$ NK cells and an increase in non-functional $CD16^{+}CD56^{-}$ NK cells[199;229]. It is thought that this phenotypic change is related to chronic NK cell stimulation within the context of HIV infection, as *in vitro* stimulation of NK cells has also been demonstrated to lead to similar alterations in NK cell phenotypes[230]. The association of NK cell functionality with HIV disease status has been corroborated by studies of NK cell function in HIV-infected SP including LTNP and EC and patients successfully treated with antiretroviral drugs. For example, SP are more likely to carry KIR/HLA genotypes that confer heightened functionality[56;74]. Studies on the effect of successful antiretroviral therapy on NK cells have revealed that successful treatment, as defined by suppression of viral replication, is associated with partial restoration of NK cell functionality and a decrease in phenotypically aberrant $CD16^{+}CD56^{-}$ NK cells[199]. Indeed, we reaffirmed this observation in chapter 3, demonstrating that CD4 T cell counts were correlated with higher ADCC activation in HAART treated patients. Studies in primate models of immunodeficiency virus infection further corroborate the results in humans. Natural hosts for SIV that do not develop AIDS, such as sooty mangabeys, exhibit enhanced levels of NK cells with high functional potential after infection, whereas pathogenic SIV infections in rhesus macaques induce a decline in highly functional NK cells[231]. Lastly, unlike HIV infection in humans, infection of

chimpanzees with HIV, which often does not lead to an AIDS-like syndrome, does not disrupt the highly functional $CD8^+$ NK cell subset[232]. It is important to note here that while it is clear that there is an association between NK function and stage of disease progression the cause and effect relationship between the two phenomena is not clear. SP, particularly EC, have low viral loads, which may preserve NK cell function. Meanwhile, the higher viral loads seen in untreated typical progressors may impair NK activity. On the other hand, potent NK activity may control viral load in HIV infected subjects.

Research evaluating the *ex vivo* activities of NK cells from humans infected with HIV and primates infected with SIV has demonstrated that reduced NK cell functional potential is associated with poor prognostic markers. These data have been corroborated by the assessment of the functional behavior of NK cells from HESN. Indeed, it has been demonstrated that HESN exposed through injection drug use exhibit higher NK cell functionality than non-exposed individuals[55]. Previous work from our research team has found a higher frequency of *KIR3DL1*h/*y+HLA-B*57* among HESN[57], a genotype associated with higher NK functional potential than other KIR/HLA genotypes in both HIV infected SP and uninfected individuals[56;226]. In combination, these observations suggest that KIR/HLA genotype influences functionality. In uninfected individuals functionally potent NK cells may be able to recognize and eliminate HIV infected cells ultimately preventing HIV infection. In infected subjects functionally potent NK cells may be able to stall HIV infected cells, thus slowing the progression to

AIDS either because they remain more functional in the context of low viremia or because they are better able to control viral replication.

### 7.3: NK cell responses to autologous HIV-infected cells reveal deficiencies in the role of direct NK cell activity in inhibition of viral replication

KIR/HLA genotype combinations are determinants of NK cell functionality[177;178;184]. As exemplified in chapter 2, NK cells from several of the KIR/HLA genotypes associated with slower progression to AIDS have higher functional potential than those not associated with favorable outcomes. Regardless, research assessing the ability of NK cells to respond to autologous HIV infected $CD4^+$ T-lymphocytes has raised many questions regarding how NK cells provide protection against HIV. Initially, studies investigating the ability of NK cells to recognize and lyse autologous HIV-infected $CD4^+$ T-lymphocytes revealed that instead of being liberated by the viral-induced downregulation of HLA-B[233], NK cells were inhibited from direct cytotoxicity and ADCC by the maintenance of HLA-C and increased HLA-E surface expression levels[126;127]. Indeed, blocking the interaction of these HLA molecules with inhibitory NK cell receptors with Abs was sufficient to allow NK cells to lyse autologous HIV-infected $CD4^+$ T-lymphocytes. Despite previous demonstrations that HIV proteins impede the ability of ligands for activating NK cell receptors to reach the cell surface[234], this research suggests that HIV-infected cells express sufficient ligands for activating receptors to allow NK cell activation, if inhibitory receptors are not

themselves ligated. More recent research has supported the notion that HIV-infected cells are sufficient to activate NK cells, especially if the NK cells are activated with cytokines prior to incubation with targets[103;104]. However, the results of these experiments do not support the notion that protective KIR/HLA combinations are protective because they endow NK cells with a better ability to respond to autologous HIV-infected $CD4^+$ T-lymphocytes. Rather they reveal that while NK cells expressing KIRs belonging to non-protective KIR/HLA combinations are capable of responding to autologous HIV-infected $CD4^+$ T-lymphocytes[103], NK cells expressing KIR3DL1, and obtained from HLA-Bw4 carriers, are incapable of degranulating upon encountering these target cells[104].

### 7.4: ADCC in HIV disease progression

The inability of KIR3DL1-expressing NK cells from HLA-Bw4 carriers to degranulate upon encountering autologous HIV-infected target cells raises the possibility that other mechanisms of NK cell activation should be considered. Aside from activation through direct interaction with target cells, NK cells can be activated by interacting with target cells coated with Abs and mediate ADCC. Although research from the cancer literature suggests that activation through interactions between CD16a and Ab constant regions can overcome KIR-mediated inhibition[218], incubation of NK cells with autologous HIV-infected $CD4^+$ T-lymphocytes in the presence of ADCC-competent Abs has demonstrated that the NK cells are inhibited by interactions between inhibitory receptors and HLA-C

and HLA-E[126]. In chapters 3 and 4 we showed that KIR3DL1$^+$ NK cells can overcome inhibitory signals delivered through interactions with HLA-Bw4 ligands to respond to autologous cells that have been coated with HIV envelope peptides and anti-HIV Abs. However, these target cells do not adequately reflect HIV-infected target cells, as they exhibit no alteration to the normal cell surface self-environment and most likely express higher levels of HIV peptide antigen than would be observed on an infected cell. As such, the differences between the results obtained for the anti-HIV peptide ADCC assay and those reported for anti-HIV ADCC against autologous infected targets most likely reflect differences in surface-bound antigen/antibody complexes. Most of the assays evaluating anti-HIV ADCC have strengths and weaknesses. These assays often use non-autologous cell lines, such as the CEM-NKr CD4$^+$ T-lymphocyte line[173], or utilize coating strategies that achieve HIV antigen levels that are higher than those achieved through physiological HIV infection and replication. Aside from the issue of whether autologous HIV-infected CD4$^+$ T-lymphocytes activate NK cells efficiently enough through ADCC to result in cytolysis of target cells, acceptance of anti-HIV ADCC as a potential mechanism of protection from disease progression faces many fundamental hurdles.

The current understanding of HIV disease pathogenesis and NK cell ontogeny calls into question the concept that anti-HIV ADCC is protective in the context of chronic HIV infection. HIV infection is characterized by constant and high-level viral replication[235]. Although these viral particles maintain surface-

bound envelope, much of the unstable envelope is released from the viral membrane into the extracellular environment. Such free-floating envelope may be able to bind CD4 on non-infected cells[145]. As such, these non-infected cells could potentially serve as targets for ADCC if they are bound by anti-HIV Abs and recognized by NK cells. Furthermore, given the paucity of HIV-infected cells in HIV-infected individuals, non-infected $CD4^+$ T-lymphocytes, with surface bound HIV envelope, likely represent a larger ADCC target population than infected cells. It is possible that this phenomenon could account for some of the large amounts of destruction of non-infected bystander cells.

A major conundrum with respect to the concept that NK cell-mediated ADCC can provide protection during chronic HIV infection is the observation that HIV infection induces high levels of autoAbs[236-238]. These autoAbs target a wide variety of cell surface receptors, such as HLA and CD4. Furthermore, target cells coated with Abs are susceptible to ADCC, and the presence of these Abs is associated with disease progression. As HIV infected cells often downregulate CD4 and HLA[233;239], these autoAbs could bind to non-infected cells, making them potential NK cell targets.

Another problem with the idea that NK cell-mediated ADCC is protective against HIV disease progression is that chronic stimulation through CD16a induces similar alterations to the NK cell phenotype as those observed during chronic HIV infection. *In vitro* studies demonstrate that chronic ADCC stimulation of NK cells results in the downregulation of CD16 and CD56[230]. Similarly, reduced

CD56 expression on NK cells is characteristic of a subset of NK cell in HIV infected individuals with progressive disease[199;229]. These observations suggest that chronic stimulation of NK cells during HIV infection could actually enhance disease progression by targeting bystander cells and weakening the ability of NK cells to attack cells altered by virus infection or transformation.

