Characterization of NK Cell Subsets Responding to HIV Infected Cells

by
Irene Lisovsky

Department of Medicine
Division of Experimental Medicine
McGill University, Montreal
June 2017

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

TABLE OF CONTENTS

ABSTRACT

Globally, over 36 million people are infected with human immunodeficiency virus type 1 (HIV-1). Some individuals exposed to HIV remain persistently seronegative. Among those who become infected a subset of approximately 5% progress to acquired immunodeficiency syndrome (AIDS) slowly, and even fewer, i.e. <1% control their viral load (VL) without treatment. Epidemiological and functional studies found that carriage of certain Natural Killer (NK) cell receptor (NKR)/human leukocyte antigen (HLA) ligand pairs is associated with slow time to AIDS in HIV-positive subjects and protection from infection in HIV-exposed seronegative subjects, implicating NK cells in HIV control. NK cells acquire anti-viral functions through a process called education, that requires signals received from inhibitory NKR (iNKRs) engaging their ligands.

This thesis investigated the mechanism by which NK cells mediate inhibition of HIV replication, particularly in NK cells from donors expressing a high expression KIR3DL1 homozygous genotype with HLA-B*57 (*h/*y+B*57), which is the KIR/HLA pair that has the strongest effect on slow time to AIDS and HIV VL control. Furthermore, we analyzed the impact of expression of the iNKRs KIR2DL3, KIR3DL1 and NKG2A on NK functionality in response to autologous HIV infected cells in NK cell populations defined by these iNKRs. We extended our analyses of NK cell responses to autologous HIV infected stimuli to iNKR/HLA pairs involved in education through the above mentioned iNKRs, taking into consideration the impact of HIV infection on downmodulation of the ligands for these iNKR. In this thesis we show that NK cell education influences the potency of NK cell mediated inhibition of HIV replication. The inhibition of viral

replication in autologous HIV-infected cells involves responses by the KIR3DL1^{+} NK cells and is, at least in part due, to the secretion of CC-chemokines by this NK cell population. Our results also show that NKG2A^{+} and KIR2DL3^{+} NK cells mediate responses to autologous HIV infected cells. We show that the responses of NK cell populations to HIV-infected cells are influenced not only by NK cell education through specific KIR/HLA pairs, but also by differential HIV mediated changes in HLA expression. Together these observations provide additional support for the importance of NK cells and populations of these cells as mediators of response to HIV-infected cells. These findings also demonstrate the dynamic role of NK cell education on aspects of NK cell functions, both in the context of broad stimulation by HLA null cells and responses to HIV-infected cells.

RÉSUMÉ

A travers le monde, 36 millions de personnes sont actuellement infectées par le virus de l'immunodéficience humaine de type 1 (VIH-1). Bien qu'ils soient également exposés au virus, un certain nombre d'individus restent séronégatif à long terme. Parmi ceux qui sont infectés, environ 5% progressent lentement vers le syndrome d'immunodéficience acquis (SIDA) et moins de 1% contrôlent leur charge virale en l'absence de tout traitement. Des études épidémiologiques et fonctionnelles ont mis en évidence le rôle potentiel des cellules Natural Killer (NK) dans le contrôle de l'infection virale. En effet, ces études ont montré que les individus qui co-expriment certains récepteurs présents à la surface des cellules NK, les NKR, en association avec certains ligands du complexe majeur d'histocompatibilité (CMH/HLA), sont protégés de l'infection ou progressent moins rapidement vers le SIDA si ils sont déjà infectés. Les cellules NK acquièrent leurs fonctions antivirales par un processus appelé « éducation » qui nécessite la signalisation à travers les NKR inhibiteurs (iNKRs) par leurs ligands.

Dans ce travail, nous avons étudié les mécanismes par lesquels les cellules NK inhibent la réplication virale, en particulier les cellules NK issues d'individus qui expriment fortement KIR3DL1 (homozygote) et HLA-B*57 (*h/*y+B*57), un des tandems récepteur/ligand le plus fortement associé à une progression lente vers le SIDA et au contrôle de la charge virale. Nous avons analysé l'impact des iNKRs KIR2DL3, KIR3DL1 et NKG2A sur la fonctionnalité des cellules NK en réponse à des cellules T $CD4^+$ autologues infectées par le virus (iCD4). Nous avons ensuite étudié cette réponse dans le cadre plus particulier des tandems iNKRs/HLA (mentionnés ci-dessus)

impliqués dans l'éducation des cellules NK, en prenant en compte l'effet du virus sur la diminution de l'expression membranaire des ligands de ces iNKRs.

Dans ce travail, nous avons alors montré que l'éducation des cellules NK influe sur leur capacité à inhiber la réplication virale. L'inhibition de la réplication virale met en jeu la réponse des cellules NK KIR3DL1+ et est, en partie, dépendent de la sécrétion de chimiokines de la famille CC par ces cellules. Nos résultats montrent également que ce sont les cellules NK NKG2A^{+} et KIR2DL3^{+} qui répondent aux iCD4, que l'éducation des cellules NK via les iNKRs mais également l'expression membranaire du HLA modulent leur capacité à répondre aux iCD4. L'ensemble de ces résultats apporte de nouveaux arguments démontrant l'importance des cellules NK en tant que médiateur de la réponse contre les cellules infectées par le VIH. Ils démontrent également le rôle dynamique de l'éducation des cellules NK sur leurs capacités fonctionnelles que ce soit dans un contexte de stimulation par des cellules n'exprimant pas de HLA ou par des cellules infectées par le virus.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr Nicole F Bernard for giving me the opportunity to pursue my PhD studies in her laboratory. I am grateful for your guidance, support and trust that permitted me to work independently and pursue my professional curiosity.

I would also like to thank past and present members of the Bernard lab for their technical expertise, collaborative support and friendly work environment. Particularly, Dr Song, Dr DaFonseca and Dr Isitman for working together on developing new assays and studies that propel this field forward.

My family and close friends, who were there to listen, advise and celebrate the successes of this journey. Thank You!

Most of all, I want to acknowledge my awesome parents, Tanya and Felix, who believed in me, encouraged me and reminded me to focus on the primary. As a token of my appreciation for your nurturing support, I would like to dedicate this thesis to you. לחיים!

LIST OF ABBREVIATIONS

aa- amino acid

Ab - antibody

ADCC - antibody dependent cytotoxicity

aKIR – activating killer immunoglobulin-like receptor

aNKR - activating natural killer cell receptor

AIDS - acquired immunodeficiency syndrome

ART - antiretroviral therapy

ARVs - antiretrovirals

CA - capsid (or p24)

CD4 - $CD4^+$ T cells

CMV - cytomegalovirus

CRF - circulating recombinant form

CSW - commercial sex workers

CTLs - cytotoxic T lymphocytes

DNA - deoxyribonucleic acid

EC - elite controllers

EIA - enzyme immunoassay

FI - fusion inhibitor

GALT - gut associated lymphoid tissue

HA - hemagglutinin

HAART - highly active antiretroviral therapy

HCV - hepatitis C virus

HESN - HIV exposed seronegative

HIV-1 - human immunodeficiency virus type - 1

HLA - human leukocyte antigens

HSC - hematopoietic stem cell

iCD4 - HIV-infected $CD4^+$ T cells

IDU - injection drug use

Ig - immunoglobulin

iKIR – inhibitory killer immunoglobulin-like receptor

iNKR - inhibitory natural killer cell receptor

IN - integrase

INI - integrase inhibitor

ITAM - immunoreceptor tyrosine-based activation motif

ITIM - immunoreceptor tyrosine-based inhibition motif

KIR - killer immunoglobulin-like receptors

LILRB - leukocyte immunoglobulin-like receptor subfamily B

LRC - leukocyte receptor complex

LTNP - long-term nonprogressors

LTR - long terminal repeats

MA - matrix (or p17)

MALT - mucosal associated lymphoid tissue

MHC - major histocompatibility complex class I

MSM - men who have sex with men

NC - nucleocapsid (or p7)

NCAM - neural cell adhesion molecule

NCR - natural cytotoxicity receptor

NK - natural killer

NKR - natural killer cell receptor

Nef - negative regulatory factor

NNRTI - non nucleoside reverse transcriptase inhibitor

NRTI - nucleotide and nucleoside reverse transcriptase inhibitor

PBMC - peripheral blood mononuclear cell

PCR - polymerase chain reaction

PI - protease inhibitor

PR - protease

Rev - regulator of expression of virion proteins

RM - rhesus macaque

RNA - ribonucleic acid

RRE - Rev response element

RT - reverse transcriptase

SIV - simian immunodeficiency virus

SLT - secondary lymphoid tissues

SP - slow progressors

Tat - trans-activating protein

Vif - viral infectivity factor

VL –viral load

Vpr - viral protein R

Vpu - viral protein U

ULBP - UL16 binding protein

URF - unique recombinant form

FORWARD

The year 2013 marked the 30th anniversary of the discovery of HIV as the causative agent of AIDS. Research conducted thus far has greatly increased our understanding of the virus, immune responses against it, as well as facilitated the development of diagnostic assays, potent antiretroviral drug therapies, and global surveillance programs. Despite our current understanding, no cure exists and efforts to produce an effective vaccine have met with limited success.

NK cells have a pivotal role in anti-viral immune responses. Epidemiological and functional studies implicate NK cells in HIV resistance and slow time to AIDS in those infected. These protective outcomes are associated with expressing certain NK cell surface receptors called KIRs and their ligands, HLA molecules. However, little is known regarding the mechanism(s) underlying this protective effect and the NK cell population(s) responsible for these responses. The objectives of this thesis are to: 1) identify the mechanism by which NK cell populations inhibit HIV replication *in vitro*, 2) characterize NK cell populations responding to HIV infected cells, 3) study the impact of expression of certain KIR-HLA pairs on NK cell responses to HLA-null and HIV infected stimuli.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Background

The emergence of immune deficiency in young homosexual men was noted in the summer of 1981 when the Center for Disease Control (Atlanta, Georgia, USA) detected an unusual clustering of patients diagnosed with *Pneumocystis carinii* pneumonia and Kaposi's sarcoma [1]. At this time, it was recognized that these men had a marked depletion of the specific subset of T-lymphocytes, $CD4^+$ helper cells [2, 3]. One year later, this disease began to afflict injection drug users and recipients of blood transfusion, both male and female of diverse sexual orientations [4]. Consequently, it quickly became apparent that the causative agent was blood borne and the disease was eventually termed AIDS.

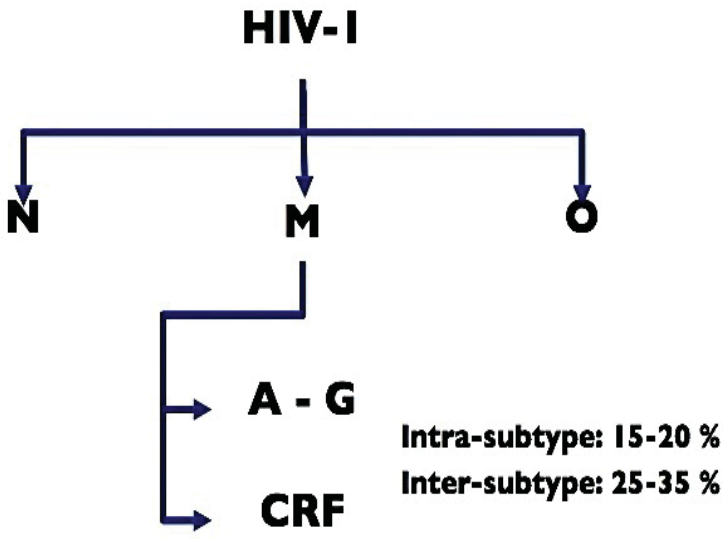


Figure 1: HIV-1 genetic classification. HIV-1 is classified based on genomic differences into groups, subtypes and circulating recombinant forms (CRFs).

Figure made by Irene Lisovsky.

The discovery of the etiological agent responsible for the development of AIDS remains a controversial topic. In 1983, Drs. Françoise Barré-Sinoussi and Luc Montagnier isolated a novel virus from patients who displayed signs and symptoms of AIDS [5]. The following year, *Science* magazine published an article authored by Robert Gallo's research team about the detection, isolation and continuous production of a retrovirus from patients with AIDS [6]. The conclusions from both studies implied that these viral variants were different. Eventually, genomic sequencing confirmed that these viruses belonged to a single viral species, which displayed great genetic sequence variation [7]. In 1986, the newly discovered virus was termed HIV [8]. In 2008, Drs. Françoise Barré-Sinoussi and Luc Montagnier were jointly awarded the Nobel Prize in Physiology or Medicine for their role in the discovery of HIV.

1.2. Global distribution and genetic diversity of HIV

HIV-1 is further divided into groups M, N and O, where group M is predominantly responsible for the HIV-1 pandemic [9]. This group of viruses accounts for the global pandemic with over 36 million infected individuals [10]. Group M has been further divided into subtypes, denoted with letters, and sub-subtypes, denoted with numerals. Subtypes and sub-subtypes currently recognized include: A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K [11, 12] (Fig.1). Advances in genome sequencing have led to the identification of HIV-1 'circulating' and 'unique' recombinant forms (CRFs and URFs, respectively), which are a result of recombination events between subtypes [9, 11].

At the nucleotide level, genetic variation within a subtype can range from 15 to 20%,

whereas diversity between subtypes can reach 25 to 35% [13, 14]. Genetic variation at the nucleotide level can translate into variation at the protein level that may result in different viral phenotypes that impact virulence, for example, transmission fitness. The high degree of HIV-1 diversity results from the lack of a proofreading mechanism of the viral enzyme reverse transcriptase (RT), rapid rate of viral replication and recombination between heterogeneous copies of viral ribonucleic acid (RNA). Such HIV-1 genetic variability can affect rates of disease progression, diagnostic tests, response to antiretroviral therapy (ART) and has been proposed to be an impediment to vaccine development [9, 15-18].

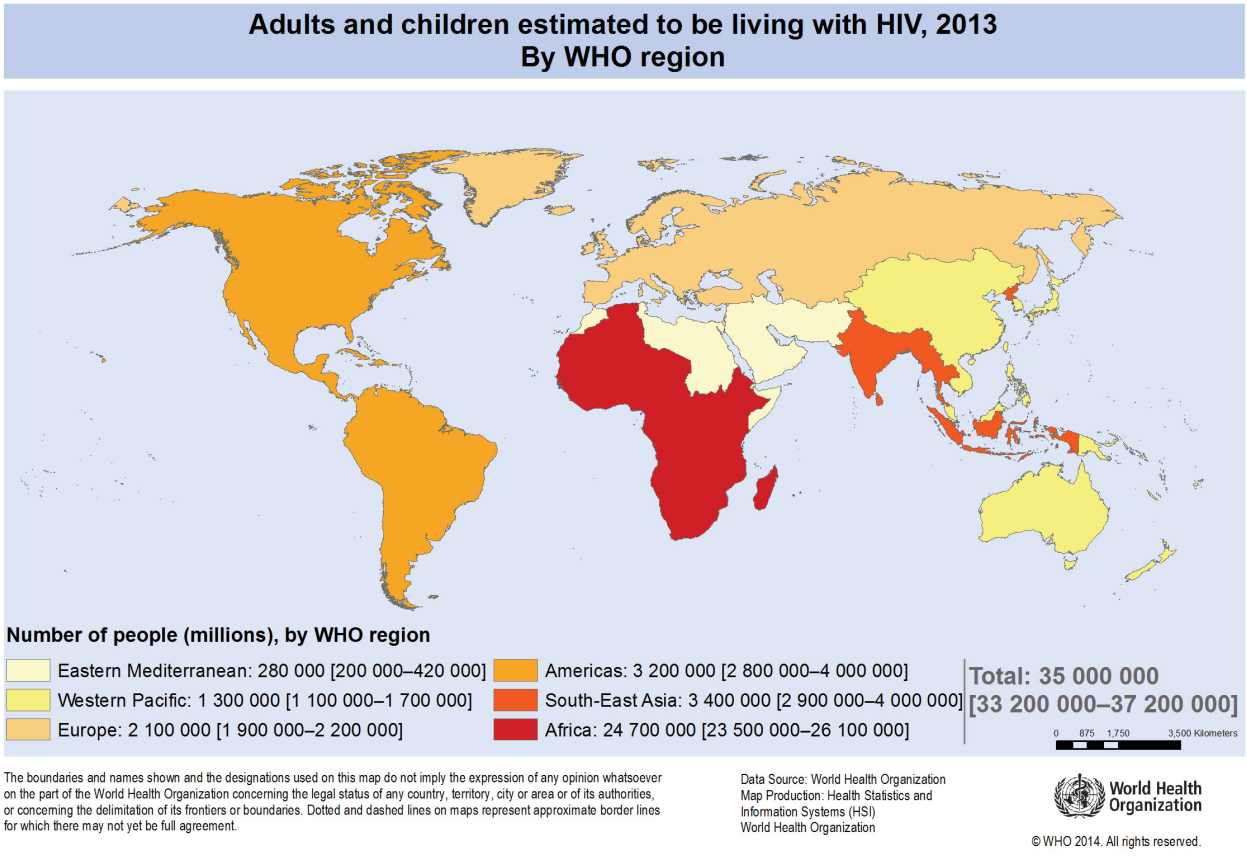


Figure 2. Global prevalence of HIV 2013. Adults and children living with HIV. Figure reprinted with permission from World Health Organization.