Furthermore, HIV infection is associated with a decline in the ability of NK cells to mediate ADCC[228]. In chapter 3 we show that this decline in ADCC potential is related to a decline in the ability of NK cells educated through KIR3DL1 to mediate ADCC. Similarly, we have also evaluated a cohort of SP using the GranToxiLux assay and observed that there was no educational advantage for mediating of ADCC for NK cells from carriers of the *h*y*+B*57* genotype, as compared to those from *HLA-Bw6* hmz (Data not shown). This is in contrast to the results reported in chapter 5 for NK cells from uninfected individuals. Together such observations are difficult to reconcile with a hypothesis that predicts education conferred functionality is responsible for protection from disease progression.

Despite epidemiological evidence linking NK cell KIR/HLA genotypes that confer enhanced functionality with protection from HIV disease progression and an association between enhanced NK cell function and slower disease progression, several studies suggest that high NK cell activity could be detrimental in the context of HIV infection. Indeed, it has been demonstrated that NK cells lyse activated uninfected $CD4^+$ T-lymphocytes during HIV infection,

and that this can contribute to the $CD4^+$ T-lymphocyte decline[197;212;214;240]. In summary, much of the experimental evidence and theoretical ideas described above point to a unifying model where NK cell activity contributes to HIV disease progression.

**7.5: Is ignorance bliss?**

Given these perspectives, how do we reconcile the studies that demonstrate correlations between heightened ADCC and protection from disease progression in HIV infected humans or SIV infected primates?[71;220] Also, how do we explain the association of NK cell functional potential with HIV disease state? Does not the observation that KIR/HLA combinations, which themselves confer heightened NK cell functionality and are protective in terms of being associated with slower time to AIDS also suggest that high NK cell functionality is beneficial after HIV infection?[56;226] Although KIR/HLA combinations do contribute to NK cell education for functional potential, they are also responsible for regulating uncontrolled autoaggressive function. Given that cells expressing protective KIR3DL1/HLA-Bw4 combinations have been demonstrated to be hypofunctional against autologous HIV-infected $CD4^+$ T-lymphocytes[104], we hypothesize that it is the regulatory component of these KIR/HLA interactions that is protective against progression to AIDS. Furthermore, we hypothesize that the heightened levels of NK cell functionality observed in individuals exhibiting slow progression

towards AIDS reflects the fact that their NK cells are not exhausted from chronic activation.

Epidemiological and *ex vivo* functional analyses have attracted attention to the potential of NK cells to control HIV disease progression. For several reasons, the associations of high *ex vivo* functionality and KIR/HLA combinations that confer high functionality with slower disease progression need to be interpreted with caution. First, the observation that those individuals with higher *ex vivo* functioning NK cells have less disease progression does not suggest that their NK cells are more functional *in vivo*. Indeed, high *ex vivo* function could reflect a low level of *in vivo* function. This suggestion is supported by the fact that chronic NK cell stimulation leads to NK cell exhaustion and alterations in CD16 and CD56 expression[230]. Similarly, the level of functionality observed in NK cells isolated from HIV infected individuals is negatively correlated with the size of the non-functional $CD16^{+}CD56^{-}$ NK cell subset[199]. This suggests that high *ex vivo* NK cell activity could be reflective of low levels of NK cell activation *in vivo.* Another point that should be considered has to do with the KIR/HLA combinations that confer enhanced NK cell functional potential. It should be noted that the increased functional potential of these cells has been demonstrated using HLA-devoid non-autologous cell lines[56;226]. As such, the levels of functionality exerted against these cell lines may not reflect the functional potential of these cells against autologous HIV-infected targets. Indeed, although *KIR3DL1/HLA-Bw4* combinations have been shown to educate

NK cells for responses against K562 (chapter 2) and ADCC targets (chapters 3-5), this KIR/HLA combination does not appear to confer NK cells with a heightened ability to lyse autologous HIV-infected $CD4^+$ T-lymphocytes[104]. Furthermore, NK cells carrying non-protective KIR/HLA combinations readily degranulate upon exposure to autologous infected $CD4^+$ T-lymphocytes[103]. Although Dr. Galit Alter and Dr. Marcus Altfeld have interpreted their experimental results as supporting the idea that heightened NK cell functionality may be desirable for slowing HIV disease progression, little evidence exists to suggest this is the case.

Recent studies support the notion that high *in vivo* NK cell functionality could be detrimental during HIV infection. For example murine studies show that high *in vivo* NK cell activation is associated with the establishment of chronic viral infections and reduced cytotoxic $CD8^+$ T-lymphocyte responses[241]. Although the exact mechanisms underlying this process have not yet been elucidated, NK cell depletion studies have confirmed a role for NK cells in suppressing anti-viral $CD8^+$ T-lymphocyte responses, which allow the infection to persist chronically. This suppression has been linked to NK cell perforin-mediated cytolysis, and this cytolysis has been demonstrated to be directed at two different cell types – activated $CD4^+$ and activated $CD8^+$ T-lymphocytes[241-243]. Other studies have demonstrated that human NK cells can kill activated uninfected autologous T-cells[244]. Cumulatively, these results challenge the concept that high *in vivo* NK cell functionality is responsible for slow HIV disease progression. NK cell mediated lysis of $CD4^+$ T-lymphocytes could contribute to HIV-associated

depletion of this cellular subset; whereas, lysis of $CD8^+$ T-lymphocytes could eliminate anti-viral CTL and allow more extensive viral replication, similar to the effects of Ab-mediated CTL depletion on viral replication[77;78].

As an alternative to the hypothesis that high *in vivo* NK cell activation slows HIV disease progression, we propose that NK cells carrying protective KIR/HLA combinations may be capable of being inhibited by KIR/HLA interactions sparing autologous infected T-lymphocytes, and activated uninfected T-lymphocytes. As such, NK cell activation would not lead to the destruction of important anti-viral adaptive immune responses or contribute to the depletion of $CD4^+$ T-lymphocytes.

Lastly, it should be highlighted that the situation in HESN subjects is quite different from that of chronic HIV infection. Exposure to HIV may involve exposure to a Trojan horse cell, which is an HIV-infected cell from the transmitting partner.[245] As these cells may be recognized as foreign by the NK cells of the exposed individual, we hypothesize that NK cell activation in the context of HIV exposure could account for why some KIR/HLA genotypes are protective against HIV infection. Furthermore, although ADCC may not be sufficient to protect from disease progression, it is possible that the presence of anti-HIV ADCC Abs could be sufficient to target Trojan horse cells or early infected autologous cells and prevent the establishment of HIV infection.

**7.6: Trojan War or guerrilla warfare: Mechanism of HIV infection may be essential to understanding KIR/HLA mediated protection from infection**

Although our interpretation of the existing data favor a model in which high level *in vivo* NK cell anti-viral function is not a determinant of slow time to AIDS and low viral load, it is highly likely that *in vivo* NK cell anti-viral functionality plays a role in preventing the establishment of HIV infection in HESN. To prevent HIV infection following exposure, rapid clearance of the virus is essential. As already reviewed, several KIR/HLA combinations have been associated with this protection[57;58]; however, how NK cells from individuals carrying these combinations mediate protection from infection is as yet unknown. Essential to elucidating the mechanism of NK cell mediated protection is a greater understanding of how HIV is transmitted between individuals.

The transmission of HIV between individuals occurs through the sharing of bodily fluids such as semen or blood. These fluids contain two forms of HIV: free virions and cell-associated (i.e., infected cells)[245]. Although both forms of the virus are capable of infecting cells *in vitro* and in *in vivo* in animal models, it is unknown which form of the virus is responsible for the majority of transmission between humans. Several studies have attempted to solve this question, and have provided reasonable evidence that cell-to-cell transmission of HIV could account for some infections. These studies have demonstrated that infection of rhesus macaques with cell associated virus requires an amount of virus that is more reflective of viral titers in semen than similar infections utilizing free

virions[246]. Moreover, analysis of the founder viruses in newly infected individuals has revealed that the majority of these have viral sequences that more closely resemble the cell-associated virus in their transmission partner's semen than the free virions[247].

These observations may help explain why certain KIR/HLA combinations are protective against HIV transmission even though they do not appear to respond to infected autologous cells. Exposed individuals with education-induced highly functional NK cells may be able to lyse and clear infected cells from HIV-infected partners that have different HLA backgrounds, which would be less likely to inhibit NK cells from exposed individuals. Along the same lines, it may also be possible to explain why these combined genotypes are not always protective. For example, if the exposed individual and the infected partner have a similar HLA background, the NK cells may be inhibited and prevented from clearing the virally infected cells. Furthermore, if the transmission occurs through free virions that infect the cells of the exposed individual, clearance may not be possible as the NK cells may be inhibited by the self HLA background.

**7.7: ADCC antibodies: Friends with benefits?**

It is at this junction where it becomes obvious that another NK cell effector function, ADCC, may provide additional protection upon HIV exposure. Activation of NK cells through ADCC provides additional activating signals that

have previously been demonstrated to overcome inhibition through KIR/HLA interactions[218]. As such, if anti-HIV Env Abs were present upon exposure, they could coat infected allogeneic and/or autologous cells and allow their lysis by NK cells. This represents a different situation than chronic infection, as the number of infected cells present in the first week or two after exposure is small, NK cells would not be diverted by the large amounts of uninfected cells carrying Env, there is a lack of autoAbs prior to infection, and NK cells would not be required to be chronically activated as they would only need to clear the small number of infected cells. Furthermore, the NK cells present at this time would have normal function as their ADCC functional potential would not have had a chance to be abrogated by HIV infection.

The idea that anti-HIV ADCC could be protective against HIV transmission has been supported by several human and primate vaccine and passive transfer studies. First and foremost, recent speculation regarding the potential for ADCC responses to mediate protection against infection was instigated by the demonstration that the BnAb b12 lost some of its ability to protect macaques from a SHIV challenge if it was modified to an ADCC non-competent Ab prior to passive administration to macaques[219]. This elegant study was followed by a macaque vaccine study that has suggested that ADCC was a correlate of protection[248]. Lastly, immune correlate analysis of samples from the RV144 Thai HIV vaccine trial, which provided a modest level of protection against HIV acquisition, points to non-neutralizing Ab activity and possibly ADCC-like activity

as important effector functions involved in protection[88]. This vaccine did not induce robust immune responses, such as BnAbs or CTL, that have traditionally been considered important for protection from HIV infection[87]. However, the vaccine regimen does induce high titers of ADCC competent Abs[90]. Furthermore, a recent analysis of immune correlates from this trial has demonstrated that ADCC does correlate with a lower incidence of HIV acquisition, but only in individuals with low levels of plasma IgA[88]. This observation has led investigators to suggest that the plasma IgA antibodies are capable of inhibiting ADCC by competing with anti-HIV IgG for binding to ADCC epitopes.

It is remarkable that the above-mentioned correlate of immunity analysis has uncovered a significant relationship between ADCC levels and rates of HIV acquisition. This analysis investigates the potential of vaccine induced Abs to mediate ADCC using NK cells from a single high-responding donor and target cells from a common cell line. A more thorough analysis should take into consideration the KIR/HLA background of the individuals receiving the vaccine. In chapters 3-5 we demonstrate that the relative ability of NK cells to mediate ADCC against both allogeneic and autologous cells is determined by the process of NK cell education. As such, even in the presence of ADCC competent Abs, it is unlikely that protection would be observed in individuals without efficient NK cell education. Merely investigating the potential of vaccine induced Abs to mediate ADCC is equivalent to investigating the effectiveness of passively transferred HLA-specific CTL in an individual with mismatched HLA. Future

analyses need to consider the role of NK cell education in anti-HIV ADCC responses. Although the recent RV144 vaccine trial analysis demonstrated ADCC as an immune correlate associated with reduced HIV acquisition, the level of significance of this factor could be underappreciated due to an incomplete analysis of all the variables involved in ADCC responses.

**7.8: Conclusion**

The data presented in this thesis clearly demonstrate that NK cell education through KIR3DL1/HLA-Bw4 interactions determines the potential of NK cells to mediate functionality upon direct and/or antibody-dependent stimulation. In particular, we showed that allelic combinations of KIR/HLA that protect against HIV infection and/or disease progression confer the greatest level of education. These observations coincide with previous demonstrations of enhanced functionality in NK cells obtained from individuals protected from HIV infection or disease progression[55;72]. Previously, such observations have led to suggestions that NK cell activation directed against HIV-infected cells could account for the association of NK cells with protection from HIV infection or disease progression[198]. However, the data presented in chapter 3 of this thesis demonstrate that NK cell functional potential is abrogated in HIV-infected individuals, in a manner that is reflective of aberrant NK cell education during HIV infection. Together with previous observations of NK cell-mediated

immunopathology in HIV-infected individuals[197;212], this finding calls into question an interpretation in which NK cell mediated activation plays a role in slow progression to AIDS. However, the data presented in chapter 3 and 5 of this thesis using cells from uninfected individuals demonstrates that NK cell education is a determinant of NK cell mediated ADCC functional potential. These data, in combination with recent suggestions that HIV vaccines be designed to utilize anti-HIV ADCC, raises the specter that HIV infection could be prevented by vaccines harnessing the potential of the innate immune system through the induction of adaptive immune responses[89]. However, the observation that ADCC is dependent upon KIR/HLA-mediated NK cell education should raise concerns about the universal applicability of such vaccines, as similar anti-viral Abs will mediate ADCC responses of variable magnitude and functionality in different individuals according to their KIR/HLA background.

In summary, the data presented in this thesis are consistent with a model whereby KIR/HLA combinations provide protection against HIV infection and disease progression via two divergent mechanisms. NK cells could provide protection against infection by recognizing and destroying allogeneic or autologous HIV-infected cells directly or through ADCC; whereas, protection from progression to AIDS would be provided by the ability of inhibitory KIR/KIR combinations to prevent NK cell-mediated immunopathology.

# Appendix: Supplementary data

Supplementary Table 1

| Subject ID~ | HAART treatment | Viral load <50 | Time on HAART (Years) | Post-HAART CD4 recovery | Current CD4 T-lymphocyte count | HLA-B Type* |
|---|---|---|---|---|---|---|
| HIV01 | Yes | Yes | 0.25 | 72 | 320 | B15:**B57** |
| HIV02 | Yes | Yes | 1.92 | 524 | 872 | **B44**;**B51** |
| HIV03 | Yes | Yes | 11 | 351 | 886 | B18; **B44** |
| HIV04 | Yes | Yes | 10.25 | 324 | 469 | **B44**;**B53** |
| HIV05 | Yes | Yes | 5.75 | 168 | 311 | **B27**;**B27** |
| HIV06 | Yes | Yes | 13.50 | 734 | 941 | B35;**B57** |
| HIV07 | No | No | n/a | n/a | 520 | B40;**B51** |
| HIV08 | Yes | Yes | 14.08 | 856 | 1060 | **B44**;B62 |
| HIV09 | No | No | n/a | n/a | 1033 | **B27**;B35 |
| HIV10 | Yes | Yes | 5.92 | 806 | 990 | **B44**;**B44** |
| HIV11 | No | No | n/a | n/a | 404 | B07;**B49** |
| HIV12 | Yes | Yes | 0.75 | 133 | 588 | B07;B18 |
| HIV13 | No | No | n/a | n/a | 454 | B08;B08 |
| HIV14 | Yes | Yes | 9.25 | 458 | 634 | B08;B40 |
| HIV15 | Yes | Yes | 13.33 | 63 | 225 | B07;B08 |
| HIV16 | Yes | Yes | 14.50 | 647 | 857 | B07;B14 |
| HIV17 | Yes | Yes | 7.5 | 418 | 599 | B14;B40 |
| HIV18 | Yes | Yes | 6.42 | 490 | 634 | B07;B40 |
| HIV19 | Yes | Yes | 9.75 | 0 | 799 | B07;B40 |
| HIV20 | Yes | Yes | 3.42 | 277 | 393 | B18;B35 |
| HIV21 | Yes | Yes | 2.83 | 473 | 360 | B08;**B44** |
| HIV22 | Yes | Yes | 0.67 | 108 | 355 | B39:**B44** |
| HIV23 | Yes | Yes | 7 | 497 | 426 | B15;**B51** |
| HIV24 | Yes | Yes | 1.17 | 69 | 403 | B08;**B57** |
| HIV25 | Yes | Yes | 1.75 | 155 | 528 | **B51;**B54 |
| HIV26 | Yes | Yes | 1.08 | 183 | 716 | **B44**;**B51** |
| HIV27 | Yes | Yes | 7.92 | 64 | 395 | **B44**;**B44** |
| HC01 | n/a | n/a | n/a | n/a | n/a | **B44**;**B51** |

| | | | | | | |
|---|---|---|---|---|---|---|
| HC02 | n/a | n/a | n/a | n/a | n/a | **B44**;**B44** |
| HC03 | n/a | n/a | n/a | n/a | n/a | B07;**B44** |
| HC04 | n/a | n/a | n/a | n/a | n/a | **B44**;**B57** |
| HC05 | n/a | n/a | n/a | n/a | n/a | **B13**;B35 |
| HC06 | n/a | n/a | n/a | n/a | n/a | B07;**B44** |
| HC07 | n/a | n/a | n/a | n/a | n/a | B40;**B44** |
| HC08 | n/a | n/a | n/a | n/a | n/a | B40;**B51** |
| HC09 | n/a | n/a | n/a | n/a | n/a | B18;**B38** |
| HC10 | n/a | n/a | n/a | n/a | n/a | B60;**B51** |
| HC11 | n/a | n/a | n/a | n/a | n/a | B07;**B44** |
| HC12 | n/a | n/a | n/a | n/a | n/a | B08,B15 |
| HC13 | n/a | n/a | n/a | n/a | n/a | B35;B35 |
| HC14 | n/a | n/a | n/a | n/a | n/a | B07;B41 |
| HC15 | n/a | n/a | n/a | n/a | n/a | B39;B40 |
| HC16 | n/a | n/a | n/a | n/a | n/a | B07;B18 |
| HC17 | n/a | n/a | n/a | n/a | n/a | B35;B55 |

˜HIV-infected subjects are coded are HIV00 and healthy control subjects are coded as HC00.