The global distribution of HIV-1 subtypes and CRFs is in constant flux and surveillance is important for understanding the HIV/AIDS pandemic. Subtype B is dominant in North and South America and Western Europe, however, it accounts for only 10% of global infections, where non-B HIV-1 subtypes constitute 90% of this pandemic [11, 19]. Non-B subtypes and CRFs are commonly found in Africa, Asia and to a lesser extent, South America. CRF01_AE and CRF02_AG account for 10% of global infections caused by non-B subtypes and are prevalent in South Asia and West Africa, respectively [9]. Currently, subtype C is the most prevalent globally and accounts for nearly 50% of all global infections [9, 12]. It is the dominant subtype in Africa, particularly, sub-Saharan Africa where about two-thirds (over 24 million) of all people living with HIV-1 reside, despite this region being home to only 10% of the world's population [12, 20] (Fig. 2). In addition, a rapid spreading of non-B subtypes is being observed and documented throughout North America and Europe [21, 22]. The disproportionate increase in subtype C prevalence compared to other HIV-1 subtypes suggests that it may be more easily transmitted and/or has a greater replication capacity.

1.3 Virology of HIV-1

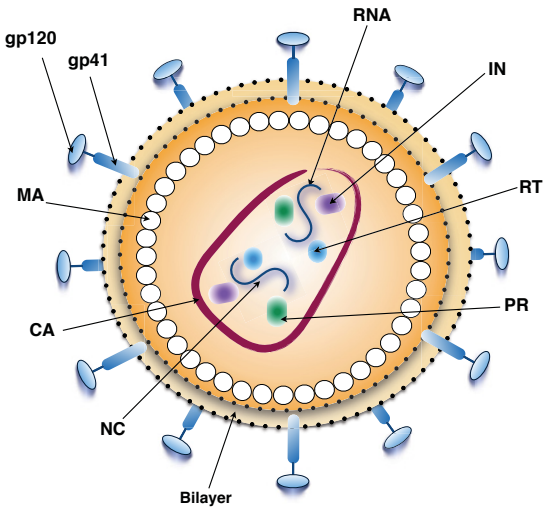
1.3.1. Viral structure

HIV-1 belongs to the *Lentivirus* genus *of the Retroviridae* family. A mature and infectious HIV-1 virion is spherical, enveloped and has a diameter of 100 to 120 nm [23]. Figure 3A depicts a characteristic structure of an HIV-1 virion. The envelope is of cellular origin (acquired upon viral budding) and it surrounds an icosahedral viral capsid containing

viral genomic ribonucleic acid (RNA), proteins and cellular derived factors. Embedded in the envelope is the transmembrane viral glycoprotein (gp160) composed of the proteins termed gp120 and gp41 [24, 25]. In direct interaction with the viral envelope proteins are the matrix proteins (MA). Structurally, MA proteins line the inside of the virion and have an important role in targeting viral polypeptides to the plasma membrane during viral assembly [26, 27]. Within the viral envelope, the icosahedral viral core is formed by the association of multiple hexameric subunits of the capsid (CA or p24) protein [26]. Within the viral core are two single stranded RNA molecules associated with the nucleocapsid (NC or p7) viral protein, which stabilizes the viral genome [28, 29]. During assembly, NC binds genomic RNA and delivers it to the core [28, 30]. Viral protein p6 is also found in the core and is important in the late stages of viral assembly, by recruiting cellular machinery required for viral budding, and incorporation of the accessory protein viral protein R (Vpr) into the virion [30, 31].

The viral core also contains a number of viral enzymes and accessory proteins necessary for viral replication within the cell. The viral enzymes found inside the virion are: protease (PR), reverse transcriptase (RT) and integrase (IN) [29, 30]. The viral accessory proteins present in the core include: viral infectivity factor (Vif), viral protein U (Vpu), Vpr, trans-activator protein (Tat), regulator of expression of virion proteins (Rev) and negative regulatory factor (Nef) [29]. These accessory proteins are essential for viral replication and immune evasion, and will be discussed in the following section.

A.



B.

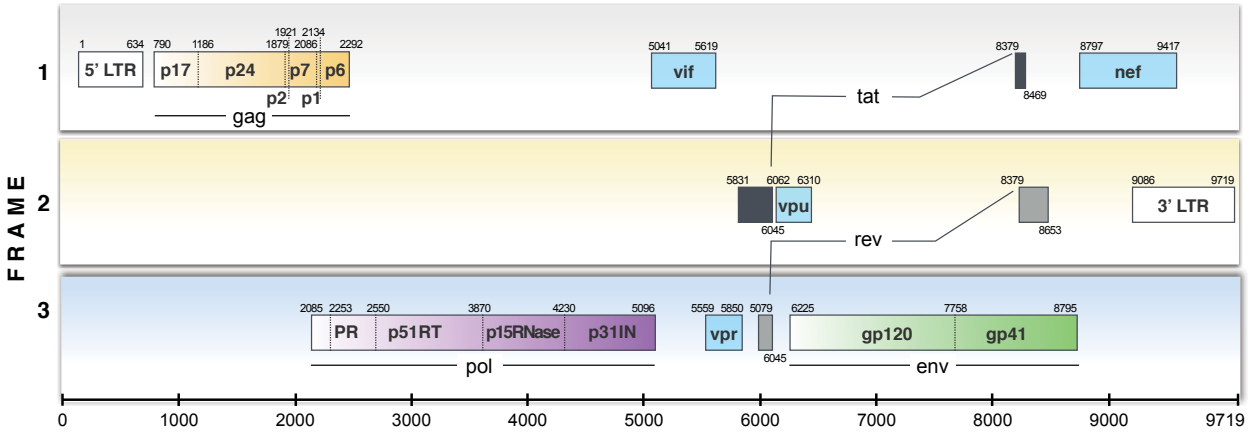


Figure 3: Structure of a mature HIV-1 virion (A). HIV genomic organization (B). The numbers at the top and bottom indicate nucleotide positioning (from HIV Sequence Database http://www.hiv.lanl.gov/content/index).

Figures made by Irene Lisovsky

1.3.2. HIV-1 genomic organization

The 9.2kb RNA genome of HIV-1 can be classified into four main regions: (1) long terminal repeats (LTR), (2) *gag-pol* genes, (3) *env* gene and (4) genes coding for accessory proteins [29]. The HIV genome contains internal RNA structures important in transcriptional activation, HIV packaging, signal polyadenylation, regulating RNA export and interaction with viral and host proteins [30, 32, 33]. A study by Watts *et al*. was the first to show that the HIV genome coded for proteins at two levels. The first level is via nucleotides that are translated into an amino acid (aa), and second level is via secondary structures involved in protein synthesis, which modulate ribosomal elongation to promote protein folding [34].

Figure 3B outlines the organization of HIV-1 genomic RNA. The LTR regions are present at either end of the RNA molecule. Each LTR contains U3, R and U5 coding sequences required for binding of cellular transcription factors and Tat-mediated activation that drives the synthesis of genomic transcripts [29, 35]. The second region, the *gag-pol* gene, encodes two polyprotein precursors: Gag, which codes for structural proteins (MA, CA, NC and p6) [23, 27, 30, 31], and Gag-Pol, which contains three viral enzymes PR (p11), RT (p51 and p66) and IN (p32) [29, 36, 37]. The function of these enzymes will be discussed in more detail in section 1.3.3. The third region, the *env* gene, encodes the gp160 glycoprotein that is cleaved into exterior gp120 and transmembrane gp41 protein components. The gp120 protein interacts with the CD4 receptor and CCR5 or CXCR4 co-receptors to promote viral attachment to susceptible cells [38]. The gp120 protein is highly variable. This is advantageous for the virus since

the virus can alter immunogenic determinants that trigger immune response, and consequently, escape immune pressure designed to mediate viral clearance [39-41]. The transmembrane component of the gp160 polyprotein, gp41, contains protein domains that facilitate viral fusion with the target cell [25]. The fourth region of the HIV-1 genome codes for the viral accessory proteins Tat, Rev, Nef, Vif, Vpr and Vpu.

These accessory proteins play an important role in viral pathogenesis, regulation of HIV genome transcription and immune evasion. Transcription from the viral LTR promoters is initiated by the binding of Tat proteins and recruitment of cellular transcription factors and the RNA polymerase enzyme [29, 35, 42]. Tat also facilitates efficient elongation of newly synthesized transcripts [42, 43]. Rev proteins, on the other hand, act post transcriptionally by binding to the Rev responsive element (RRE) and promoting the transport of unspliced viral RNA through the nuclear membrane to the cytoplasm, where *Gag*, *Gag-Pol*, *env*, *vif*, *vpr* and *vpu* are translated [44, 45]. The regulatory protein Vpr is required for efficient viral replication in non-dividing cells, such as monocytes and macrophages [46-49]. Vpr's roles in HIV-1 replication include nuclear import of the pre-integration complex (viral deoxyribonucleic acid (DNA) with viral and cellular proteins) [50, 51], cell cycle arrest at the G2 phase and apoptosis [52, 53]. In addition, Vpr is also packaged into the virion through an interaction with the Gag derived protein p6 [54]. The accessory proteins Vif, Nef and Vpu have an important role in pathogenesis and immune evasion. HIV-1 Vif counteracts the host restriction factor enzymes called APOBEC by polyubiquitinating them and sending them for proteosomal degradation, which reduces incorporation of APOBEC into HIV-1 virions [55-57]. In fact, when *vif* is

knocked out the resultant virus is almost completely non-infectious in cell culture experiments in comparison to the wild type virus (all genes present) [55, 56]. Furthermore, in *vif* knockouts, APOBEC_3G and 3F enzymes are incorporated into the virion as it buds off infected cells. When viruses containing these enzymes infect neighbouring susceptible cells APOBEC cytidine deaminates viral reverse transcribed DNA resulting in silent and non-silent mutations, which can have a negative impact on viral infectivity [58, 59]. Nef and Vpu promote viral pathogenesis and immune evasion by down-regulating cell surface molecules such as CD4, major histocompatibility complex class I (MHC I) and MHC II [60-62]. In addition, the *nef* gene has been associated with more robust HIV disease progression [63, 64]. Moreover, Vpu proteins assemble into ion conductive pores and enhance virus release by down-regulating a host restriction factor, tetherin, from the cell membrane [65, 66]. Although there are a number of proposed models for tetherin's antiviral activity and how Vpu counteracts this activity, the exact molecular mechanisms remain unknown and need to be elucidated.

1.3.3. Replication cycle

Attachment of the HIV-1 envelope protein gp120 to the target cell surface CD4 receptor marks the start of infection. The interaction between gp120 and CD4 causes a conformational change in the viral protein, exposing a "hidden" domain that acts as a binding site for either CCR5 or CXCR4 co-receptors [38, 67]. The binding to the co-receptor induces additional conformational changes that expose the fusion domain of gp41, leading to membrane fusion and release of viral components into the cytoplasm

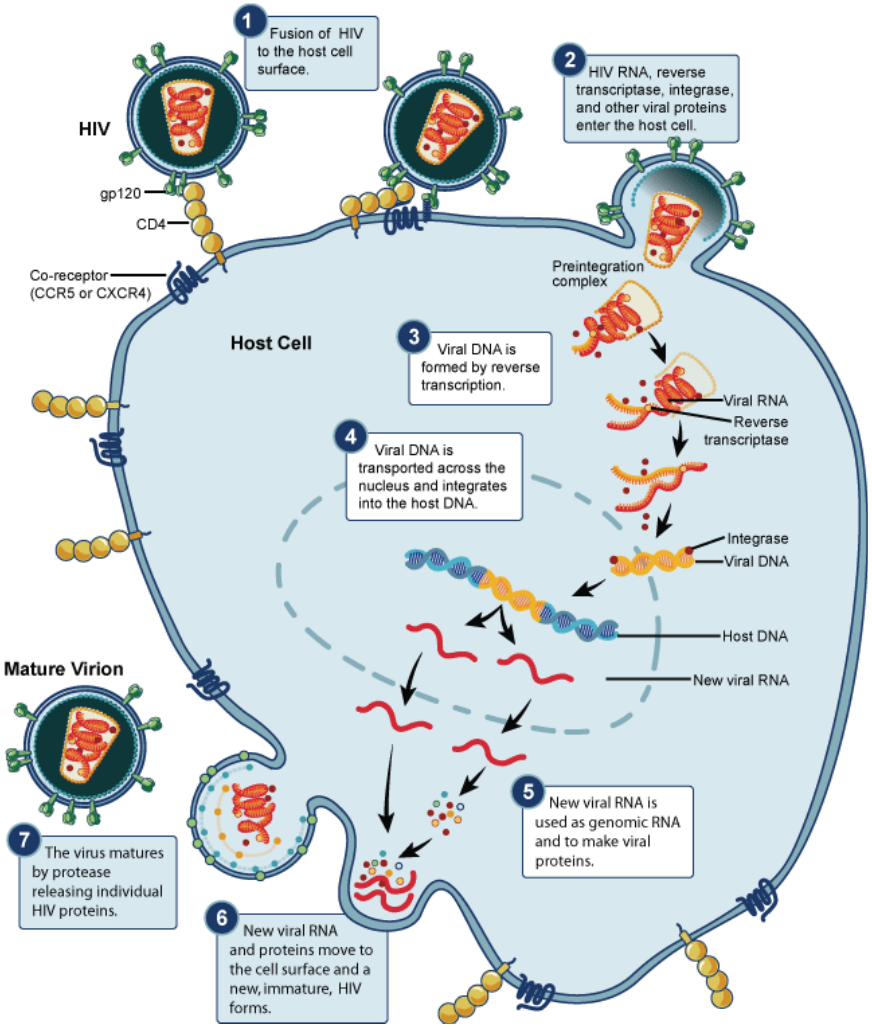


Figure 4: HIV-1 replication cycle.

Figure reprinted with permission from NIAID http://www.niaid.nih.gov

[24, 67, 68]. Viral uncoating involves cellular factors and viral proteins MA, Nef and Vif [29]. The HIV-1 genome is reverse transcribed from single stranded RNA to full length double stranded DNA by the viral RT [69-71] (Fig. 4). Vpr facilitates the transport of the proviral DNA to the nuclear membrane and the DNA enters the nucleus through a nuclear pore [72-74].

Inside the nucleus, the viral enzyme IN ‘inserts’ the linear double stranded DNA into the host chromosome [75, 76] (Fig. 4). Following integration, cellular transcription factors bind to the viral LTRs and initiate viral transcription utilizing cellular RNA polymerase II [77]. At this stage of the replication cycle, only multiply spliced mRNAs are produced leading to the translation of the regulatory proteins Tat, Nef and Rev. Once a sufficient level of the protein Rev is attained, Rev binds to the RRE present on single-spliced and unspliced viral RNA transcripts and directs their transport to the cytoplasm resulting in production of other viral proteins and genomic RNA [29, 45, 78].