*HLA-Bw4 carrying alleles are bolded.

**References**


(1) Kornfeld H, Vande Stouwe RA, Lange M, Reddy MM, Grieco MH: T-lymphocyte subpopulations in homosexual men. N Engl J Med 1982; 307(12):729-731.

(2) Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California: MMWR Morb Mortal Wkly Rep 1981; 30(25):305-308.

(3) Gottlieb MS, Schroff R, Schanker HM et al.: Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med 1981; 305(24):1425-1431.

(4) Vieira J, Frank E, Spira TJ, Landesman SH: Acquired immune deficiency in Haitians: opportunistic infections in previously healthy Haitian immigrants. N Engl J Med 1983; 308(3):125-129.

(5) Small CB, Klein RS, Friedland GH et al.: Community-acquired opportunistic infections and defective cellular immunity in heterosexual drug abusers and homosexual men. Am J Med 1983; 74(3):433-441.

(6) Feigal EG: AIDS-associated malignancies: research perspectives. Biochim Biophys Acta 1999; 1423(1):C1-C9.

(7) Moll B, Emeson EE, Small CB et al.: Inverted ratio of inducer to suppressor T-lymphocyte subsets in drug abusers with opportunistic infections. Clin Immunol Immunopathol 1982; 25(3):417-423.

(8) Van de Perre P, Rouvroy D, Lepage P et al.: Acquired immunodeficiency syndrome in Rwanda. Lancet 1984; 2(8394):62-65.

(9) Clumeck N, Sonnet J, Taelman H et al.: Acquired immunodeficiency syndrome in African patients. N Engl J Med 1984; 310(8):492-497.

(10) Lederman MM, Ratnoff OD, Scillian JJ, Jones PK, Schacter B: Impaired cell-mediated immunity in patients with classic hemophilia. N Engl J Med 1983; 308(2):79-83.

(11) Rubinstein A, Sicklick M, Gupta A et al.: Acquired immunodeficiency with reversed T4/T8 ratios in infants born to promiscuous and drug-addicted mothers. JAMA 1983; 249(17):2350-2356.

(12) Gallo RC, Salahuddin SZ, Popovic M et al.: Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 1984; 224(4648):500-503.

(13) Popovic M, Sarngadharan MG, Read E, Gallo RC: Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 1984; 224(4648):497-500.

(14) Sarngadharan MG, Popovic M, Bruch L, Schupbach J, Gallo RC: Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. Science 1984; 224(4648):506-508.

(15) Schupbach J, Popovic M, Gilden RV et al.: Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. Science 1984; 224(4648):503-505.

(16) UNAIDS. World AIDS day report 2011. Vol. 2011; 2011.

(17) UNAIDS. Report on global AIDS epidemic 2010. Vol. 2010; 2010.

(18) Barre-Sinoussi F, Chermann JC, Rey F et al.: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 1983; 220(4599):868-871.

(19) Levy JA: HIV and the pathogenesis of AIDS. 3rd Edition. ASM Press, Washington, 2007.

(20) Klatzmann D, Barre-Sinoussi F, Nugeyre MT et al.: Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science 1984; 225(4657):59-63.

(21) von BH, Andreesen R, Esser R et al.: Infection of monocytes/macrophages by HIV in vitro. Res Virol 1990; 141(2):225-231.

(22) Freed EO: HIV-1 replication. Somat Cell Mol Genet 2001; 26(1-6):13-33.

(23) Greene WC, Peterlin BM: Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. Nat Med 2002; 8(7):673-680.

(24) Hansasuta P, Rowland-Jones SL: HIV-1 transmission and acute HIV-1 infection. Br Med Bull 2001; 58:109-127.

(25) Lamptey PR: Reducing heterosexual transmission of HIV in poor countries. BMJ 2002; 324(7331):207-211.

(26) Prejean J, Song R, Hernandez A et al.: Estimated HIV incidence in the United States, 2006-2009. PLoS One 2011; 6(8):e17502.

(27) Gomez C, Hope TJ: The ins and outs of HIV replication. Cell Microbiol 2005; 7(5):621-626.

(28) Arhel N: Revisiting HIV-1 uncoating. Retrovirology 2010; 7:96.

(29) Mansky LM, Temin HM: Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol 1995; 69(8):5087-5094.

(30) Gao F, Chen Y, Levy DN et al.: Unselected mutations in the human immunodeficiency virus type 1 genome are mostly nonsynonymous and often deleterious. J Virol 2004; 78(5):2426-2433.

(31) Baeuerle PA, Henkel T: Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994; 12:141-179.

(32) Charania MR, Crepaz N, Guenther-Gray C et al.: Efficacy of structural-level condom distribution interventions: a meta-analysis of U.S. and international studies, 1998-2007. AIDS Behav 2011; 15(7):1283-1297.

(33) Leroy V, Sakarovitch C, Cortina-Borja M et al.: Is there a difference in the efficacy of peripartum antiretroviral regimens in reducing mother-to-child transmission of HIV in Africa? AIDS 2005; 19(16):1865-1875.

(34) Dabis F, Bequet L, Ekouevi DK et al.: Field efficacy of zidovudine, lamivudine and single-dose nevirapine to prevent peripartum HIV transmission. AIDS 2005; 19(3):309-318.

(35) Wodak A, Cooney A: Do needle syringe programs reduce HIV infection among injecting drug users: a comprehensive review of the international evidence. Subst Use Misuse 2006; 41(6-7):777-813.

(36) Wood E, Tyndall MW, Montaner JS, Kerr T: Summary of findings from the evaluation of a pilot medically supervised safer injecting facility. CMAJ 2006; 175(11):1399-1404.

(37) New pope reaffirms abstinence: AIDS Patient Care STDS 2005; 19(8):541-542.

(38) Small D: An appeal to humanity: legal victory in favour of North America's only supervised injection facility: Insite. Harm Reduct J 2010; 7:23.

(39) Duesberg PH: Human immunodeficiency virus and acquired immunodeficiency syndrome: correlation but not causation. Proc Natl Acad Sci U S A 1989; 86(3):755-764.

(40) Duesberg PH: Retroviruses as carcinogens and pathogens: expectations and reality. Cancer Res 1987; 47(5):1199-1220.

(41) Nattrass N: Defending the boundaries of science: AIDS denialism, peer review and the Medical Hypotheses saga. Sociol Health Illn 2011; 33(4):507-521.

(42) Chen LF, Hoy J, Lewin SR: Ten years of highly active antiretroviral therapy for HIV infection. Med J Aust 2007; 186(3):146-151.

(43) Richman DD, Morton SC, Wrin T et al.: The prevalence of antiretroviral drug resistance in the United States. AIDS 2004; 18(10):1393-1401.

(44) Wainberg MA, Zaharatos GJ, Brenner BG: Development of antiretroviral drug resistance. N Engl J Med 2011; 365(7):637-646.

(45) Nunez M: Clinical syndromes and consequences of antiretroviral-related hepatotoxicity. Hepatology 2010; 52(3):1143-1155.

(46) Scherzer R, Estrella M, Li Y et al.: Association of tenofovir exposure with kidney disease risk in HIV infection. AIDS 2012; 26(7):867-875.

(47) D'Ascenzo F, Cerrato E, Biondi-Zoccai G et al.: Acute coronary syndromes in human immunodeficiency virus patients: a meta-analysis investigating adverse event rates and the role of antiretroviral therapy. Eur Heart J 2012; 33(7):875-880.

(48) Nguyen ML, Farrell KJ, Gunthel CJ: Non-AIDS-Defining Malignancies in Patients with HIV in the HAART Era. Curr Infect Dis Rep 2010; 12(1):46-55.

(49) Horton RE, McLaren PJ, Fowke K, Kimani J, Ball TB: Cohorts for the study of HIV-1-exposed but uninfected individuals: benefits and limitations. J Infect Dis 2010; 202 Suppl 3:S377-S381.

(50) Miyazawa M, Lopalco L, Mazzotta F et al.: The 'immunologic advantage' of HIV-exposed seronegative individuals. AIDS 2009; 23(2):161-175.

(51) Clerici M, Levin JM, Kessler HA et al.: HIV-specific T-helper activity in seronegative health care workers exposed to contaminated blood. JAMA 1994; 271(1):42-46.

(52) Legrand FA, Nixon DF, Loo CP et al.: Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. PLoS One 2006; 1:e102.

(53) Letvin NL, Mascola JR, Sun Y et al: Preserved $CD4^+$central memory T cells and survival in vaccinated SIV-challenged monkeys. Science 2006; 312: 1530-1533.

(54) Tudor D, Derrien M, Diomede L et al.: HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4(+) cell infection: an IgA gene and functional analysis. Mucosal Immunol 2009; 2(5):412-426.

(55) Scott-Algara D, Truong LX, Versmisse P et al.: Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. J Immunol 2003; 171(11):5663-5667.

(56) Boulet S, Song R, Kamya P et al.: HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. J Immunol 2010; 184(4):2057-2064.

(57) Boulet S, Kleyman M, Kim JY et al.: A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. AIDS 2008; 22(12):1487-1491.

(58) Boulet S, Sharafi S, Simic N et al.: Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. AIDS 2008; 22(5):595-599.

(59) Long BR, Ndhlovu LC, Oksenberg JR et al.: Conferral of enhanced natural killer cell function by KIR3DS1 in early human immunodeficiency virus type 1 infection. J Virol 2008; 82(10):4785-4792.

(60) Mandalia S, Westrop SJ, Beck EJ et al.: Are long-term non-progressors very slow progressors? Insights from the Chelsea and Westminster HIV cohort, 1988-2010. PLoS One 2012; 7(2):e29844.

(61) Blankson JN: Effector mechanisms in HIV-1 infected elite controllers: highly active immune responses? Antiviral Res 2010; 85(1):295-302.

(62) Kloosterboer N, Groeneveld PH, Jansen CA et al.: Natural controlled HIV infection: preserved HIV-specific immunity despite undetectable replication competent virus. Virology 2005; 339(1):70-80.

(63) Lambotte O, Boufassa F, Madec Y et al.: HIV controllers: a homogeneous group of HIV-1-infected patients with spontaneous control of viral replication. Clin Infect Dis 2005; 41(7):1053-1056.

(64) Betts MR, Nason MC, West SM et al.: HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood 2006; 107(12):4781-4789.

(65) Thobakgale CF, Streeck H, Mkhwanazi N et al.: Short communication: CD8(+) T cell polyfunctionality profiles in progressive and nonprogressive pediatric HIV type 1 infection. AIDS Res Hum Retroviruses 2011; 27(9):1005-1012.

(66) Jansen CA, Kostense S, Vandenberghe K et al.: High responsiveness of HLA-B57-restricted Gag-specific CD8+ T cells in vitro may contribute to the protective effect of HLA-B57 in HIV-infection. Eur J Immunol 2005; 35(1):150-158.

(67) Altfeld M, Kalife ET, Qi Y et al.: HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1. PLoS Med 2006; 3(10):e403.

(68) Mothe B, Llano A, Ibarrondo J et al.: CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. PLoS One 2012; 7(1):e29717.

(69) Braibant M, Agut H, Rouzioux C et al.: Characteristics of the env genes of HIV type 1 quasispecies in long-term nonprogressors with broadly neutralizing antibodies. J Acquir Immune Defic Syndr 2008; 47(3):274-284.

(70) Euler Z, van Gils MJ, Bunnik EM et al.: Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. J Infect Dis 2010; 201(7):1045-1053.

(71) Lambotte O, Ferrari G, Moog C et al.: Heterogeneous neutralizing antibody and antibody-dependent cell cytotoxicity responses in HIV-1 elite controllers. AIDS 2009; 23(8):897-906.

(72) O'Connor GM, Holmes A, Mulcahy F, Gardiner CM: Natural Killer cells from long-term non-progressor HIV patients are characterized by altered phenotype and function. Clin Immunol 2007; 124(3):277-283.

(73) Vieillard V, Fausther-Bovendo H, Samri A, Debre P: Specific phenotypic and functional features of natural killer cells from HIV-infected long-term

nonprogressors and HIV controllers. J Acquir Immune Defic Syndr 2010; 53(5):564-573.

(74) Martin MP, Qi Y, Gao X et al.: Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet 2007; 39(6):733-740.

(75) Sodora DL, Ross TM: Simian immunodeficiency virus pathogenesis. Curr HIV Res 2009; 7(1):1.

(76) Li J, Lord CI, Haseltine W, Letvin NL, Sodroski J: Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. J Acquir Immune Defic Syndr 1992; 5(7):639-646.

(77) Mueller YM, Do DH, Boyer JD et al.: CD8+ cell depletion of SHIV89.6P-infected macaques induces CD4+ T cell proliferation that contributes to increased viral loads. J Immunol 2009; 183(8):5006-5012.

(78) Jin X, Bauer DE, Tuttleton SE et al.: Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 1999; 189(6):991-998.

(79) Parren PW, Marx PA, Hessell AJ et al.: Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human

immunodeficiency virus at serum levels giving complete neutralization in vitro. J Virol 2001; 75(17):8340-8347.

(80) Veazey RS, Shattock RJ, Pope M et al.: Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat Med 2003; 9(3):343-346.

(81) Trkola A, Kuster H, Rusert P et al.: Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat Med 2005; 11(6):615-622.

(82) McCarthy M: AIDS vaccine fails in Thai trial. Lancet 2003; 362(9397):1728.

(83) Francis DP, Heyward WL, Popovic V et al.: Candidate HIV/AIDS vaccines: lessons learned from the World's first phase III efficacy trials. AIDS 2003; 17(2):147-156.

(84) Buchbinder SP, Mehrotra DV, Duerr A et al.: Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 2008; 372(9653):1881-1893.

(85) Uberla K: HIV vaccine development in the aftermath of the STEP study: re-focus on occult HIV infection? PLoS Pathog 2008; 4(8):e1000114.

(86) Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S et al.: Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 2009; 361(23):2209-2220.

(87) Walker BD, Burton DR: Toward an AIDS vaccine. Science 2008; 320(5877):760-764.

(88) Haynes BF, Gilbert PB, McElrath MJ et al.: Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med 2012; 366(14):1275-1286.

(89) Wren L, Kent SJ: HIV Vaccine efficacy trial: glimmers of hope and the potential role of antibody-dependent cellular cytotoxicity. Hum Vaccin 2011; 7(4):466-473.

(90) Karnasuta C, Paris RM, Cox JH et al.: Antibody-dependent cell-mediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDSVAX B/E prime-boost HIV-1 vaccine trial in Thailand. Vaccine 2005; 23(19):2522-2529.

(91) Robertson MJ, Ritz J: Biology and clinical relevance of human natural killer cells. Blood 1990; 76(12):2421-2438.

(92) Doherty DG, Norris S, Madrigal-Estebas L et al.: The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. J Immunol 1999; 163(4):2314-2321.

(93) Kuijpers TW, Baars PA, Dantin C et al.: Human NK cells can control CMV infection in the absence of T cells. Blood 2008; 112(3):914-915.

(94) Waldhauer I, Steinle A: NK cells and cancer immunosurveillance. Oncogene 2008; 27(45):5932-5943.

(95) Shi FD, Zhou Q: Natural killer cells as indispensable players and therapeutic targets in autoimmunity. Autoimmunity 2011; 44(1):3-10.

(96) Marcenaro E, Carlomagno S, Pesce S, Moretta A, Sivori S: NK/DC crosstalk in anti-viral response. Adv Exp Med Biol 2012; 946:295-308.

(97) Pelletier S, Drouin C, Bedard N et al.: Increased degranulation of natural killer cells during acute HCV correlates with the magnitude of virus-specific T cell responses. J Hepatol 2010; 53(5):805-816.

(98) Parham P, Norman PJ, Abi-Rached L, Guethlein LA: Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. Philos Trans R Soc Lond B Biol Sci 2012; 367(1590):800-811.

(99) Cheng TP, French AR, Plougastel BF et al.: Ly49h is necessary for genetic resistance to murine cytomegalovirus. Immunogenetics 2008; 60(10):565-573.

(100) Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL: Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science 2002; 296(5571):1323-1326.

(101) Sirianni MC, Vincenzi L, Topino S et al.: NK cell activity controls human herpesvirus 8 latent infection and is restored upon highly active antiretroviral therapy in AIDS patients with regressing Kaposi's sarcoma. Eur J Immunol 2002; 32(10):2711-2720.

(102) Golden-Mason L, Cox AL, Randall JA, Cheng L, Rosen HR: Increased natural killer cell cytotoxicity and NKp30 expression protects against hepatitis C virus infection in high-risk individuals and inhibits replication in vitro. Hepatology 2010; 52(5):1581-1589.