A ribosomal frameshift is responsible for the translation of the *gag-pol* gene into the polyproteins Gag and Gag-Pol [79]. These polyproteins migrate to the cellular membrane to begin virion assembly, which is directed by the Gag polyprotein (Fig. 4) [80]. The *env* gene is translated into gp160 which becomes glycosylated in the endoplasmic reticulum and inserted into the plasma membrane [41]. In addition, full length viral RNA and cellular factors associate with the assembling immature viral core [81-83]. The budding of the virion activates the PR enzyme that auto-catalytically cleaves the polyproteins Gag and Gag-Pol into structural proteins and enzymes, respectively [36]. This cleavage leads to new stabilizing interaction between viral proteins and virion maturation, which result in an infectious viral particle [29, 81, 82].

1.4 Pathogenesis of HIV-1

1.4.1. Establishment of HIV infection

Infection by *Lentiviruses* is characterized by a lengthy period of incubation and clinical disease presentation. However, recent studies on simian immunodeficiency virus (SIV) in rhesus macaques, as a model for HIV-1 transmission in humans, highlight the importance of early events on establishment of a productive viral infection [84]. Earlier studies on heterosexual and mother-to-child transmission measured a lower degree of genetic diversity in viral isolates from newly infected compared to transmitting individuals [85, 86]. With advancements in sequencing technologies, about 80% of productive HIV infections due to heterosexual transmission were initiated by a single founder virus or infected cell [87, 88]. Moreover, the founder viruses do not have a loss of replicative capacity and are often phenotypically more resistant to CCR5 or entry inhibitors and neutralizing antibodies compared to virus isolates from individuals in chronic phase infection [88, 89].

In vivo studies of SIV infection in non-human primate have been an invaluable source of information about very early events in viral transmission and establishment of infection because due to practical and ethical reasons these studies cannot be done in humans [90]. Based on information from SIV infected rhesus macaque (RM) animal models, the genetic transmission bottle neck occurs days after crossing the mucosal barrier, after which the virus establishes a founder population that expands and disseminates to the lymph nodes resulting in systemic infection [91].

1.4.2. Course of infection

HIV infection and disease can be divided into three main stages: acute, chronic and AIDS. HIV infection begins with the eclipse phase that is part of the acute stage. The eclipse phase, which has been studied in female RMs, lasts a few days. The virus establishes small foci of infected cells in the submucosa of the female genital tract. At this stage the viral load (VL) produced by these localized foci is below the level of detection of sensitive tests currently available. The eclipse phase ends when virus reaches the draining lymph nodes and systemic viral dissemination occurs. Primary infection is characterized by high VL in the blood. Some individuals experience fever, diarrhea, rash, sore throat, fatigue and lymphadenopathy [92-94]. The VL peak occurs

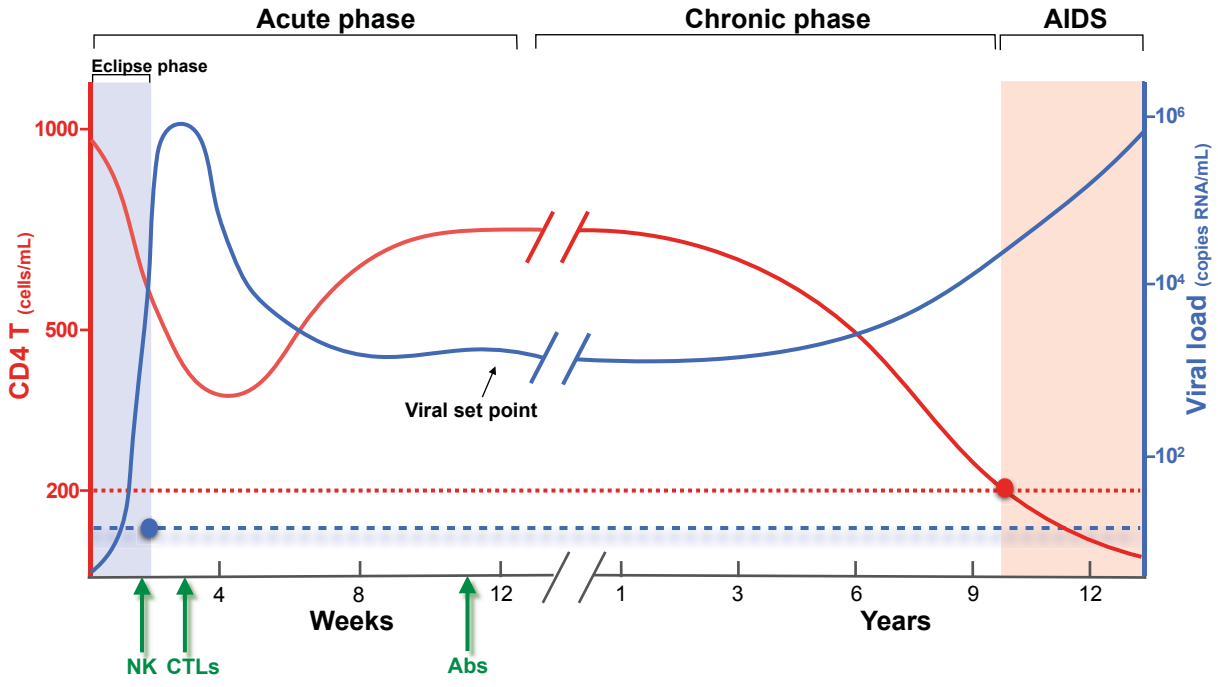


Figure 5: Course of HIV-1 infection.

Figure made by Irene Lisovsky

approximately 2 weeks after infection [95]. This state of high viremia resolves to a lower VL called the set point. The VL set point is predictive of the rate of progression to AIDS [96, 97]. In other words, the higher the VL, the worse the prognosis. By the end of the eclipse phase latent viral reservoirs (discussed in section 1.4.3.) are established and eradication of the virus using currently available means is practically impossible. The only known case to date of complete HIV-clearance is the 'Berlin-patient', discussed in section 1.4.5.

About 2 to 3 weeks following infection, the virus begins to be cleared by specific cytotoxic T lymphocytes (CTLs) and T helper cells that provide the necessary cytokines to CTLs. The virus is not only present in the blood, but undergoes rapid replication in lymphoid tissues. Numerous studies point to the mucosal associated lymphoid tissue (MALT) of the gut (GALT) as the most active site of HIV replication in early infection [98-101]. The GALT is a major site of the body's $CCR5^{+}CD4^{+}$ memory T cells, the main targets of HIV replication. The loss of $CCR5^{+}CD4^{+}$ memory T cells is a consequence of acute HIV infection, however initially, it does not compromise the regenerative capacity of the immune system [102].

During the acute phase of infection, seroconversion occurs and anti HIV antibodies (Ab) directed against envelope (Env) and p24 antigens emerge [103, 104]. These Abs contribute to the efforts of cell-mediated immunity in viral control [105, 106]. However, the immune system is a 'step-behind' the virus. Production of effective neutralizing antibodies (nAbs) and CTLs are accompanied by rapid escape from recognition due to

the virus' rapid mutation rate, particularly in the Env proteins, against which most nAbs are directed.

The chronic asymptomatic phase can last for years [107]. During this stage of infection, persistent viral replication continues to take place. CD4 cell counts fall slowly by an annual average of 60 cells/mm^3. During this phase a dynamic balance is established between rapid $CD4^+$ T cell clearance and robust $CD4^+$ T cell proliferation [107-109]. The constant immune activation driven by HIV infection eventually exhausts the capacity of the immune system to regenerate. When $CD4^+$ T cell counts fall below 200 cells/mm^3 the individual is highly susceptible to opportunistic infections that their immune system does not have the capacity to control [110]. At this stage, AIDS-defining diseases emerge.

HIV is diagnosed using a 4^{th} generation HIV enzyme immunoassay (EIA) that detects anti-p24 IgM and IgG Abs [111]. When positive, a confirmatory HIV western blot is performed. The acute to early chronic phases of HIV infection can be separated into Fiebig stages I to VI based on the results of these diagnostic tests [95, 97]. Fiebig stage I starts about 10 days post infection. It is defined by a positive viral RNA PCR assay, while HIV-p24 antigen and anti-p24 Ab tests are negative. Stage II is defined by a positive p24 antigen ELISA test, which can be detected as early as 15 days post infection, in the absence of a positive anti-p24 Ab test. Stage III is defined by a positive ELISA detecting HIV p24 specific IgM and IgG Abs that can appear around 3 weeks post infection. Stage IV is defined by positive HIV EIA results accompanied by an indeterminate HIV western blot. At Fiebig stage V, the western blot become reactive for

p24, p41 and/or gp120/160, but is negative for a band that corresponds to p31 (IN). The last stage, VI, is characterized by the same criteria as stage V, but with a fully reactive HIV western blot that includes a positive p31 band.

1.4.3. Antiretroviral therapy (ART) and resistance

The goal of ART is to reduce plasma VL, which correlates directly with rate of HIV disease progression [96, 97]. ARTs target viral enzymes that control and execute specific steps of the viral replication cycle. Fusion and entry inhibitors block the entry of HIV into susceptible target cells by interfering with the fusion event between the viral Env and cell membrane [112]. RT inhibitors include nucleoside and nucleotide analogues (NRTI) that act as DNA chain terminators and inhibit reverse transcription of viral RNA into DNA [113]. A second class of RT inhibitors, non-nucleoside RT inhibitors (NNRTIs), bind to a small hydrophobic pocket within RT; this alters the enzyme's flexibility and blocks its ability to synthesize DNA (allosteric inhibitors) [114]. Integration of viral DNA into the host chromosome is blocked by integrase inhibitors (INIs), which are the newest ART drug class that exhibit a high barrier for resistance [115]. Lastly, protease inhibitors (PIs) target the HIV-1 PR and interfere with virus maturation [116, 117]. In the presence of a PI, viral particles are produced, however, they are non infectious since Gag and Gag-Pol polypeptides are not cleaved into their mature form.

Among the first ARTs used to treat HIV infected subject were RTIs and PIs; these proved successful in decreasing VL. However, this success was short-lived as HIV drug

resistant mutants rapidly emerged. To overcome the rapidly emerging drug resistance seen with ART mono-therapy, a combination of drugs from different antiretroviral classes was given as treatment in 1996 [118]. This new course of triple-therapy is called highly active antiretroviral therapy (HAART). The use of HAART has proven to be very effective in controlling viremia, progression of HIV disease and in prolonging survival [118-122]. However, the long term success of HAART is also impeded by the development of resistance [14]. Furthermore, HIV-1 genetic diversity, global prevalence and design of ART based on subtype B information, highlight the need to focus on HIV non-B subtypes and the impact of genetic differences on disease progression, drug efficacy and evolution of drug resistance [123, 124].

1.4.4. Latency

Productive HIV-1 infection, as described in the previous section, occurs when the virus infects activated $CD4^+$ T cells, replicates and buds off to begin a new round of infection. However, part of the HIV-1 replication cycle includes integration of its viral genome into the host chromosomes, which establishes a productive or latent viral infection. Interestingly, even though latently infected cells are rare in HIV infected individuals, they pose a major obstacle for curing HIV. There are a number of approaches and tools available to evaluate the size of the latent reservoir, each having advantages and limitations (reviewed in [125]). A recent modeling study shows variation in measuring the size of the latent reservoir depending on the method used in evaluation [126]. This further complicates efforts to eradicate the virus.

Seeding of the latent reservoir begins early in infection when HIV infects activated $CD4^+$ T cells that undergo a burst of proliferation in response to these viral antigens and develop into an effector phenotype [127]. Most of the expanded effector cells die, however, a small subset of HIV infected CD4 T cells transition into a long lived resting memory state [128, 129]. These unique cells have a different gene expression profile that is non-permissive for HIV replication. This results in a stably integrated and transcriptionally silent virus that can survive in the host for a long period of time.

HIV latency can occur even during successful antiretroviral therapy (ART), when the patient's viremia is undetectable [130, 131]. The latent HIV-1 reservoir may consist of both wild type and drug resistant viruses. Few cells in the reservoir harbor replication competent virus [132-134]. Nevertheless, this reservoir shows minimal decline and seems to be sufficient to sustain lifelong HIV-1 persistence in infected individuals who receive ARTs [135, 136]. Thus, latency is one of the major obstacles to HIV-1 eradication. Furthermore, intensification of ART does not impact the size of latent reservoirs. Reservoir persistence is due to its residence in long-lived resting memory $CD4^+$ T cells, rather than continuous reseeding of the reservoir by new rounds of infection.

Strategies to 'shock and kill' latent reservoirs have been on the forefront of research in the past few years (discussed in [137]). Attempts have been made to purge latent reservoirs with chemical agents that stimulate gene transcription, such as histone deacetylase inhibitors, inhibitors of bromodomain proteins and protein kinase C

activators, which can reverse latency. Following a successful purge and reactivation of active viral transcription and protein production, potent ART can potentially be used to kill these virus producing targets. Although this approach has been the focus of many studies in the past few years, results have not been promising. Thus the “shock and kill” strategy may not be a realistic approach to reverse latency in all infected cells in order to cure HIV.

1.4.5. Protection and control of HIV-1 infection

- *CCR5 Delta 32* mutation

A genetic host factor that has been implicated in protection from HIV-1 infection and slow disease progression is a 32 basepair deletion in the *CCR5* gene (*CCR5Δ32*). Individuals homozygous for this mutation encode a truncated CCR5 co-receptor that is no longer expressed on the cell surface. This makes their $CD4^+$ cells resistant to infection with R5-tropic HIV-1, which represent the majority of transmission fit viruses [138-141]. Individuals heterozygous for *CCR5Δ32* express lower surface levels of CCR5 than do *CCR5* wild type homozygotes, permitting viral entry but slowing the propagation of infectious virions into susceptible neighboring cells. *CCR5Δ32* heterozygotes achieve an AIDS diagnosis an average of 2 to 3 years later than *CCR5* wild type homozygotes [138, 142, 143].

These discoveries have sparked the interest of the scientific and pharmaceutical communities in CCR5 as a therapeutic target, leading to the development of the fusion

inhibitor (FI) Enfuviritide in 2003 and CCR5-antagonist Maraviroc in 2005. Moreover, resistance to HIV infection due to homozygosity for the CCR5Δ32 mutation provided the basis for the remarkable story of Timothy Ray Brown, also known as “The Berlin patient”, who is currently the only human cured of HIV. Brown, who was an HIV-positive patient on successful ARV treatment, developed acute myeloid leukemia (AML) and consequently underwent an allogeneic $CD34^{+}$ hematopoietic stem cell (HSC) transplant from a donor who was homozygous for CCR5Δ32 [144]. Following the receipt of a second transplant, required due to rebound of AML, the leukemia was resolved and HIV RNA fell to below detection levels (<50copies/mL) in the absence of ART [144]. Brown has maintained undetectable levels of HIV replication and trace amounts of replication-deficient viral genetic material for 7 years without ART, i.e. since the second transplant.

The success of this single case, at this stage, cannot be translated for wider clinical usage mainly due to the risk associated with the pre-transplantation regimen and the treatment burden involved. Furthermore the probability of finding donors homozygous for CCR5Δ32 deletion is low due to the prevalence of this genotype, which is estimated at 1% among Caucasians; the frequency of this genotype in other populations is even lower [145, 146]. Nevertheless, the successful suppression of HIV, in the absence of ART, following reconstitution with HSCs from a *CCR5Δ32* homozygous donor, strengthens the rationale for therapies that rely on HSCs with disrupted CCR5. The lack of CCR5 does not appear to have a negative impact on life expectancy and is associated with a reduced risk of certain inflammatory conditions. However, lack of CCR5 may increase susceptibility to more uncommon infections [147, 148]. Some

strategies that have been tried in an attempt to replicate the above success in treating HIV infection have included the use of lentiviral vectors containing siRNAs targeting *CCR5*, ribozymes that specifically interrupt *CCR5* mRNA processing and zinc-finger nucleases that introduce site-specific interruptions in the CCR5 gene, which result in a mutated and non-functional receptor. These approaches have been explored in *in vivo* mouse models with promising results [149-152].

• HIV exposed seronegative (HESN) subjects: Cohort studies and protective immune responses

Individuals that have been repeatedly exposed to HIV without becoming infected are called HIV exposed seronegative (HESN) individuals [153]. The homozygous *CCR5Δ32* genotype only accounts for a minority of HESN cases. This may be due to its low prevalence, especially in the non-Caucasian populations, which constitute the main platform for epidemiological studies involving high-risk behaviour [139, 154]. A number of HESN cohorts throughout the world have been assembled and studied to better understand what genetic and environmental factors, as well as, immune responses contribute to protective mechanisms against HIV-1. These cohorts differ in their HIV exposure routes and fall into either sexually or non-sexually exposed groups. Modes of non-sexual transmission include injection drug use (IDU), mother-to-child transmission, breast feeding and exposure to contaminated blood products. Mother-to-child cohorts are limited, well controlled and monitored. Together with advancements in ART, they have contributed invaluable information and progress on interventions designed to

eliminate this form of HIV transmission [155-157]. Important information about resistance to HIV infection comes from IDU cohorts. IDU have a higher risk of infection compared to those exposed sexually [158]. In IDU, other risky behaviours such as unprotected sex that can accompany drug-use may make it difficult to identify the precise route and level of exposure in this group. It is worth noting that intravenous exposures to the peripheral immune system bypass the mucosal route.

High risk sexual transmission studies have been done on commercial sex workers (CSW), men who have sex with men (MSM) and HIV discordant couples. The majority of the CSW cohorts are based in Asia and Africa. One of the largest and best studied is the Pumwani sex worker cohort based in Nairobi, Kenya. The HESN women recruited to this cohort remain uninfected for a defined number of years despite having repeated high frequency unprotected sex that can reach up to 15 clients per day [159-161]. In contrast to CSW cohorts, high HIV infection rates and disease burden are well documented in MSM cohorts in resource-rich countries [162-164]. MSM have about a 10 fold greater risk of infection compared to CSWs due to unprotected receptive anal intercourse compared to vaginal sexual exposure. The third group of HESNs are the seronegative partners in HIV serodiscordant couples. These cohorts are distributed globally and include both heterosexual and homosexual couples. Participants of these cohorts have a lower risk of exposure compared to CSW and thus seronegative partners are epidemiologically classified as “high risk” instead of “resistant”, where the greater risk of transmission is with receptive, rather than insertive intercourse. The discordant couples cohorts can be considered more controlled compared to CSWs and

MSM, since the details of exposure, as well as, the virus isolate information is easier to collect. It is important to consider that physiological differences between types of cohorts, routes of exposure, life style and other possible concurrent infections may be important factors influencing specific mechanisms of protection, even though some correlates of protection and immune responses are likely to be common among the different types of cohorts.

Immune responses that correlate with protection in HESN still remain incompletely understood despite many studies on this topic. The proposed immune correlates of protection in HESN include cellular, humoral and innate immune responses. The first evidence that HIV-specific cellular immunity may be involved in protection from infection in HESN was documented in 1989 by Ranki et al. who detected HIV-specific T cell responses to HIV envelope and core proteins in HIV negative sexual partners of HIV-positive men [165]. Other groups confirmed the finding of both HIV-specific T helper (Th) and CTL in other HESN population studies [166-170]. Cells isolated from HESN, compared to HIV infected individuals, were found to secrete greater levels of CCL4 and CCL5 chemokines and IFN-γ and IL-2 type 1 cytokines [171-174]. These findings, in combination with other studies, were interpreted as support for the hypothesis that Th1 immune responses were more protective against HIV infection than Th2 responses [175]. Some studies have found an opposite trend where CD4 cells from HIV infected subjects, compared to HESN, produced higher levels of IFN-γ following stimulation with HIV derived proteins [176-178]. Lower immune activation and cytokine production was also measured in the Nairobi CSW cohort, which was termed “immune quiescence”

and thought to contribute to host resistance to HIV [179]. The current thinking on this topic is that, if present, HIV-specific cellular responses in HESN are of low magnitude and breadth, and may represent markers of previous HIV exposure, rather than a mechanism related to their protection from infection.

Humoral immune responses have also been identified in HESN. HIV-specific IgA antibodies were isolated from mucosal sites and the peripheral circulation of participants in several HESN cohorts [172, 180-182]. These antibodies can neutralize the virus, however, since they are not consistently present suggests that frequency of viral exposure may be a factor driving their presence [183, 184]. The specificity of IgA antibodies detected in HESN individuals is different than that of HIV infected subjects. Antibodies from HESN have been reported to be specific for an extra-cellular epitope of gp41 [185].

Acute HIV infection is characterized by a rapid rise in VL in the first 2 weeks of infection, often surpassing one million copies/ml plasma. The timing of VL decline after this peak implicates an important role for innate immune responses that precede T cells responses, all of which are involved in achieving the eventual VL set point. Factors that may be involved include the restriction factor APOBEC3G, which is present at higher levels in HESN compared to HIV-infected individuals and healthy controls [186]. NK cells from a Vietnamese cohort of HESN IDU compared to seroconverters and healthy controls had a higher functional potential in terms of cytotolytic activity and secreted IFN-γ, TNF-α. CCL3, CCL4 and CCL5 [187]. The importance of NK cell receptors and

functional responses to HIV infection and protection from infection is further exemplified in epidemiological and genetic studies that are discussed in detail in section 1.8 of this thesis.

• Slow progressors (SP): Control of HIV infection

While studying HESN informs our understanding of mechanisms and factors involved in protection from HIV infection, it is also imperative to understand correlates of viral control and slow disease progression in those infected. Five to 15% of HIV infected individuals exhibit a slow HIV disease course, maintaining either high CD4 counts and/ or low VLs without ART. Such individuals are termed slow progressors (SP) [188, 189]. These SP can further be subdivided into long-term nonprogressors (LTNP), who maintain a healthy range of CD4 T cell counts for over 10 years, and elite controllers (EC), who represent <1% of HIV infected individuals and have VLs below the level of detection (<50 HIV RNA copies/mL plasma) in the absence of ART [190, 191]. A third group of viral controllers (VC) maintain VLs <3000 HIV RNA copies/ml plasma without treatment. The definition of HIV control, whether LTNP, EC or VC, has been a topic of debate as multiple definitions have been suggested based on results from population based studies [192-194]. A recent study focused on examining existing definitions of HIV EC to identify what definition, if any, best characterizes this unique group of HIV infected subjects. This study found that HIV control can be lost with increasing time from infection. Furthermore, the group suggested two possible definitions that best define

ECs: 1) maintenance of undetectable HIV RNA levels for > 6 months, and 2) having >90% of VL measurements fall below 400 copies of HIV RNA/mL for > 10 years [195]. Mechanisms of control of viral infection in SP are thought to include both host and viral factors. The route of HIV acquisition is not a factor associated with HIV disease control in SP [196, 197]. While virus isolated from some SPs can contain mutations and/or deletions in key viral proteins that can lead to reduced viral replication capacity, no particular mutation accounts for the SP phenotype [64, 198-200]. Furthermore, carrying mutations that were associated with attenuated viruses did not preclude progression to AIDS in untreated subjects [201, 202]. Moreover, replication competent viruses with limited genetic diversity (in the *env* gene) have been isolated from HIV controllers [203, 204]. Overall, these findings suggest that while viral factors can influence HIV control in SP, predominant control of HIV replication is achieved by the host.

Heterozygosity for the *CCR5Δ32* mutation is observed among the different SP cohort studies and may account for a slower disease course due to restricted expression of the viral entry co-receptor CCR5 in some subjects [205-208]. Epidemiological and functional studies of SP have found a higher frequency of protective HLA, such as HLA-B*57 and B*27, in SP than progressors or healthy controls [209-211]. GWAS studies have shown that genes involved in virus control map to the MHC class I region (e.g. HLA-B*57 and a region upstream of HLA-C [212]. In addition, CD8 T cell responses from SP restricted by these protective HLA alleles show better VL control compared to cells from typical progressors [213]. SP individuals, compared to typical progressors, have poly-functional CD8 T cell responses characterised by high proliferative and cytotoxic capacity,

secretion of different cytokines and ability to respond to different viral variants [214-217]. HIV specific CD4 T cell responses measured by multifunctional cytokine secretion are higher in controllers than HIV-progressors [193, 218]. However, despite these findings, other studies show that many SP lack protective HLA alleles and specific CD8 T cell responses [203]. In addition, nearly half the controllers lack HIV specific cytokine producing CD4 T cells [219, 220]. These finding suggest that other mechanisms than T cell responses may be involved in maintaining long-term HIV control. Studies on humoral immunity in HIV SP have found that HIV specific neutralizing antibodies are of low titre and insufficient to mediate viral control [193, 203, 221]. Moreover, EC have lower *env* sequence diversity, less N-linked glycans and shorter variable loops (factors influencing antibody (Ab) sensitivity and functional potential) than ART treated or untreated progressors [222]. Ackerman *et al*. reported that EC differ from VC and progressors in having non-neutralizing HIV-specific antibodies able to mediate a more coordinated Fc-dependent innate immune response to HIV [223]. These intriguing findings will require more investigation in order to determine whether humoral immunity mediated by innate Fc receptor dependent activities play a role in HIV control in some EC.

Though it is apparent that no single factor can account for and explain control of HIV infection seen in all SP, the involvement of innate immune responses, particularly NK cells, may contribute to HIV control. NK cells in controllers are closer in number, distribution, receptor phenotype to healthy controls than progressors [224-227]. HLA antigens that were strongly implicated in HIV control interact, not only with T cell

receptors, but also engage cell surface receptors on NK cells. Specific combinations of HLA and NK cell surface receptors are associated with greater NK cell function. The importance of such genetic combinations on NK cell functional responses in control of HIV infection is further highlighted in epidemiological and genetic studies that are discussed in detail in section 1.8 of this thesis.

1.5 NK cells

1.5.1. Development and differentiation

NK cells are a subset of lymphocytes that compose 10-15% of peripheral blood lymphocytes. Peripheral blood NK cells differentiate from HSC common lymphoid progenitors in bone marrow (BM) and continue their maturation in the periphery in secondary lymphoid tissues (SLT), such as spleen, tonsils, and lymph nodes [228-230]. In the BM, NK cells express surface makers, such as CD34, CD45RA, and CD10, which they lose upon migration to SLT where they continue to differentiate, into cells expressing CD56, also knows as a neural cell adhesion molecule (NCAM). $CD56^{Bright}$ NK cells appear first and eventually differentiate into $CD56^{Dim}$ NK cells [231, 232]. The more mature $CD56^{Dim}$ NK cells are found in the peripheral circulation and spleen [233, 234]. The less differentiated $CD56^{Bright}$ population makes up less than 10% of the peripheral NK cells and predominantly localize to the lymph nodes and tonsils [235-237].

During development, NK cells acquire functional potential through a process termed ‘NK cell education’ (discussed in section 1.6.3). Education confers NK cells with properties such as tolerance to self and functional potential, which are important in the maintenance of immune homeostasis. Studies conducted using samples from humans diagnosed with NK cell deficiency diseases have highlighted their role in distinguishing between self and non-self antigens and mediating the clearance of transformed and virally infected cells. Two types NK cell deficiencies exist in humans: Classical NK cell deficiency is characterized by reduced numbers of NK cells. Functional NK deficiency is characterized by normal NK cell numbers with compromised functionality [238, 239]. Individuals exhibiting either of these deficiencies consistently present with severe viral infections, and are particularly susceptible to infections caused by herpes viruses.

1.5.2. Effector functions

NK cells mediate immune responses against virally-infected and transformed cells [240, 241]. They contribute to innate immune defenses directly by eliciting functions such as cytotoxicity (often measured via CD107a expression) and secretion of cytokines (e.g. IFN-γ, TNF-α) and chemokines (e.g. CCL3/4/5). The $CD56^{Bright}$ NK cells are less differentiated than the $CD56^{Dim}$ cells [231, 232]. The functional response profile of $CD56^{Bright}$ NK cells is dominated by chemokine/cytokine secretion. The functional profile of $CD56^{dim}$ NK cells was predominantly characterized by degranulation, as measured by CD107a expression [242]; however, recent studies have also provided strong evidence of chemokine and cytokine secretion that challenge this dichotomization [243-245]. NK

cells can also mediate Ab-dependent cellular cytotoxicity (ADCC) of virally infected and transformed cells. In the context of HIV, ADCC involves the recognition of HIV Env (gp120) on infected cells by Env-specific antibodies whose Fc region binds to the CD16 receptor on NK cells. CD16 engagement can also lead to Ab-induced activation of NK cells, where additional NK cell functions, such as secretion of cytokines/chemokines and degranulation, are induced.

NK cells also contribute in a number of ways to shaping adaptive immune responses through their interactions with dendritic cells (DC) and T cells. When DC cells get activated by pathogen associated molecular patterns they produce IL-12, IL-15 and IL-18 that promote the activation of NK cells, which in turn produce cytokines and chemokines that further promote the activation and maturation of DCs [246, 247]. This cytokine 'cross-talk' between NK and DC cells also maintains balanced maturation status of the DC compartment; when a high ratio of immature DC to NK cells occurs, NK cells kill the immature cells in a process termed 'DC editing' [248-250]. Maintaining a balanced maturity ratio is important for proper DC cell function. Activated NK cells can also contribute to adaptive immune responses by increasing the pool of IFN-γ, which is the primary driver of Th1 mediated immune responses that leads to elimination of virally infected or transformed cells by CTL.

1.5.3. Memory-like NK cells

Classically, innate immunity is assumed to be rapidly induced and to not exhibit

immunological memory. The generation of memory B and T cells depends on production of antigen-specific receptors through the recombination of antigen receptor genes mediated by RAG proteins [251]. New concepts of immune memory have begun to take shape based on the discovery of mechanisms that alter gene-expression patterns, including epigenetic changes and micro RNA production [252, 253]. NK cells memory-like responses have been induced by viruses [254], haptens [255], and proinflammatory cytokines [256, 257]. In mice, Ly49H^{+} NK cells undergo expansion upon binding mouse cytomegalovirus (MCMV) encoded m157 during infection, which is followed by contraction phase that establishes a “memory” pool for this antigen [254, 258]. In mice, these NK cells also persist after the infection, displaying some phenotypic characteristics of classic memory T cells. They are long lived, self-renewing and can mount a heightened immune response upon secondary exposure to this pathogen [254]. Similarly, human NK cells can also achieve a memory-like phenotype by activation with a combination of IL-12, IL-15 and IL-18 cytokines. This priming enhanced IFN-γ NK cell responses to restimulation with IL-12 and IL-15 cytokines. Interestingly, these memory-like IFN-γ responsive NK cells had a less differentiated NK cell receptor phenotype configuration. They were CD56^{Dim}CD57^{-}NKG2A^{+}, where CD57 is a differentiation marker and NKG2A is an inhibitory NKR (discussed in section 1.6.1) [257]. Furthermore, following hematopoietic cell transplantation from human CMV (HCMV) seropositive donors, NKG2C^{+} NK cells, not only expanded, but also exhibited enhanced IFN-γ production upon exposure to a secondary HCMV challenge compared to NKG2C^{+} NK cells from HCMV seronegative donors [259].

The mechanisms that underlie and drive the generation of memory are poorly understood. A recent study was the first to present compelling evidence for a potential mechanism through which MCMV induces the generation of NK memory-like cells [260]. The process requires mitophagy, a process by which damaged mitochondria are degraded via autophagy during the contraction phase of the NK cell response to MCMV. Several mitochondrial receptors (such as BNIP3 and BNIP3L) are found to be important players in this process. Thus it appears that clearing cells responding to a stimulus of damaged mitochondria is key to the generation of memory-like NK cells [260]. Previously, mitophagy was shown to be important in the formation of memory CD8 T cells and the importance of this process in generation of memory-like NK cells further highlights the plasticity NK cells exhibit as mediators of immune responses [261].