(103) Alter G, Heckerman D, Schneidewind A et al.: HIV-1 adaptation to NK-cell-mediated immune pressure. Nature 2011; 476(7358):96-100.

(104) Alter G, Martin MP, Teigen N et al.: Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. J Exp Med 2007; 204(12):3027-3036.

(105) Li Y, Zhang T, Ho C et al.: Natural killer cells inhibit hepatitis C virus expression. J Leukoc Biol 2004; 76(6):1171-1179.

(106) Fauriat C, Long EO, Ljunggren HG, Bryceson YT: Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood 2010; 115(11):2167-2176.

(107) Lanier LL: NK cell recognition. Annu Rev Immunol 2005; 23:225-274.

(108) Puppo F, Brenci S, Bosco O et al.: Downregulation of HLA class I antigen expression in CD4+ T lymphocytes from HIV type 1-infected individuals. AIDS Res Hum Retroviruses 1997; 13(17):1509-1516.

(109) Baba T, Hanagiri T, Takenoyama M et al.: Identification of a lung cancer antigen evading CTL attack due to loss of human leukocyte antigen (HLA) class I expression. Cancer Sci 2010; 101(10):2115-2120.

(110) Williams AP, Bateman AR, Khakoo SI: Hanging in the balance. KIR and their role in disease. Mol Interv 2005; 5(4):226-240.

(111) Bauer S, Groh V, Wu J et al.: Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999; 285(5428):727-729.

(112) Lanier LL: Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008; 9(5):495-502.

(113) Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L: Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. Immunol Today 2000; 21(5):228-234.

(114) Nimmerjahn F, Ravetch JV: Fcgamma receptors as regulators of immune responses. Nat Rev Immunol 2008; 8(1):34-47.

(115) Parham P, Norman PJ, Abi-Rached L, Guethlein LA: Variable NK cell receptors exemplified by human KIR3DL1/S1. J Immunol 2011; 187(1):11-19.

(116) Renedo M, Arce I, Rodriguez A et al.: The human natural killer gene complex is located on chromosome 12p12-p13. Immunogenetics 1997; 46(4):307-311.

(117) Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE: NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. J Exp Med 1997; 185(4):795-800.

(118) Lanier LL, Corliss B, Wu J, Phillips JH: Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity 1998; 8(6):693-701.

(119) Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH: Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. J Immunol 1996; 157(11):4741-4745.

(120) Braud VM, Allan DS, O'Callaghan CA et al.: HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 1998; 391(6669):795-799.

(121) Tripathi P, Agrawal S: The role of human leukocyte antigen E and G in HIV infection. AIDS 2007; 21(11):1395-1404.

(122) Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE: HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. J Immunol 1998; 160(10):4951-4960.

(123) Nattermann J, Nischalke HD, Hofmeister V et al.: HIV-1 infection leads to increased HLA-E expression resulting in impaired function of natural killer cells. Antivir Ther 2005; 10(1):95-107.

(124) Kaiser BK, Pizarro JC, Kerns J, Strong RK: Structural basis for NKG2A/CD94 recognition of HLA-E. Proc Natl Acad Sci U S A 2008; 105(18):6696-6701.

(125) Martini F, Agrati C, D'Offizi G, Poccia F: HLA-E up-regulation induced by HIV infection may directly contribute to CD94-mediated impairment of NK cells. Int J Immunopathol Pharmacol 2005; 18(2):269-276.

(126) Ward JP, Bonaparte MI, Barker E: HLA-C and HLA-E reduce antibody-dependent natural killer cell-mediated cytotoxicity of HIV-infected primary T cell blasts. AIDS 2004; 18(13):1769-1779.

(127) Bonaparte MI, Barker E: Inability of natural killer cells to destroy autologous HIV-infected T lymphocytes. AIDS 2003; 17(4):487-494.

(128) Glienke J, Sobanov Y, Brostjan C et al.: The genomic organization of NKG2C, E, F, and D receptor genes in the human natural killer gene complex. Immunogenetics 1998; 48(3):163-173.

(129) Obeidy P, Sharland AF: NKG2D and its ligands. Int J Biochem Cell Biol 2009; 41(12):2364-2367.

(130) Wu J, Song Y, Bakker AB et al.: An activating immunoreceptor complex formed by NKG2D and DAP10. Science 1999; 285(5428):730-732.

(131) Cerboni C, Neri F, Casartelli N et al.: Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. J Gen Virol 2007; 88(Pt 1):242-250.

(132) Arreygue-Garcia NA, Daneri-Navarro A, del Toro-Arreola A et al.: Augmented serum level of major histocompatibility complex class I-related chain A (MICA) protein and reduced NKG2D expression on NK and T cells in patients with cervical cancer and precursor lesions. BMC Cancer 2008; 8:16.

(133) Moretta A, Bottino C, Vitale M et al.: Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 2001; 19:197-223.

(134) Campbell KS, Yusa S, Kikuchi-Maki A, Catina TL: NKp44 triggers NK cell activation through DAP12 association that is not influenced by a putative cytoplasmic inhibitory sequence. J Immunol 2004; 172(2):899-906.

(135) Pende D, Parolini S, Pessino A et al.: Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med 1999; 190(10):1505-1516.

(136) Pessino A, Sivori S, Bottino C et al.: Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. J Exp Med 1998; 188(5):953-960.

(137) Nattermann J, Feldmann G, Ahlenstiel G et al.: Surface expression and cytolytic function of natural killer cell receptors is altered in chronic hepatitis C. Gut 2006; 55(6):869-877.

(138) Arnon TI, Achdout H, Levi O et al.: Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. Nat Immunol 2005; 6(5):515-523.

(139) Li Y, Wang Q, Mariuzza RA: Structure of the human activating natural cytotoxicity receptor NKp30 bound to its tumor cell ligand B7-H6. J Exp Med 2011; 208(4):703-714.

(140) Arnon TI, Lev M, Katz G et al.: Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol 2001; 31(9):2680-2689.

(141) Mandelboim O, Lieberman N, Lev M et al.: Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 2001; 409(6823):1055-1060.

(142) Vankayalapati R, Wizel B, Weis SE et al.: The NKp46 receptor contributes to NK cell lysis of mononuclear phagocytes infected with an intracellular bacterium. J Immunol 2002; 168(7):3451-3457.

(143) Peltz GA, Grundy HO, Lebo RV et al.: Human Fc gamma RIII: cloning, expression, and identification of the chromosomal locus of two Fc receptors for IgG. Proc Natl Acad Sci U S A 1989; 86(3):1013-1017.

(144) Ravetch JV, Perussia B: Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. J Exp Med 1989; 170(2):481-497.

(145) Iannello A, Debbeche O, Samarani S, Ahmad A: Antiviral NK cell responses in HIV infection: II. viral strategies for evasion and lessons for immunotherapy and vaccination. J Leukoc Biol 2008; 84(1):27-49.

(146) Marquez ME, Millet C, Stekman H et al.: CD16 cross-linking induces increased expression of CD56 and production of IL-12 in peripheral NK cells. Cell Immunol 2010; 264(1):86-92.

(147) Bowles JA, Weiner GJ: CD16 polymorphisms and NK activation induced by monoclonal antibody-coated target cells. J Immunol Methods 2005; 304(1-2):88-99.

(148) Hatjiharissi E, Xu L, Santos DD et al.: Increased natural killer cell expression of CD16, augmented binding and ADCC activity to rituximab among individuals expressing the Fc{gamma}RIIIa-158 V/V and V/F polymorphism. Blood 2007; 110(7):2561-2564.

(149) Koene HR, Kleijer M, Algra J et al.: Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood 1997; 90(3):1109-1114.

(150) Niwa R, Hatanaka S, Shoji-Hosaka E et al.: Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 Is independent of FcgammaRIIIa functional polymorphism. Clin Cancer Res 2004; 10(18 Pt 1):6248-6255.

(151) Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS: Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immunol 2007; 44(7):1524-1534.

(152) Dillman RO: Cancer immunotherapy. Cancer Biother Radiopharm 2011; 26(1):1-64.

(153) Clynes R, Takechi Y, Moroi Y, Houghton A, Ravetch JV: Fc receptors are required in passive and active immunity to melanoma. Proc Natl Acad Sci U S A 1998; 95(2):652-656.

(154) Weng WK, Levy R: Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 2003; 21(21):3940-3947.

(155) Older Aguilar AM, Guethlein LA, Adams EJ et al.: Coevolution of killer cell Ig-like receptors with HLA-C to become the major variable regulators of human NK cells. J Immunol 2010; 185(7):4238-4251.

(156) Middleton D, Gonzelez F: The extensive polymorphism of KIR genes. Immunology 2010; 129(1):8-19.

(157) Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH: Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature 1998; 391(6668):703-707.