1.6 NK receptors (NKR) and functional potential

1.6.1. NKR families

NK cells express a variety of cell surface receptors that permit the cell to distinguish self from non-self antigens/cells and thus act both in tolerogenic and immunogenic capacities. These receptors belong to different families which have distinct structural features and functions. Even though NKRs are notably diverse, receptors can be dichotomized into activating or inhibitory forms. Activating NK receptors (aNKRs) associate non-covalently with transmembrane segments (via oppositely charged aa's) of adapter molecules that contain immunoreceptor tyrosine-based activation motifs (ITAMs) [262]. The ITAM intracellular signaling subunits get activated by kinase

phosphorylation and consequently initiate a mitogen-activated protein signaling cascade [263-265]. The cytoplasmic domain of iNKRs' contains one or more immunoreceptor tyrosine-based inhibition motifs (ITIMs) and upon ligand binding these tyrosine residues get phosphorylated and initiate a signaling cascade that predominantly relies on the recruitment of phosphotases to interfere with NK cell responses linked to aNKR [262, 266]. In general, the ligands for iNKRs are MHC-I molecules, while the ligands for aNKRs are stress induced molecules and pathogen-derived antigens. Some of the activating Killer immunoglobulin-like receptors (aKIR) also bind MHC-1 but at lower affinities than their iKIR counterparts [267, 268]. The key receptor families pertinent to NK cell function in the context of HIV are described in the section below.

- CD16 (FcγRIIIa)

This activating transmembrane receptor belongs to the Fcγ receptor family, contains ITAM motifs and is co-expressed primarily on $CD56^{Dim}$ NK cells. Together with the CD56 marker, it can also be considered as a marker of NK cell maturity; where, the $CD56^{Bright}CD16^{-}$ population is the least mature, $CD56^{Bright}CD16^{+}$ is a functional intermediate and $CD56^{Dim}CD16^{+}$ is the mature functional population [269]. The CD16 receptor on NK cells recognizes and binds the constant region, or fragment crystallizable (Fc) part of Abs of certain IgG subclasses [270, 271]. When the same antibody's antigen combining site binds to an epitope on a virally infected or transformed cell, NK cell activation ensues. This can trigger ADCC, killing the Ab-coated target cells, and Ab-induced activation of NK cells that results in secretion of cytokines/

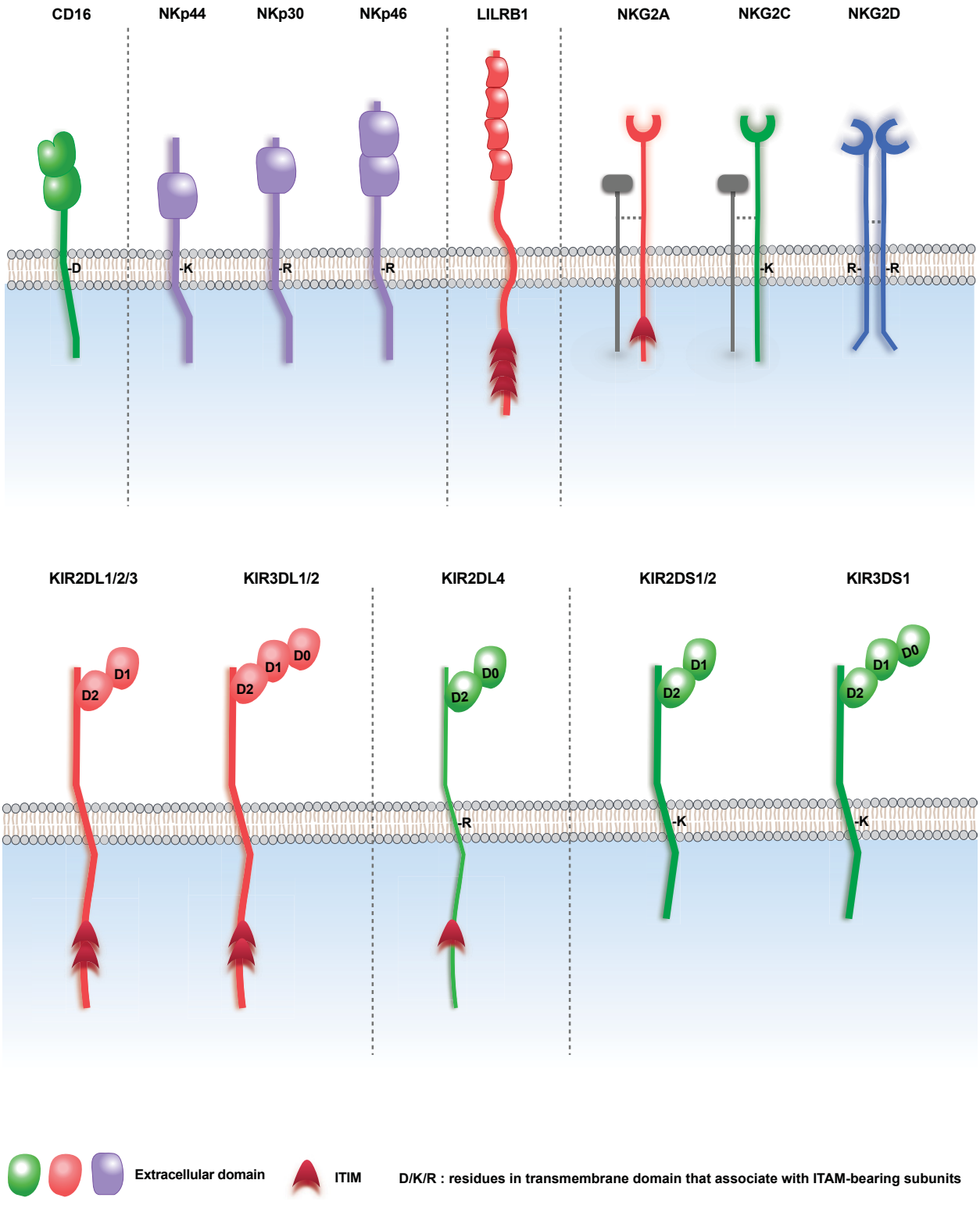


Figure 6: NK receptors. Inhibitory (red) and activating (green, purple and blue) NK receptors. CD94, which dimerizes with NKG2A and NKG2C is in grey. Figure made by Irene Lisovsky.

chemokines and degranulation. Furthermore, the engagement of other FcγR variants on immune cells such as monocytes, neutrophils and DCs by IgG Fc can activate these cell types [272-274]. These cells can contribute to immune responses by opsonizing and phagocytosing virally infected cells and acting as antigen presenting cells, altogether leading to Ab-dependant cell-mediated viral inhibition [275-280].

In the context of responses to HIV, ADCC occurs when Env-specific antibodies bind gp120 Env or Env derived epitopes on virally infected cells and their Fc region binds CD16 on NK cells. A single nucleotide polymorphism (SNP) at aa 158 of the CD16 protein can influence the potency of ADCC. A valine at position 158, rather than a phenylalanine at this position, enhances the binding avidity of CD16 for IgG and increases ADCC [281, 282]. NK cell mediated ADCC of HIV infected cells has been a focus of extensive research since it was found to be a correlate of protection from infection in a secondary post-hoc analysis of the results of the RV144 HIV vaccine trial, the only vaccine trial to date to confer modest (~31%) protection against HIV infection [283, 284]. Interestingly, recent studies point out a number of additional mechanisms of Fc-Ab mediated NK activation that are important to preferential responses in protecting and controlling HIV infection [104, 271, 285]. NK cell mediated Ab-dependent activation, as measured by secretion of cytokines and chemokines, has been observed in response to autologous HIV infected cells and gp120-coated target CD4 cell-line [286-288].

• Natural Cytotoxicity Receptors (NCR)

NKp30, NKp44 and NKp46 are type-I glycoproteins belonging to the immunoglobulin (Ig) superfamily [289]. NKp30 and NKp44 are both encoded on chromosome 6, while NKp46 is encoded on chromosome 19 and is part of the leukocyte receptor complex (LRC). These receptors associate with ITAM bearing adaptor proteins: NKp44 with DAP12, NKp30 with CD3ζ and NKp46 with CD3ζ and FcRεIγ [290]; they function as aNKR. Their respective ligands are not well characterized and are thought to be cellular or pathogen-derived molecules expressed by normal cells under stress conditions and infection, respectively. All three receptors recognize heparan sulfate proteoglycans [291]. NKp30 can also bind B7-H6, a tumor associated molecule and HCMV derived pp65 protein [292, 293]. NKp44 and NKp46 bind influenza hemagglutinin (HA) [294, 295].

NKp30 and NKp46 are expressed at low levels on resting cells and are upregulated upon NK cell activation. In contrast, NKp44 is expressed upon NK cell activation. NCRs represent specific NK cell markers, whose engagement induces tumor and virus infected cell lysis. Recognition of influenza HA by NKp44 results in enhanced killing of virally infected cells [294]. Furthermore, in the context of HIV, gp41 induces the expression of an NKp44 ligand that results in activation of $NKp44^{+}$ NK cells and cytotoxicity against CD4 T-cells in HIV-infected individuals [296]. The NKp30 receptor is involved in DC-NK cross-talk by meditating ‘DC-editing’, a process in which immature DC cells are killed by NK cells to maintain a lower immature DC to NK cell ratio [248]. Reduced surface expression of NCR and impaired NK cytotoxicity has also been

observed in HIV infected patients [224].

• Leukocyte immunoglobulin-like receptors (LILRs)

LILRs are a large family of genes located on chromosome 19 within the LRC. Of the thirteen LILR genes only one, LILRB1 (also referred to as LIR1, CD85j and ILT2), can be found on the surface of NK cells, while the others are expressed on B cells and monocytes [297]. LILRB1 is a transmembrane protein that functions as an iNKR since its cytoplasmic tail contains ITIM. LILRB1 binds with a low affinity to the conserved α3 domain that is present in a majority of cellular MHC-I molecules, including HLA-A, B, C, E, F and G [298, 299]. Thus, LILRB1 is thought to sense overall changes in MHC-I cell surface expression resulting from viral infection or transformation. This iNKR also interacts with the HCMV derived glycoprotein UL18, though with an approximately 1000-fold higher binding affinity compared to the MHC-I ligands [300, 301].

• NKG2

The NKG2 gene family is located within the NK cell complex on human chromosome 12p12.3-p13.2 [302]. This genetically conserved family encodes both activating (NKG2C, NKG2D, NKG2E) and inhibitory receptors (NKG2A, and its splice variant NKG2B) [303]. An amino acid residue in the transmembrane of the activating receptors NKG2C and NKG2E, but not NKG2D, interacts with adaptor proteins containing ITAM. The inhibitory receptors in this group contain intracellular ITIMs that upon

phosphorylation initiate a signaling cascade leading to NK cell inhibition/tolerance. NKG2 and CD94 are transmembrane proteins belonging to the C-type lectin like family [251]. The CD94 gene is closely linked to the NKG2 gene cluster. The CD94 protein forms heterodimers with NKG2A, NKG2C, NKG2E and NKG2F receptors. These heterodimers are held together on the NK cell surface with disulfide bonds. The non-classical MHC-I HLA-E molecules serve as ligands for all these NKG2 receptors with the exception of NKG2D [304]. HLA-E surface expression is dependent on the availability of peptides derived from the leader sequence of various HLA-class I antigens [305, 306]. Accordingly, HLA-E surface expression correlates with the overall expression of HLA-class I molecules on cells. The iNKR NKG2A binds HLA-E with a higher affinity than the NKG2 aNKR counterparts. Similarly to LILRB1, NKG2A can also sense and respond to overall changes in MHC-I levels on target cells due to virus infection or transformation.

The expression levels of NKG2A, together with CD56 and CD57, can define peripheral blood NK cells at different stages of maturation. As NK cells mature, the intensity of CD56 expression transitions from bright to dim, CD57 expression is upregulated, NKG2A expression declines and expression of other iNKRs (i.e. KIRs) increases [243, 307]. Accordingly the more immature peripheral NK cells are $CD56^{Bright}CD57^{-}NKG2A^{+}iKIR^{-}$, while the most mature peripheral NK cells are $CD56^{Dim}CD57^{+}NKG2A^{-}iKIR^{+}$.

NKG2D has little homology with the other NKG2 family members. It forms homodimers,

linked by disulfide bonds, on the surface of NK cells [308]. The ligands for NKG2D are MIC-A, MIC-B, and the glycoprotein UL16-binding proteins (ULBP) 1-4, which have structural similarities with MHC-class I molecules [251]. Upon ligand binding, an arginine residue in the transmembrane domain of NKG2D associates with the transmembrane region of the DAP-10 adaptor protein and initiates an intracellular signaling cascade [308]. Engagement of NKG2D with its ligands on target cells activates NK cells robustly and results in target cell killing. HIV infection upregulates the ligands for NKG2D and thus the virus has evolved a number of strategies to evade NK cell activation through this aNKR [309-311]. HIV Nef promotes downmodulation of the stress ligands ULBP1 and 2 [312]. Furthermore, Vif counteracts A3G by routing it to a proteasomal degradation pathway [313], thereby, avoiding activation of the DNA damage pathway and upregulation of NKG2D ligands.

• Killer immunoglobulin-like receptors (KIRs)

The KIR family is encoded by the KIR gene cluster within the LRC on human chromosome 19. Currently, 15 different KIR genes and 2 pseudogenes (*KIR2DP1* and *KIR3DP1*) have been identified [314, 315]. The KIR gene region is polygenic, meaning that the number of genes present differs from one person to another [315, 316]. Each of the loci are also polymorphic. KIR gene regions can be broadly classified into two groups termed haplotype A or B based on gene content [317]. With few exceptions all KIR gene haplotypes include 4 framework genes: *KIR3DL3* at the centromeric end *KIR3DL2* at the telomeric end, with *KIR3DP1* and *KIR2DL4* in the middle of the KIR

region [318-320]. Haplotype A consists of the framework genes plus *KIR2DL1*, *KIR2DL3*, *KIR3DL1*, *KIR2DS4* and *KIR2DP1*. *KIR2DS4* is the only locus encoding an aKIR but many of the alleles at this locus encode a truncated protein that is not expressed on the cell surface [321, 322], Thus many individuals homozygous for KIR haplotype A express no aKIR. KIR B haplotypes vary more broadly with respect to the number and combination of genes present. They can include the framework genes plus *KIR2DL2*, *KIR2DL5A*, *KIR2DL5B*, *KIR2DS1*, *KIR2DS2*, centromeric and telomeric *KIR2DS3* and *KIR2DS5* and *KIR3DS1* [323, 324].

Although the KIR region displays a high level of plasticity due to equal and unequal recombination events, most KIR haplotypes are made up of combinations of one of 4 centromeric and one of 2 telomeric gene motifs, each exhibiting a high level of within-motif linkage disequilibrium between genes [325]. There is one haplotype A centromeric and telomeric motif; the others are haplotype B motifs. Individuals homozygous for haplotype A have only centromeric and telomeric A motifs. KIR B haplotypes consist of all other combinations of centromeric and telomeric motifs, where they can be characterized as BB homozygous or BA heterozygous. A positive correlation was discovered between BB homozygosity and enhanced clinical benefit following MHC-I mismatched hematopoietic cell transplantation for the treatment of myelogenous leukemia [326]. The specific structural and functional features of KIRs will be explored in detail in the following section (1.6.2).

1.6.2. KIR structure, signaling and ligands

The KIR gene family encodes type I transmembrane glycoproteins that belong to the Ig superfamily. The KIR gene products have either two or three extracellular domains designated as KIR2D or KIR3D. The KIR3D extracellular domains are of the D0, D1 and D2 type followed by a short stem, transmembrane domain and cytoplasmic tail [251]. Most of the KIR2D molecules lack a D0 domain and begin their extracellular N-terminus with D1 and D2 Ig-like domains [251, 320]. Exceptions to this are KIR2DL4 and KIR2DL5, which have extracellular domains of the D0-D2 type[327, 328]. The nature of the cytoplasmic tail determines the activity of the KIR. Long (L) cytoplasmic domains have one or two ITIMs and such receptors act in an inhibitory fashion [262, 266]. Short (S) cytoplasmic tails, without ITIMs, are characteristic of aKIRs, which associate with ITAM containing adaptor proteins [264].

Inhibitory KIRs (iKIRs) recognize MHC-I proteins (HLA-A, HLA-B, and HLA-C). KIR2DL1 recognizes HLA-C group 2 (C2) antigens that have a lysine at position 80. The remaining HLA-C variants, which have an asparagine at this position, form the C1 group originally reported to interact with KIR2DL2 and KIR2DL3 [329-331]. The KIR2DL2 and KIR2DL3 receptors can also bind certain C2 and two HLA-B allotypes [332]. In general, KIR2DL2 and KIR2DL3 bind these C2 antigens with a lower affinity than KIR2DL1 and the KIR2DL2 receptor binds C1 and C2 ligands with a higher affinity than does KIR2DL3 [332].

There are 2 lineages of KIR3DL1 receptors, the *015-like family which is often expressed on the cell surface at high levels and the *005-like family expressed on the cell surface at lower levels [333]. KIR3DL1 homozygous genotypes can be dichotomized into two groups referred to as **h/*y* and **l/*x* [334]. The **h/*y* genotypes include high expression alleles (**h*) with no low expression alleles (**l*) and the **l/*x* genotypes include at least one **l* allele [333, 334]. The **h/*y* genotypes are distinguished from the **l/*x* genotypes by encoding KIR3DL1 combinations expressed on the cell surface at different levels. Consequently, the KIR3DL1-specific monoclonal antibody (mAb) clone DX9 stains KIR3DL1 from **l/*x* carriers with a positive-dim intensity and from **h/*y* carriers with a positive-bright intensity [333]. KIR3DL1 interacts with a subset of HLA-A and –B antigens that belong to the Bw4 group [335, 336]. The Bw4 antigens differ from the remaining Bw6 variants at aa's 77-83 of the HLA heavy chain [337]. Bw4 variants can have an isoleucine at position 80 (80I) or a threonine (80T) at this position of the HLA heavy chain. An initial report suggested that 80I Bw4 antigens bound KIR3DL1 with a higher affinity than 80T variants [335]. However, subsequent findings showed that certain KIR3DL1 allotypes preferentially recognize 80T variants [338]. A recent study also points out an aa 283 of KIR3DL1 as having an important role in recognition of Bw4 subtypes [339]. The co-carriage of *KIR3DL1*h/*y* and *HLA-B*57* alleles is particularly effective at educating NK cells for functional potential, suggesting that high expression KIR3DL1 and the HLA-Bw4*80I HLA-B*57 receptor-ligand combination interact with a particularly high affinity [340-343] (discussed in sections 1.6.3. and 1.6.4.).

KIR3DL2 has been shown to recognize certain HLA-A3 and A11 allotypes, as well as, free HLA-B27 heavy (H)-chains and H-chain dimers. The high affinity interaction of KIR3DL2 with HLA-B*27 free H chain and dimers has been proposed to promote the expansion of T cells in ankylosing spondylitis, a disease associated with carriage of HLA-B27 [344-347].

The specificity of ligands for aKIR are not well characterized despite the high degree of genetic homology between respective iKIR and aKIR. For example, KIR3DL1 and KIR3DS1 have over 95% aa homology in the extracellular domain. Earlier studies failed to show binding between KIR3DS1 and HLA-Bw4 antigens [348]. More recently, O'Connor *et al.* showed that the R166 residue of KIR3DS1, compared to the shorter leucine 166 found in KIR3DL1, plays an important role in preventing interactions with many peptide/HLA allotype complexes [349]. This study also found that certain HIV derived peptides can also overcome the effect of R166 to allow interaction between KIR3DS1 with HLA-B*57:01 [349]. Structural studies of KIR2DS receptors have shown more progress in identifying key aa residues important in regulating binding between KIR2DS1/KIR2DS2 and HLA-C molecules. KIR2DS1 has been shown to bind HLA-C2 ligands but with half the affinity of KIR2DL1. This is due KIR2DS1 having a lysine at position 70, while KIR2DL1 has a threonine at this position [350-352]. Similarly, mutating the naturally occurring tyrosine at position 45 to the phenylalanine of KIR2DS2 enables binding to HLA-C1 [353]. A study by Moesta *et al.* shows that even when binding occurs between KIR2DS2 and the HLA-C1 allotype HLA-C*16, it is of very low affinity [351]. Alleles at the *KIR2DS4* locus are evenly balanced between those that

encode a full length receptor that is cell surface expressed and those that have a 22 base pair deletion, resulting in a truncated protein not expressed on the cell surface [268, 322]. Expressed KIR2DS4 is able to bind weakly to certain HLA-C1, -C2 and HLA-A*11 alleles [268, 354]. Furthermore, an amino acid motif at residues 71 and 72 share homology with KIR3DL2. This was likely introduced by a gene conversion event between *KIR3DL2* and an ancestral *KIR2DS4* gene, which conferred KIR2DS4 with its ability to bind HLA-A*11 [268].

KIR2DL4, like KIR2DL5 and unlike the other KIRs, has a hybrid D0-D2 type domain structure. It carries a single ITIM that display weak inhibitory activity, but can also act in an activating capacity due to association with the FcRεIγ adapter protein [355, 356]. KIR2DL4 is not expressed on the surface of resting peripheral NK cells. Rather, it is maintained in an endosomal compartment upon stimulation with IL-2 [356-358]. Activation of KIR2DL4^{+} NK cells by soluble Abs results in robust cytokine secretion, particularly IFN-γ, but not cytotoxic responses [358].

1.6.3. NK cell education and functional potential

The state of activation of NK cells is determined by an ontogenic process known as education or licensing, which requires the interaction of iNKRs with their cognate HLA ligands on neighbouring cells. The education process generates a functional and self-tolerant NK cell repertoire in a given individual [359-361]. NK cells expressing iNKRs to co-expressed MHC-I ligands gain functional potential. NK cells with no iNKR to co-

expressed ligand, remain uneducated and are hyporesponsive [359, 362]. For example, KIR3DL1$^+$ NK cells that develop in carriers of *Bw4* alleles are educated because they express a KIR/HLA combination that can interact and transmit educating signals to the NK cell through this iKIR. In carriers of only *Bw6* alleles, there are no ligands for KIR3DL1. In this setting KIR3DL1$^+$ NK cells expressing no other INKR to self ligands remain uneducated and hyporesponsive.

Education is not an on/off switch as functionality can be tuned by the number of iNKRs engaged, the strength of interactions between iNKRs and their ligands and whether aNKRs are also engaged [363-366]. Certain aNKRs such as KIR2DS1 shares a high degree of homology with its iNKR partner, KIR2DL1; both KIR2DS1 and KIR2DL1 interact with C2 HLA variants [365]. Engagement of KIR2DS1 tunes down the activity of NK cells expressing this receptor when from donors homozygous for the ligand HLA-C2, providing an example of how aNKR can also contribute to the education process [365]. In general, when the ligand for an aNKR has been identified, the resulting receptor-ligand interaction is usually not as strong as the interaction of the same ligand with the iNKR counterpart. Consequently, the impact of aNKR, in contrast to iNKR, signaling on NK cell education is more modest. An example of the correlation between the strength of the interaction between NKR and their ligands and impact on education is illustrated by differences in the functionality of KIR3DL1$^+$ NK cells from carriers of the highly expressed (*h*/y**) *KIR3DL1* homozygous genotype combination versus the low expression **l/*x* combination from individuals who co-carry the high affinity binding ligand HLA-B*57. KIR3DL1$^+$ NK cells from *h*/y**+B*57 carriers receive stronger

educating signals than those from *I*/x*+B*57* carriers, and consequently exhibit a higher frequency of functional KIR3DL1^{+} NK cells upon stimulation with HLA-null cells [367].

Recent evidence suggest that NK cell education is a dynamic event that begins with NK cell development in the BM and can be further tuned in the periphery during adulthood in response pathogens and environmental changes [368, 369]. Studies in mice show that adoptive transfer of mature responsive NK cells to a host lacking donor self MHC renders these NK cells anergic [370]. Responsiveness can also be gained by formerly hyporesponsive NK cells if they are transferred to a host expressing the cognate MHC ligand for cell surface expressed iNKRs [371]. These studies demonstrate that NK cell education and function are dynamic processes, where changes to the peripheral environment have important effects on NK cell effector responses.

1.6.4. Evaluation of NK cell functional potential

NK cell functionality can be assessed using a variety of stimuli including cell lines, Abs, cytokines and virally infected cells. However the optimal and most common way to test the impact of NK cell education on NK functional potential is using HLA-null cell lines as stimuli. The HLA-null 721.221 (henceforth 721) and K562 cell lines are commonly used to activate NK cells educated through the interaction of iNKRs with HLA ligands. K562 is a human erythromyeloblastoid leukemia cell line derived from the pleural effusion of a chronic myeloid leukemia patient [372]. 721 is a B lymphoblastoid cell line that was generated by γ-ray-induced mutagenesis in the HLA complex [373]. Their ability to

activate NK cells is dependent on the fact that they each express a set of ligands for aNKR and on their lack of MHC-I expression, which abrogates negative signaling through iNKRs. 721 and K562 cell lines differ in their expression of ligands for aNKRs [293, 374-378] (Tremblay-McLean A personal communication). Given the differences in the origins of these HLA-null cell lines, the ligands for aNKRs they express and NK cell activation pathways for cytolysis and cytokine/chemokine secretion that aNKR binding these ligands initiate, it is probably not surprising that these cell lines can induce different functional patterns of NK cell activation. Previous work form our lab showed that 721 activates a significantly higher frequency of NK cells and KIR3DL1^{+} NK cells than K562 [379]. The NK cell functional subsets that are stimulated to a higher degree by 721 than K562 include those secreting IFN-γ and/or CCL4. On the other hand, the functional subsets that include CD107 expression contribute to a higher proportion of the total NK cell response following stimulation with K562 than 721.

1.7 NK cells in infectious diseases

1.7.1. HCMV

HCMV is highly prevalent among humans in both developing and developed countries. HCMV results in asymptomatic infection in healthy individuals, however, the virus can cause severe disease in immunocompromised people (e.g. those diagnosed with AIDS) or individuals undergoing an organ or HSC transplantation. HCMV has a number of genes that enable the virus to evade the immune system. For example, HCMV encoded

MHC-I homologs inhibit NK cell function via maintenance of interactions with iNKRs. The viral protein UL18 binds the inhibitory LILRB1 receptor on NK cells and inhibits their function [380]. A peptide within the HCMV UL40 protein upregulates surface levels of HLA-E that on one hand dampen the activation of $NKG2A^+$ NK cells, and on the other hand, engage with aNKR NKG2C and trigger activation of $NKG2C^+$ NK cells [381]. Interestingly, following primary infection with HCMV, peripheral $NKG2C^+$ NK cells expand from about 2% of NK cells in seronegative people to over 25% in those who are seropositive [382]. HCMV infection also upregulates the cellular stress molecules MIC-A, MIC-B and ULBPs that engage the aNKR NKG2D and promote activation of $NKG2D^+$ NK cells [383, 384].

1.7.2. Hepatitis C virus (HCV)

Globally, about 170 million individuals are infected with HCV and unresolved infections can lead to chronic disease and further complications, such as liver cirrhosis and eventually to hepatocellular carcinoma. The incubation period for acute HCV infection is 6-10 weeks and about 20% of newly infected individuals can clear the infection in the acute phase. These people differ from those who develop chronic HCV infection, by having robust and broad cellular and humoral immune responses. However, prior to the onset of adaptive immune responses, innate immune effector cells, like NK cells, are thought to be important in clearing the virus [385, 386]. NK cells secrete IFN-γ, which directly inhibits HCV replication in a non-cytopathic manner [387]. This is an important mechanism of viral control since due to the high rate of HCV replication almost all

hepatocytes become infected and cytotoxic elimination of HCV infected cells would result in substantial liver damage. In addition, in healthy individuals, NK cells represent about 30% of the lymphocytes found in the liver, while in HCV infected individuals, their numbers can rise to as high as 90%. Epidemiological studies have also implicated the carriage of specific KIR/HLA combinations in viral clearance. Intravenous drug users carrying the alleles for KIR2DL3 and homozygous for HLA-C1, the primary ligand for this receptor, exhibit a higher frequency of viral clearance that prevents the establishment of chronic infection [388, 389]. This KIR/HLA pair was also enriched in HCV-exposed seronegative aviremic individuals [390]. Moreover, chronically infected individuals had a higher frequency of HLA-C2 alleles compared to healthy controls [391]. In general, higher frequencies of KIR2DL1/2/3 and KIR2DS1/2^{+} NK cells were observed in individuals who resolved their HCV infection compared to those with acute or chronic HCV infection [386].

1.7.3. Influenza

Globally, an estimated 250-500 thousand deaths per year are attributed to seasonal influenza infection [392]. Clearance of acute infection and subsequent resistance to reinfection are associated with humoral responses, which are directed against the viral HA and neuraminidase glycoproteins, and cytotoxic CD8 T cells, which kill virally infected cells [393, 394]. The early induction of innate immune responses, particularly those mediated by NK cells, has been highlighted in a number of studies as being important in controlling and limiting influenza infection. The NCRs NKp44 and NKp46

directly bind viral HA on influenza infected cells and mediate cell lysis [294, 295]. Moreover, blocking NKp46 with Abs results in impaired NK cell responses to influenza [395, 396]. Certain KIR/HLA combinations have also been shown to play a role in responses to influenza. For example, *in vitro* studies have shown that KIR2DL3^{+} NK cells from donors with the cognate ligand HLA-C1, compared to HLA-C2 homozygotes, had stronger CD107a and IFN-γ responses to influenza [397]. However, despite convincing *in vitro* studies no epidemiological studies have reported a protective KIR2DL3 mediated effect at the population level. In contrast, the percentage of individuals who co-carry KIR2DL2/3 with HLA-C1 was increased in persons with severe influenza infection [398]. In addition, the frequency of KIR3DS1, KIR2DS5 and KIR2DL5 was reported to be associated with severe pandemic (H1N1) influenza [399].

The importance of NK cell activity in the clearance of influenza is implicated by the fact that influenza uses several strategies to evade NK mediated immune responses. The virus mutates its HA molecule glycosylation pattern, which confers resistance to lysis mediated by NCRs [400, 401]. Furthermore, the virus promotes the clustering of MHC-I molecules into lipid raft microdomains on virus infected cells, which increases inhibitory input to NK cells expressing the iKIRs using these MHCs as ligands [402].

1.8 NK cells in HIV-1 infection

In a typical immune response to a viral infection the activation of NK cells occurs without prior sensitization, before induction of T cell mediated immune responses. The immune response is a sequential process that begins with the secretion of type-I interferon (IFN-α) that results in activation of innate effector cells, such as macrophages and DCs, to secrete cytokines, including IL-15 that drives the proliferation of NK cells. The timing of NK cell responses suggests that they may have a role in initial control of HIV infection [403, 404]. The initial decline in HIV viremia following the VL peak precedes the establishment of adaptive immune responses [403]. This concept is further supported by studies that implicate NK cells in resistance to HIV, such as the enhanced NK cell functional responses (IFN-γ, TNF-α, CCL3/4/5) measured in a well studied Vietnamese cohort of HESN IDUs [187]. The importance of NK cell function in the context of HIV infection is further highlighted by the development of HIV sequence polymorphisms that allow the virus to evade KIR2DL2^{+} NK cell anti-viral pressure [405].

HIV infection is associated with dramatic changes in the peripheral NK cell compartment resulting in a marked reduction of CD3^{-}CD56^{+} NK cells [406-408] and emergence of a new hypofunctional CD3^{-}CD56^{-}CD16^{+} NK cell population [409, 410]. Despite this imbalance within the NK cell compartment, overall NK cell numbers remain stable throughout the course of HIV-1 disease. Successful ART leads to the normalization of the ratio of hypofunctional CD3^{-}CD56^{-} to functional CD3^{-}CD56^{+} NK cell populations, though CD3^{-}CD56^{-} NK cells persist at higher levels than those measured in healthy individuals. Skewed immuno-physiological ratios are also observed in both the CD4 and CD8 T cell compartment during HIV infection. They are indicative of immune dysfunction

caused by the virus and correlate with an accelerated speed of disease progression to AIDS [411-414]. Similarly to what is observed with the NK cell compartment, ART can normalize the CD4:CD8 ratio in some cases, and T cell homeostasis in both adults and children. However this normalization may not reflect complete recovery of immune cells with these phenotypes as T cell dysregulation may persist [415-417].