(158) Chan HW, Miller JS, Moore MB, Lutz CT: Epigenetic control of highly homologous killer Ig-like receptor gene alleles. J Immunol 2005; 175(9):5966-5974.

(159) Morvan M, Willem C, Gagne K et al.: Phenotypic and functional analyses of KIR3DL1+ and KIR3DS1+ NK cell subsets demonstrate differential regulation by Bw4 molecules and induced KIR3DS1 expression on stimulated NK cells. J Immunol 2009; 182(11):6727-6735.

(160) Chan HW, Kurago ZB, Stewart CA et al.: DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. J Exp Med 2003; 197(2):245-255.

(161) Beziat V, Descours B, Parizot C, Debre P, Vieillard V: NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. PLoS One 2010; 5(8):e11966.

(162) Uhrberg M, Valiante NM, Shum BP et al.: Human diversity in killer cell inhibitory receptor genes. Immunity 1997; 7(6):753-763.

(163) Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P: The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. J Exp Med 1995; 181(3):1133-1144.

(164) Wan AM, Ennis P, Parham P, Holmes N: The primary structure of HLA-A32 suggests a region involved in formation of the Bw4/Bw6 epitopes. J Immunol 1986; 137(11):3671-3674.

(165) Sanjanwala B, Draghi M, Norman PJ, Guethlein LA, Parham P: Polymorphic sites away from the Bw4 epitope that affect interaction of Bw4+ HLA-B with KIR3DL1. J Immunol 2008; 181(9):6293-6300.

(166) Thananchai H, Gillespie G, Martin MP et al.: Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. J Immunol 2007; 178(1):33-37.

(167) O'Connor GM, Yamada E, Rampersaud A et al.: Analysis of binding of KIR3DS1*014 to HLA suggests distinct evolutionary history of KIR3DS1. J Immunol 2011; 187(5):2162-2171.

(168) Gillespie GM, Bashirova A, Dong T et al.: Lack of KIR3DS1 binding to MHC class I Bw4 tetramers in complex with CD8+ T cell epitopes. AIDS Res Hum Retroviruses 2007; 23(3):451-455.

(169) Gardiner CM, Guethlein LA, Shilling HG et al.: Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. J Immunol 2001; 166(5):2992-3001.

(170) Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P: The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. J Immunol 2003; 171(12):6640-6649.

(171) Mousavi T, Poormoghim H, Moradi M et al.: Inhibitory killer cell immunoglobulin-like receptor KIR3DL1 in combination with HLA-B Bw4iso protect against ankylosing spondylitis. Iran J Immunol 2010; 7(2):88-95.

(172) Al OS, Middleton D, Marshall E et al.: Associations between genes for killer immunoglobulin-like receptors and their ligands in patients with solid tumors. Hum Immunol 2010; 71(10):976-981.

(173) Cox JH: HIV-1-Specific Antibody-Dependent Cellular Cytotoxicity (ADCC). Methods Mol Med 1999; 17:373-381.

(174) Fernandez NC, Treiner E, Vance RE et al.: A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. Blood 2005; 105(11):4416-4423.

(175) Cerboni C, Zingoni A, Cippitelli M et al.: Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK- cell lysis. Blood 2007; 110(2):606-615.

(176) Kim S, Poursine-Laurent J, Truscott SM et al.: Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 2005; 436(7051):709-713.

(177) Kim S, Sunwoo JB, Yang L et al.: HLA alleles determine differences in human natural killer cell responsiveness and potency. Proc Natl Acad Sci U S A 2008; 105(8):3053-3058.

(178) Anfossi N, Andre P, Guia S et al.: Human NK cell education by inhibitory receptors for MHC class I. Immunity 2006; 25(2):331-342.

(179) Hoglund P, Brodin P: Current perspectives of natural killer cell education by MHC class I molecules. Nat Rev Immunol 2010; 10(10):724-734.

(180) Brodin P, Lakshmikanth T, Johansson S, Karre K, Hoglund P: The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood 2009; 113(11):2434-2441.

(181) Fauriat C, Ivarsson MA, Ljunggren HG, Malmberg KJ, Michaelsson J: Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. Blood 2010; 115(6):1166-1174.

(182) Joncker NT, Raulet DH: Regulation of NK cell responsiveness to achieve self-tolerance and maximal responses to diseased target cells. Immunol Rev 2008; 224:85-97.

(183) Brodin P, Karre K, Hoglund P: NK cell education: not an on-off switch but a tunable rheostat. Trends Immunol 2009; 30(4):143-149.

(184) Parsons MS, Zipperlen K, Gallant M, Grant M: Killer cell immunoglobulin-like receptor 3DL1 licenses CD16-mediated effector functions of natural killer cells. J Leukoc Biol 2010; 88(5):905-912.

(185) Sun JC, Lanier LL: Tolerance of NK cells encountering their viral ligand during development. J Exp Med 2008; 205(8):1819-1828.

(186) Yu J, Heller G, Chewning J et al.: Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. J Immunol 2007; 179(9):5977-5989.

(187) Sun JC: Re-educating natural killer cells. J Exp Med 2010; 207(10):2049-2052.

(188) Elliott JM, Wahle JA, Yokoyama WM: MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. J Exp Med 2010; 207(10):2073-2079.

(189) Joncker NT, Shifrin N, Delebecque F, Raulet DH: Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. J Exp Med 2010; 207(10):2065-2072.

(190) Moga E, Canto E, Vidal S et al.: Interleukin-15 enhances rituximab-dependent cytotoxicity against chronic lymphocytic leukemia cells and overcomes transforming growth factor beta-mediated immunosuppression. Exp Hematol 2011; 39(11):1064-1071.

(191) Mocellin S, Panelli M, Wang E et al.: IL-10 stimulatory effects on human NK cells explored by gene profile analysis. Genes Immun 2004; 5(8):621-630.

(192) Gamero AM, Ussery D, Reintgen DS, Puleo CA, Djeu JY: Interleukin 15 induction of lymphokine-activated killer cell function against autologous tumor cells in melanoma patient lymphocytes by a CD18-dependent, perforin-related mechanism. Cancer Res 1995; 55(21):4988-4994.

(193) Chehimi J, Starr SE, Frank I et al.: Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus-infected patients. J Exp Med 1992; 175(3):789-796.

(194) Rook AH, Kehrl JH, Wakefield LM et al.: Effects of transforming growth factor beta on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. J Immunol 1986; 136(10):3916-3920.

(195) Weigent DA, Stanton GJ, Johnson HM: Interleukin 2 enhances natural killer cell activity through induction of gamma interferon. Infect Immun 1983; 41(3):992-997.

(196) Martin MP, Gao X, Lee JH et al.: Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet 2002; 31(4):429-434.

(197) Vieillard V, Strominger JL, Debre P: NK cytotoxicity against CD4+ T cells during HIV-1 infection: a gp41 peptide induces the expression of an NKp44 ligand. Proc Natl Acad Sci U S A 2005; 102(31):10981-10986.

(198) Fauci AS, Mavilio D, Kottilil S: NK cells in HIV infection: paradigm for protection or targets for ambush. Nat Rev Immunol 2005; 5(11):835-843.

(199) Mavilio D, Benjamin J, Daucher M et al.: Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. Proc Natl Acad Sci U S A 2003; 100(25):15011-15016.

(200) De MA, Fogli M, Costa P et al.: The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). Eur J Immunol 2003; 33(9):2410-2418.

(201) Cooper MA, Fehniger TA, Caligiuri MA: The biology of human natural killer-cell subsets. Trends Immunol 2001; 22(11):633-640.

(202) Bjorkstrom NK, Ljunggren HG, Sandberg JK: CD56 negative NK cells: origin, function, and role in chronic viral disease. Trends Immunol 2010; 31(11):401-406.

(203) Tyler DS, Stanley SD, Nastala CA et al.: Alterations in antibody-dependent cellular cytotoxicity during the course of HIV-1 infection. Humoral and cellular defects. J Immunol 1990; 144(9):3375-3384.

(204) Valentin A, Rosati M, Patenaude DJ et al.: Persistent HIV-1 infection of natural killer cells in patients receiving highly active antiretroviral therapy. Proc Natl Acad Sci U S A 2002; 99(10):7015-7020.

(205) Peruzzi M, Azzari C, Rossi ME, De MM, Vierucci A: Inhibition of natural killer cell cytotoxicity and interferon gamma production by the envelope protein of HIV and prevention by vasoactive intestinal peptide. AIDS Res Hum Retroviruses 2000; 16(11):1067-1073.

(206) Douek D: HIV disease progression: immune activation, microbes, and a leaky gut. Top HIV Med 2007; 15(4):114-117.

(207) Leonard R, Zagury D, Desportes I et al.: Cytopathic effect of human immunodeficiency virus in T4 cells is linked to the last stage of virus infection. Proc Natl Acad Sci U S A 1988; 85(10):3570-3574.