1.8.1. Genome-wide association studies (GWAS) and HIV infection outcomes

GWAS and epidemiological studies on the association of host genetic determinants with HIV outcomes are invaluable tools for the discovery of candidate SNPs whose gene products may function in immune responses that play a role in protection from HIV infection and/or disease progression, in those infected. Several GWAS have revealed significant associations between SNPs that encode variations within MHC class I region genes on chromosome 6 and HIV viral control [212, 213, 403, 418]. The first GWAS study by Fellay J *et al*. was published in 2007 where over 30,000 HIV infected individuals were screened. Following exclusion of subjects with incomplete data or unclear VL set-points, the analysis was based on 486 individuals. They identified two SNPs linked to HLA-B*57 and HLA-C that served as major genetic determinants of VL set point [212]. In addition the group identified a third SNP located over 1M base-pair away from the MHC complex in a gene that encodes RNA polymerase; this SNP explains about 6% of variations in disease progression, as defined in this study by CD4 counts <350 cells/mm^3, and was also found to be associated with VL control, though to a lesser degree than disease progression. Collectively these three SNPs explained about 14% of the VL set point variation and 10% of the variation in disease progression.

In a follow-up study two years later, the group validated their findings in a larger cohort of HIV infected individuals (n >2500) [419]. Of particular interest, the two SNPs reported previously to be associated with HIV disease outcomes were reconfirmed to be the strongest determinants of variations in VL using a more stringent analysis. In addition, the third SNP identified located outside the MHC complex was not significantly associated with viremia in this more comprehensive analysis, yet the effect on progression remained significant. This suggests that there are different mechanisms involved in modulating HIV disease progression and VL set point. In another large-scale GWAS of HIV controllers (SP) and typical-progressors Pereyra *et. al.* identified a link between the SNPs associated with VL control and key amino acids in the HLA-B peptide binding groove. Altogether, information from these important studies suggest that different mechanisms are in place to control for VL set point and disease progression, however at this stage these mechanisms still remain elusive.

HLA-B loci exhibit the greatest degree of diversity among HLA genes [420]. Two of the HLA-B alleles associated with slow disease progression are *HLA-B*57* and *B*27*, where the former has the strongest predictive effect on HIV disease outcomes [193, 421, 422]. The mechanism(s) by which *B*57* and *B*27* confer protection are not fully understood. Carriers of the *B*57* allele are found at a higher frequency in SP, particularly EC, than in either the general HIV infected or HIV uninfected population [193, 421]. In addition, *B*57* carriers who will go on to become HIV controllers, may present less frequently with symptoms of acute infection and achieve lower peaks of VL during acute infection [423]. In contrast, experimental evidence from several studies suggest that CD8 T cell

responses directed at HIV epitopes recognized in the context of B*57 and B*27 leads to viral escape compromising fitness and the appearance of compensatory mutations to restore fitness. Genotyping of virus isolated from HIV infected B*57-positive individuals reveals frequently observed escape mutation in three to four distinct immunodominant epitopes within the Gag polypeptide that are restricted by B*57 [424-427]. Reduction in replication capacity of these escape viral variants was confirmed in both *in vitro* and *in vivo* fitness assays. In *B*27* carriers, the virus is also subjected to restrictive pressure and undergoes a sequential acquisition of mutations that result in a viral variant that has lost the capacity to be restricted by B*27 and that has a replicative capacity comparable to wild-type virus [428]. The appearance of this viral escape variant results in rapid disease progression [429-431]. However, despite these findings, no polymorphisms within *B*57* or *B*27* distinguish carriers that become controllers and do not progress from those who do. Furthermore, many SP carry neither *B*57* nor *B*27* and about a third of SP who carry these alleles co-carry alleles that are considered to be either neutral or deleterious. This suggests that the 'HLA effect' may be a less stringent immunological barrier to HIV control than previously thought [432].

Multiple studies report an association between carriage of *HLA-B*35* alleles and susceptibility to AIDS [433-435], partly due to poor recognition of B*35-restricted Gag epitopes by CD8 T cells [436]. *B*35* alleles can be dichotomized into two groups. The B*35-Px group that includes *B*35:02*, *B*35:03*, *B*35:04* and *B*53:01* associates with poor HIV outcomes and susceptibility and the B*35-Py allotypes *B*35:01* and *B*35:08*, which do not [437]. A potential mechanism underlying the accelerated disease

progression associated with carriage of the Px-variants may involve interactions with and impairment of DC function. In the presence of the same HIV-1 peptides, B*35:03-Px allotypes binds the inhibitory DC receptor Ig-like transcript 4 (ILT4) with greater affinity than do B*35-Py variants [438]. Moreover, in carriers of *B*35:03* poor DC functional responses were measured compared to those in carriers of the *B*35:01*-Py alleles [438].

Based on results from GWASs, a SNP upstream of the HLA-C locus had the second strongest association with control of HIV VL in individuals of European American origin. This region, which is located 35kb upstream of HLA-C, plays a role in determining HLA-C expression levels. The -35CC genotype results in higher HLA-C cell surface expression than the -35TT genotype [439]. The work of Thomas *et al*. found a significant correlation between carriage of the -35CC genotype and control of HIV VL in a cohort of over 1600 HIV infected subjects [440]. A follow-up study by Kulkarni *et al.* has identified the mechanism involved in regulating the levels of HLA-C translation and surface expression [441]. A cellular miRNA can bind a region in the 3' untranslated region of low-surface expression HLA-C transcript eliciting a post-transcriptional regulation; while high expression HLA-C allelic transcripts escape this regulatory step. This miRNA is in strong linkage disequilibrium with the -35kb SNP identified in GWAS, however, currently there is no explanation for a direct causal effect between the two [441]. In a recent study, the association between higher HLA-C expression levels and HIV control is thought to have a beneficial effect on enhanced HIV peptide presentation in the context of HLA-C isoforms to CD8 T cells [442]. Thus, the linkage between HLA-C expression

and HIV VL control appears to be related to the potency of T cell recognition of HLA-C-HIV peptide complexes. NK cells also recognize HLA-C through their KIR2D receptors. Whether NK cells also contribute to HIV control through their interaction with HLA-C has not been reported.

1.8.2. KIR/HLA combinations in HIV-1 infection

The impact of HLA antigens on HIV control is known to be mediated through the recognition of HIV epitope MHC class I complexes by $CD8^+$ T cells. These complexes are also recognized by iKIR on NK cells. Epidemiological and functional studies have implicated iNKRs in combination with certain HLA class I variants in protection from HIV infection and slow disease progression, in those infected. Martin *et al*. were the first to report that carriage of the combined genotype KIR3DS1 with HLA-Bw4-80I was associated with a protective effect on HIV disease progression in terms of slower time to AIDS [443]. This combined KIR/HLA genotype was later also shown to correlate with lower VLs and protection from opportunistic infection compared to a control group of Bw6 homozygotes with HLA antigens that are unable to bind KIR3DS1 [444]. Homozygosity for KIR3DS1 was shown to be enriched in an HESN cohort [445], which highlights the possibility of $KIR3DS1^+$ NK cells being important in protection from infection and slow disease progression. These findings suggest that functionality of $KIR3DS1^+$ NK cells in response to HIV infection is beneficial in restricting HIV from establishing a productive infection and/or dissemination to susceptible target cells. However, in spite of the mounting evidence for the importance of KIR3DS1 and HLA-Bw4-80I co-carriage on HIV pathogenesis and NK cell function, which suggests

interaction between this receptor-ligand pair, providing convincing evidence of binding between KIR3DS1 and HLA-Bw4 molecules has been challenging [446]. A recent study showed that mutating leucine to arginine at position 166 of KIR3DS1 prevents interactions with many peptide/HLA allotype complexes, but permits certain HIV derived peptides to overcome the effect of R166 and form interactions between KIR3DS1 with HLA-B*57:01 [349].

Another KIR/HLA combination that has been associated with both protection from infection and slow time to AIDS is KIR3DL1/HLA-Bw4. The high expression 3DL1 homozygous genotype **h/*y* co-carried with *HLA-B*57* (**h/*y+B*57*) encodes a receptor ligand combination that has the strongest effect on slow time to AIDS and HIV viral load control, compared to Bw6 homozygotes [334]. Research from Dr Bernard's group discovered an enrichment of this compound KIR/HLA genotype in a cohort of HESN, suggesting an association with protection from HIV infection [341]. Moreover, in healthy individuals, a significantly higher frequency of tri-functional NK cells (expressing CD107a and secreting IFN-γ, TNF-α) from **h/*y+B*57* donors contributed to responses to HLA-null cell stimulation, compared to NK cells from donors of non-protective genotypes (e.g. either carrying KIR3DL1 or HLA-B*57 alone or who were Bw6 homozygotes) [343]. The B*27 isoform belongs to the Bw4-80T group that can also interact with KIR3DL1. Interestingly, it was the combination of B*27 with the KIR3DL1*l/*x low expression genotype, rather than with the high expression *h/*y genotype, that was more potently associated with slow time to AIDS and VL control [334]. The exact reason or mechanisms behind this finding are currently unknown. One possibility that

explains this observation is that stronger binding occurs between B*27 and the receptors encoded by *l/*x genotypes compared to those encoded by *h/*y genotypes. Another possibility may be an aa polymorphism within B*27 and KIR3DL1 that enhances receptor-ligand binding affinity [339].

While epidemiological and genetic studies support a role for NK cells in control of HIV infection, the mechanisms that underlie the protective effect of NK cells are not fully understood. The first functional evidence that KIRs and HLA influence NK cell functions relevant to HIV control came in 2007. Alter *et al.* showed that KIR3DS1$^+$ NK cells from individuals who co-carry a Bw4-80I ligand inhibited HIV replication more robustly than carriers of either the KIR receptor or HLA ligand alone, or neither [447]. In this study KIR3DS1$^+$ and KIR3DL1$^+$ NK cells from *KIR3DL1/S1* heterozygotes who were Bw4*80I carriers, were tested for responses to autologous HIV infected CD4 cell stimulation. KIR3DS1$^+$ NK cells degranulated more robustly to this stimulus than did their KIR3DL1$^+$ counterparts. Work from Dr. Bernard's group has demonstrated that NK cells from **h/*y+B*57* carriers, compared to those from *Bw6* homozygotes, have a superior functional potential and ability to inhibit HIV replication through mechanisms that involve secretion of CC-chemokines [448]. This study is presented as Chapter 2 of this thesis.

Association of aNKR-HLA, such as KIR3DS1-HLA-Bw4*80I, with favorable outcomes implies that engagement of this activating receptor is beneficial in HIV infection. At first, the association of an iNKR with protective and favorable disease outcomes may seem counter-intuitive; however, the KIR3DL1-Bw4 axis behaves in accordance with the

principles of NK cell education. NK cells from donors co-carrying the **h/*y-B*57* combination receive the strongest signal possible through the KIR3DL1 receptor during NK education. The educating impact of an iNKR-MHC I interaction is directly related to how active the NK cell will become when it receives the right activating stimulus [449]. Thus KIR3DL1$^+$ NK cells from carriers of the **h/*y-B*57 combination* develop a higher frequency and level of function compared to NK cells expressing receptors not able to, or interacting more weakly with, self HLA antigens [343, 364, 366, 367, 449]. When NK cells that were potently educated through KIR3DL1 are stimulated by autologous *in vitro* HIV-infected CD4 cells, on which HLA-Bw4 has been down modulated by HIV Nef, inhibitory signals received through KIR3DL1 are lost [450]. Together with concurrent stimulatory input received through activating receptor these cells become activated by the HIV-infected CD4 cells according to how potently they were educated during the course of their development.

The region upstream of HLA-C, which plays a role in determining HLA-C expression levels, was associated with HIV control in individuals of European American origin based on results from GWAS studies (discussed in section *1.8.1.*). While it is assumed that the mechanism underlying this association is related to the potency of CD8$^+$ T cell recognition of HLA-C-HIV peptide complexes, KIR2D NK cells can also recognize HLA-C molecules. Whether NK cells also play a role in HIV control through KIR2D-HLA-C dependent interactions has not been reported. A recent study analyzed NK cell responses from HIV infected individuals, focusing on the KIR2D-HLA-C axis. Functional responses to the 721 HLA-null cell line of KIR2DL1$^+$ and KIR2DL2/3$^+$ NK cells from HIV

infected individuals were greater in donors co-carrying the respective HLA-C2 and C1 ligands for these receptors, compared to those from subjects not expressing ligands for these iKIRs [451]. These results suggest that the functional potential measured for KIR2DL1 and KIR2DL2/3 NK cells from HIV infected subjects behaves in accordance to what is expected based on NK cell education, where educated, compared to 'uneducated', NK cells have a greater functional potential. However, what this study didn't address and still remains unknown is whether KIR2DL-HLA-C carriage impacts protective immunity to HIV or slow disease progression. In Chapter 4 of this thesis we investigated the impact of carriage of KIR2DL3 and HLA-C1 on NK cell responses to autologous HIV infected cells *in vitro*.

1.8.3. NK cell populations responding to HIV-1

NK cells have various effector functions against HIV infected cells ranging from cytolysis, via the release of perforin and granzymes, release of chemokines that compete with HIV for binding the CCR5 co-receptor, release of cytokines that shape immune responses and Ab mediated NK cell functions, including ADCC (Figure 7). The functional potential of NK cells is determined by a process of NK cell education (discussed in section 1.6.3.), which relies on key interactions between iKIRs and their respective HLA ligands. In humans, both iKIRs and HLA molecules are polygenic and can be present in different combinations in different individuals and within a person. This creates a diverse repertoire of NK cell populations with different functional profiles to various stimuli. Therefore, which NK cell population(s) will be responsive and which

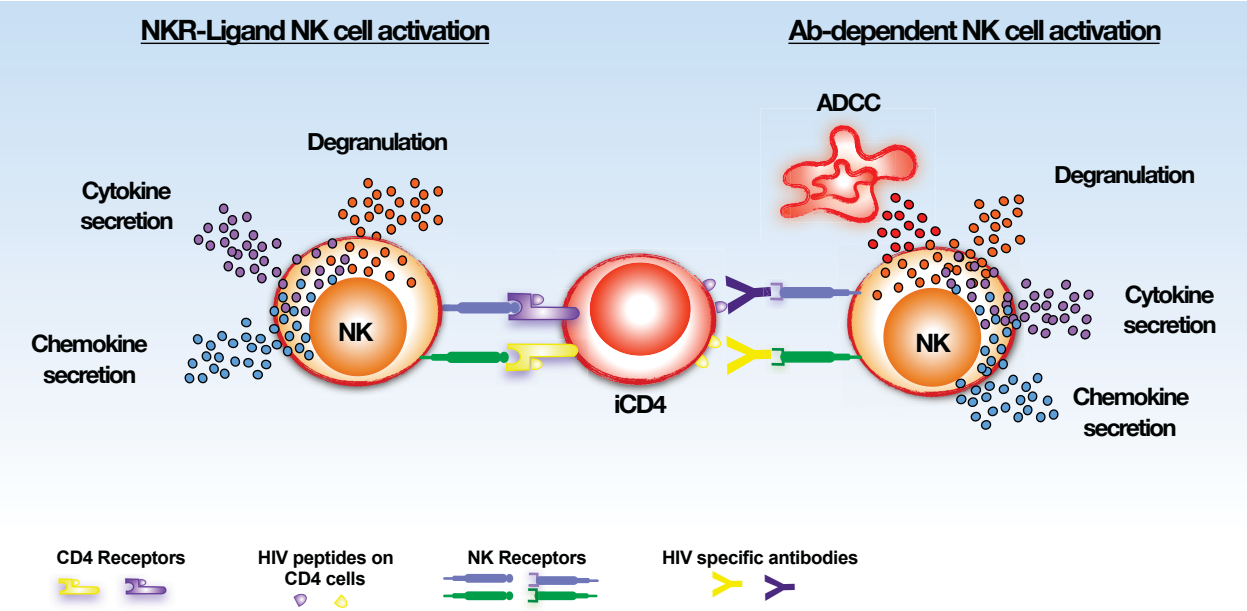


Figure 7: Mechanisms by which NK cells can respond to HIV-infected cells. The iCD4 cell can be recognized by NK cells directly, via engagement with NKR (both inhibitory and activating), or indirectly via an HIV-specific antibo iCD4- HIV-infected CD4 cell. Figure made by Irene Lisovsky.

functions will be stimulated depends on two important factors: the nature of the KIR/HLA environment NK cells developed in, and the nature of the stimulus, particularly the configuration of cell surface ligands for stimulatory and inhibitory NKR on the cells interacting with NK cells.