(208) Zarling JM, Ledbetter JA, Sias J et al.: HIV-infected humans, but not chimpanzees, have circulating cytotoxic T lymphocytes that lyse uninfected CD4+ cells. J Immunol 1990; 144(8):2992-2998.

(209) Gougeon ML, Garcia S, Heeney J et al.: Programmed cell death in AIDS-related HIV and SIV infections. AIDS Res Hum Retroviruses 1993; 9(6):553-563.

(210) Finkel TH, Tudor-Williams G, Banda NK et al.: Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med 1995; 1(2):129-134.

(211) Fausther-Bovendo H, Vieillard V, Sagan S, Bismuth G, Debre P: HIV gp41 engages gC1qR on CD4+ T cells to induce the expression of an NK ligand through the PIP3/H2O2 pathway. PLoS Pathog 2010; 6:e1000975.

(212) Vieillard V, Le GR, Dausset J, Debre P: A vaccine strategy against AIDS: an HIV gp41 peptide immunization prevents NKp44L expression and CD4+ T cell depletion in SHIV-infected macaques. Proc Natl Acad Sci U S A 2008; 105(6):2100-2104.

(213) Vieillard V, Costagliola D, Simon A, Debre P: Specific adaptive humoral response against a gp41 motif inhibits CD4 T-cell sensitivity to NK lysis during HIV-1 infection. AIDS 2006; 20(14):1795-1804.

(214) Curriu M, Fausther-Bovendo H, Pernas M et al.: Viremic HIV infected individuals with high CD4 T cells and functional envelope proteins show

anti-gp41 antibodies with unique specificity and function. PLoS One 2012; 7(2):e30330.

(215) Qi Y, Martin MP, Gao X et al.: KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. PLoS Pathog 2006; 2(8):e79.

(216) Lopez-Vazquez A, Mina-Blanco A, Martinez-Borra J et al.: Interaction between KIR3DL1 and HLA-B*57 supertype alleles influences the progression of HIV-1 infection in a Zambian population. Hum Immunol 2005; 66(3):285-289.

(217) Gaudieri S, DeSantis D, McKinnon E et al.: Killer immunoglobulin-like receptors and HLA act both independently and synergistically to modify HIV disease progression. Genes Immun 2005; 6(8):683-690.

(218) Lang P, Pfeiffer M, Handgretinger R et al.: Clinical scale isolation of T cell-depleted CD56+ donor lymphocytes in children. Bone Marrow Transplant 2002; 29(6):497-502.

(219) Hessell AJ, Hangartner L, Hunter M et al.: Fc receptor but not complement binding is important in antibody protection against HIV. Nature 2007; 449(7158):101-104.

(220) Banks ND, Kinsey N, Clements J, Hildreth JE: Sustained antibody-dependent cell-mediated cytotoxicity (ADCC) in SIV-infected macaques correlates with delayed progression to AIDS. AIDS Res Hum Retroviruses 2002; 18(16):1197-1205.

(221) Baum LL, Cassutt KJ, Knigge K et al.: HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. J Immunol 1996; 157(5):2168-2173.

(222) Pollara J, Hart L, Brewer F et al.: High-throughput quantitative analysis of HIV-1 and SIV-specific ADCC-mediating antibody responses. Cytometry A 2011; 79(8):603-612.

(223) Gomez-Roman VR, Florese RH, Patterson LJ et al.: A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity. J Immunol Methods 2006; 308(1-2):53-67.

(224) Chung AW, Rollman E, Center RJ, Kent SJ, Stratov I: Rapid degranulation of NK cells following activation by HIV-specific antibodies. J Immunol 2009; 182(2):1202-1210.

(225) Johansson SE, Rollman E, Chung AW et al.: NK cell function and antibodies mediating ADCC in HIV-1-infected viremic and controller patients. Viral Immunol 2011; 24(5):359-368.

(226) Kamya P, Boulet S, Tsoukas CM et al.: Receptor-ligand requirements for increased NK cell polyfunctional potential in slow progressors infected with HIV-1 coexpressing KIR3DL1*h/*y and HLA-B*57. J Virol 2011; 85(12):5949-5960.

(227) Taner SB, Pando MJ, Roberts A et al.: Interactions of NK cell receptor KIR3DL1*004 with chaperones and conformation-specific antibody reveal a functional folded state as well as predominant intracellular retention. J Immunol 2011; 186(1):62-72.

(228) Forthal DN, Landucci G, Keenan B: Relationship between antibody-dependent cellular cytotoxicity, plasma HIV type 1 RNA, and CD4+ lymphocyte count. AIDS Res Hum Retroviruses 2001; 17(6):553-561.

(229) Tarazona R, Casado JG, Delarosa O et al.: Selective depletion of CD56(dim) NK cell subsets and maintenance of CD56(bright) NK cells in treatment-naive HIV-1-seropositive individuals. J Clin Immunol 2002; 22(3):176-183.

(230) Jewett A, Cavalcanti M, Giorgi J, Bonavida B: Concomitant killing in vitro of both gp120-coated CD4+ peripheral T lymphocytes and natural killer cells in the antibody-dependent cellular cytotoxicity (ADCC) system. J Immunol 1997; 158(11):5492-5500.

(231) Pereira LE, Johnson RP, Ansari AA: Sooty mangabeys and rhesus macaques exhibit significant divergent natural killer cell responses during both acute and chronic phases of SIV infection. Cell Immunol 2008; 254(1):10-19.

(232) Rutjens E, Mazza S, Biassoni R et al.: CD8+ NK cells are predominant in chimpanzees, characterized by high NCR expression and cytokine production, and preserved in chronic HIV-1 infection. Eur J Immunol 2010; 40(5):1440-1450.

(233) Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D: HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature 1998; 391(6665):397-401.

(234) Fausther-Bovendo H, Sol-Foulon N, Candotti D et al.: HIV escape from natural killer cytotoxicity: nef inhibits NKp44L expression on CD4+ T cells. AIDS 2009; 23(9):1077-1087.

(235) Ho DD, Neumann AU, Perelson AS et al.: Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 1995; 373(6510):123-126.

(236) Zaitseva MB, Moshnikov SA, Kozhich AT et al.: Antibodies to MHC class II peptides are present in HIV-1-positive sera. Scand J Immunol 1992; 35(3):267-273.

(237) Keay S, Wecksler W, Wasserman SS, Margolick J, Farzadegan H: Association between anti-CD4 antibodies and a decline in CD4+ lymphocytes in human immunodeficiency virus type 1 seroconverters. J Infect Dis 1995; 171(2):312-319.

(238) Golding H, Robey FA, Gates FT, III et al.: Identification of homologous regions in human immunodeficiency virus I gp41 and human MHC class II beta 1 domain. I. Monoclonal antibodies against the gp41-derived peptide and patients' sera react with native HLA class II antigens, suggesting a role for autoimmunity in the pathogenesis of acquired immune deficiency syndrome. J Exp Med 1988; 167(3):914-923.

(239) Garcia JV, Miller AD: Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature 1991; 350(6318):508-511.

(240) Vieillard V, Crouzet J, Boufassa F et al.: Specific Anti-gp41 Antibodies Predict HIV-1 Disease Progression. J Acquir Immune Defic Syndr 2012.

(241) Lang PA, Lang KS, Xu HC et al.: Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. Proc Natl Acad Sci U S A 2012; 109(4):1210-1215.

(242) Waggoner SN, Cornberg M, Selin LK, Welsh RM: Natural killer cells act as rheostats modulating antiviral T cells. Nature 2012; 481(7381):394-398.

(243) Biron CA: Yet another role for natural killer cells: cytotoxicity in immune regulation and viral persistence. Proc Natl Acad Sci U S A 2012; 109(6):1814-1815.

(244) Nielsen N, Odum N, Urso B, Lanier LL, Spee P: Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. PLoS One 2012; 7(2):e31959.

(245) Anderson DJ, Politch JA, Nadolski AM et al.: Targeting Trojan Horse leukocytes for HIV prevention. AIDS 2010; 24(2):163-187.

(246) Salle B, Brochard P, Bourry O et al.: Infection of macaques after vaginal exposure to cell-associated simian immunodeficiency virus. J Infect Dis 2010; 202(3):337-344.

(247) Zhu T, Wang N, Carr A et al.: Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: evidence for viral compartmentalization and selection during sexual transmission. J Virol 1996; 70(5):3098-3107.

(248) Brocca-Cofano E, McKinnon K, Demberg T et al.: Vaccine-elicited SIV and HIV envelope-specific IgA and IgG memory B cells in rhesus macaque peripheral blood correlate with functional antibody responses and reduced viremia. Vaccine 2011; 29(17):3310-3319.