Few studies have been done that address what NK cell population(s) respond to HIV infected cells and their functional profiles. Results from epidemiological and GWAS studies (discussed in sections 1.8.1. and 1.8.2.) provide information on what KIRs, and subsequently population(s), may be important in the context of protection from infection or slow disease progression. These results further highlight the impact that a specific KIR/HLA environment has on shaping protective functional response profiles against

HIV. However, these types of studies do not identify the exact population(s) that respond to HIV infected cells and the mechanisms behind these responses. For example, carriage of the KIR3DS1 homozygous genotype has been implicated in protection from infection, while carriage of the KIR3DS1-80I combined genotype has been implicated in slow disease progression. These findings highlight the importance of KIR3DS1$^+$ NK cells in immune responses to HIV. However the first functional proof that indeed KIR3DS1$^+$ NK cells mediated inhibition of HIV replication in autologous HIV infected cells came in 2007. Alter *et al.* showed that KIR3DS1$^+$ NK cells (by flow cytometry gating and by cell sorting) from donors with the Bw4-80I combined genotype, inhibited HIV replication better and produced more CD107a than these cells from Bw6 homozygous controls [447].

In large epidemiological studies the KIR3DL1 homozygous **h/*y+B*57* combination has also been shown to be important in protection from infection and slow disease progression. Furthermore, a functional study from our group using NK cells from carriers of this combined genotype found that KIR3DL1$^+$ NK cells from **h/*y+B*57* donors were more functional than those from Bw6 homozygotes, in response to stimulation with HLA-null K562 cells [343]. These findings suggest that KIR3DL1$^+$ NK cells, particularly from carriers of a B*57 allele, are a likely candidate to mediate anti-HIV functional responses. The functional and mechanistic role of KIR3DL1$^+$ NK cells from **h/*y+B*57* donors in inhibition of HIV viral replication was confirmed by our group, which is presented as Chapter 2 in this thesis [448].

The influence of NK cell populations defined by other KIRs or other NKRs in mediating anti-HIV responses important in protection from infection and improved disease control is not known. Functional and mechanistic studies characterizing responses of KIR3DS1^{+} and KIR3DL1^{+} NK cells to HIV did not take into account the influence of other NKRs expressed on these KIR^{+} NK cells. Results from the previous KIR3DL1 studies [334, 343, 367] highlight the importance of education on NK cell mediated responses to HIV infected cells. We hypothesize that education can have a predictive and additive effect on which NK cell populations are likely to be responsive to HIV infected cells. Accordingly, the co-expression of additional iNKRs can potentially enhance the functional responses of NK cell populations defined by these multiple iNKRs to virally infected cells. NKG2A is an iNKR, whose ligand is HLA-E, and both receptor and ligand are expressed in all people. Our group focused on understanding the impact NKG2A and KIR3DL1 expression has in the four NK cell populations defined by these receptors, i.e. NKG2A$^{+/-}$KIR3DL1$^{+/-}$, on responses to HIV infected cells. We hypothesized that NKG2A^{+}KIR3DL1^{+} NK cells from donors with HLA-E and Bw4 would have the greatest functional potential to HLA null cell stimulation of the four populations examined and this functional superiority would extend to responses to HIV infected cells. Findings from this study are presented and discussed in this thesis in Chapter 3.

Epidemiological studies have found an important association between HLA-C and control of HIV infection [212, 440]. HLA-C molecules can interact with receptors on T cells and KIR2D^{+} NK cells. Functional outcomes of interactions between HLA-C and T cells have been described. However, similar studies have not been done for KIR2D NK

cells in the context of HIV. A recent study investigated an expansion of KIR2DL1^{+}, KIR2DL2^{+} and KIR2DL3^{+} NK cells in HIV infected individuals [451]. These NK cells also had functional responses to 721 cells that were in line with expectations based on their education status. For example KIR2DL1^{+} NK cells from donors homozygous for C2 allele were more responsive to 721 than cells those from C1/C2 heterozygotes and C1 homozygotes. Our group has investigated the functional responses of KIR2DL3^{+} NK cells to HIV infected cells. We also studied the influence of additional iNKRs, KIR3DL1 and NKG2A, on the functionality of KIR2DL3^{+} CD56^{dim} NK cells. Both objectives are presented in Chapter 4. We hypothesised that not only NK cell education, but also environmental factors driven by HIV infection can influence the engagement of a particular NK cell population. HIV infected CD4 (iCD4) cells downmodulate HLA-A and B, but not HLA-C and –E; Therefore, iCD4 may interact with NK cell subsets expressing and educated through KIR2DL3 differently from those expressing and educated through KIR3DL1 and/or NKG2A. Results from this study are presented and discussed in this thesis as Chapter 4. A recent study by Apps *et al.* demonstrated that Vpu from primary HIV isolates, but not lab-adapted strains, can downregulate HLA-C [452]. The virus source used in this thesis is a lab-adapted R5-tropic molecular clone, therefore this theme was not explored, yet remains a possible focus for future studies.

CHAPTER 2

HIV Protective KIR3DL1/S1-HLA-B Genotypes Influence NK Cell-Mediated Inhibition of HIV Replication in Autologous CD4 Targets

Song R, **Lisovsky I**, Lebouché B, Routy JP, Bruneau J and Bernard NF

This chapter was adapted from an article published in PLoS Pathogens, 2014 Jan. 10(1):1585-94.

Contribution of authors: SR and IL designed the research, conducted experiments, analyzed data, interpreted and prepared the manuscript. LB, RJP and BJ provided clinical samples used in this study. NFB designed the research, analyzed data, supervised the research and prepared the manuscript.

2.1 RATIONALE AND RESEARCH OBJECTIVES

Previous studies have shown the important role NK cells have in protection from HIV infection and disease control in those infected. The first functional study on KIR3DS1^{+} NK cells showed that NK cells inhibited HIV replication and KIR/HLA pairs were important determinants of this activity. In this chapter our primary objective was to ascertain the mechanism(s) by which NK cells mediated inhibition of HIV replication. Given the importance of NK cell education in determining NK cell functional potential, we chose to investigate the anti-HIV functionality of KIR3DL1^{+} compared to KIR3DL1^{-} NK cells from carriers who either co-expressed, or not, an HLA-B ligand for this NKR. In this chapter we included NK cells from carriers encoding one of the strongest KIR3DL1/ HLA-Bw4 receptor-ligand pairs (*h/*y+B*57), a weaker KIR3DL1/HLA-Bw4 pair (*l/ *x+B*57) and no KIR3DL1/HLA-Bw4 ligand pair (i.e. Bw6, controls).

2.2 ABSTRACT

Carriage of the genetic combination encoding a high expression inhibitory KIR3DL1 with its ligand, HLA-B*57 (*h/*y+B*57) is associated with slower time to AIDS and better HIV viral load control than being a Bw6 homozygote (Bw6hmz). NK cells from *h/*y+B*57 carriers receive potent educational signals through HLA-B*57-KIR3DL1 ligation leading to high functional potential, unlike NK cells from Bw6hmz that serve as negative controls. To better understand the impact of KIR/HLA combinations on NK cell mediated anti-viral activity we measured NK cell mediated inhibition of HIV replication in autologous infected CD4 (iCD4) cells by longitudinally assessing p24 supernatant levels and intracellular p24 frequency in CD4 targets. Forty-seven HIV uninfected subjects were studied: *h/*y+B*57, *l/*x+B*57, 3DS1+*80I and Bw6hmz. NK cells from *h/*y+B*57 carriers, like those from 3DS1+*80I subjects, inhibited HIV replication in autologous iCD4 cells better than those from Bw6hmz and *l/*x+B*57 carriers. Cell contact between NK and iCD4 cells was required to activate NK cells to inhibit viral replication in a non-contact dependent fashion through secretion of CC chemokines. iCD4 stimulated NK and 3DL1^{+} cells from *h/*y+B*57 carriers produced higher levels of CCL3 and CCL4 than those from Bw6hmz or *l/*x+B*57 carriers. We conclude that education has a role in NK-mediated inhibition of HIV replication in autologous iCD4 cells that is partially due to a block at the level of HIV entry into new targets by secreted CC-chemokines.

2.3 INTRODUCTION

NK cells function in innate immune responses to transformed and virally infected cells. They can exert their anti-viral effects soon after encountering infected targets without prior sensitization [1]. NK cell function is determined by integration of signals from activating and inhibitory cell surface receptors, which include Killer Immunoglobulin-like Receptors (KIR) [2]. Among these are inhibitory KIR3DL1 (3DL1) and activating KIR3DS1 (3DS1) receptors, which are encoded by alleles at the same KIR3DL1/S1 locus [3]. 3DL1 receptors can be classified into those expressed on NK cell surfaces at high levels (*h) low levels (*l) or *004, which is only transiently expressed [4–7]. 3DL1 homozygous genotypes can be dichotomized into *h/*y and *l/*x groups where *h/*y genotypes encode receptors expressed on the NK cell surface at higher levels than those encoded by *l/*x genotypes [6].

Epidemiological studies have found that several 3DL1 homozygous genotypes co-carried with a subset of HLA-B and –A alleles belonging to the HLA-Bw4 group are associated with slower time to AIDS and viral load (VL) control [7]. HLA-Bw4 antigens differ from the remaining HLA-Bw6 (Bw6) antigens by amino acids at positions 77–83 [8]. The genotype combinations that confers the highest degree of protection in terms of time to AIDS and VL control is 3DL1*h/*y co-carried with HLA-B*57 (*h/*y+B*57) [7]. Subjects with this combined genotype are more frequent among HIV Exposed Seronegative (HESN) than HIV susceptible individuals, implicating carriage of this genotype combination in reducing HIV infection risk [9]. NK cells from carriers of *h/*y+B*57 have more potent NK cell functional potential as defined by HLA-null cell

induced secretion of IFN-γ and TNF-a and expression of CD107a, a marker for degranulation, than those from carriers of the receptor or ligand alone, including those from carriers of the *l/*x+B*57 KIR/HLA genotype and Bw6 homozygotes (Bw6hmz) [9,10]. Bw6 antigens do not interact with 3DL1 receptors and are thus unable to gain functional potential through education via this inhibitory receptor [11,12]. NK cell education is an ontological process that depends on the interaction of inhibitory NK receptors, such as 3DL1, with their MHC class I (MHC-1) ligands. The strength of educational signals received during NK cell development determines NK cell functional potential [11, 13, 14]. Thus, NK cells from *l/*x+B*57 carriers may be less functional than those from *h/*y+B*57 positive subjects since the former express less 3DL1 than the later and thus receive lower level educational signals upon interaction with the same ligand [6,10,15].

The KIR/HLA combination 3DS1 co-expressed with a Bw4 antigen having an isoleucine at position 80 of the HLA heavy chain (3DS1+*80I) is also associated with slower time to AIDS and VL control [16,17]. NK cells from carriers of the 3DS1+*80I genotype inhibit viral replication in autologous HIV-infected CD4 (iCD4) T cells more potently than those from individuals carrying the receptor or ligand alone, or neither [18]. Together, these functional studies suggest that the association of certain KIR/HLA genotypes with either protection from HIV infection in HESN subjects or slow time to AIDS and VL control in those who are HIV infected, is linked to NK cell function.

How NK cells inhibit viral replication in autologous CD4 T cells is not completely

understood. One possibility is through the secretion of the CC-chemokines CCL3, CCL4, and CCL5 upon activation following recognition of autologous HIV iCD4 cells. These chemokines can suppress HIV replication by competing with the virus for binding the CCR5 co-receptor and blocking HIV entry into CD4 cells [19,20]. In this report we investigated whether NK cells from individuals with *h/*y+B*57 genotype inhibited HIV replication in autologous iCD4 cells better than those from *l/*x+B*57 carriers and Bw6hmz. The cell contact requirement for inhibition of viral replication was assessed. We also measured the production of CC-chemokines by NK cells stimulated with autologous HIV iCD4 cells. We determined whether CC-chemokine secretion levels differed based on the KIR/HLA genotype and evaluated the effect of CC-chemokine neutralization on NK cell mediated inhibition of HIV replication.

2.4 MATERIALS AND METHODS

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. It was approved by the Institutional Review Boards of the Comite´ d'E´ thique de la Recheche du Centre Hospitalier de l'Universite´ de Montre´al and the Research Ethics Committee of the McGill University Health Centre - Montreal General Hospital. All participants provided written informed consent for the collection of samples and subsequent analysis.

Study population

We studied 47 HIV seronegative individuals, including *h/*y+B*57 (n=7), 3DS1+*80I (n=12), Bw6hmz (n=11), *l/*x+B*57 (n=4) positive and other KIR/HLA genotypes (n=13). Characteristics of study participants are shown in Table 1.

Genotyping

All subjects were typed for MHC-I alleles by sequence based typing using kits from Atria Genetics, Inc. (South San Francisco, CA). Assign 3.5+ software was used to interpret sequence information for allele assignment (Conexio Genetics). KIR3DL1/S1 generic genotyping was performed by PCR using 2 pairs of primers specific for either 3DL1 or 3DS1 alleles as previously described [29]. 3DL1 allotyping was done by sequencing 3DL1 exons as previously described [9]. The *h/*y genotype refers to a 3DL1 homozygous genotype with no *l alleles. Bw6hmz lacked Bw4 alleles at the HLA-A and -B loci.

Cell purification

Peripheral blood mononuclear cells (PBMC) were isolated from blood by density gradient centrifugation (Ficoll-Paque; Pharmacia) and cryopreserved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) with 90% fetal bovine serum (FBS; Wisent, Inc.). CD4 T cells were isolated from thawed PBMC by positive selection kit (STEMCELL Technologies, Inc.). The purity of the CD4 cell population was verified by flow cytometry (average 95.3%). NK cells were isolated from thawed PBMC by negative selection (STEMCELL Technologies, Inc.) and yielded an average purity of 97.2%.

Inhibition of viral replication assay

Purified CD4 cells (10^6/ml) were stimulated with 1 µg/ml PHA-P (MP Biomedicals) and 100 international units (IU)/ml of IL-2 (Chiron Corp.) overnight in RPMI medium containing 2 mM L-glutamine, 100 IU/ml Penicillin, 100mg/ml Streptomycin (cRPMI) (all from Wisent) supplemented with 10% FBS, (Wisent, [R10]) at 37ºC in a 5% CO2 humidified incubator. Stimulated CD4 cells were then washed three times with cRPMI supplemented with 2% FBS (R2), and cultured in R10 with 100 IU IL-2 for 3 days. On day 4, CD4 cells were infected at a multiplicity of infection of 0.01 with HIV-1JR CSF in R10 for 4 hrs and washed three times with R2. Equal numbers (3.0 to 4.0x10^4) of these iCD4 cells were plated at NK:iCD4 ratios of 10:1, 1:1 or alone for 10 days in 300 µl of R10; 100 IU/ml IL-2. Supernatants were collected by removing supernatants and replenishing wells with 300 µl of fresh R10; 100 IU/ml IL-2 on days 3, 7 and 10 for assessment of p24 levels and on days 1, 2 and 3 for assessment of CC-chemokine levels. For some experiments CD4 cells were collected on days 3, 7 and 10 for

intracellular Gag p24 staining. Cells were stained with an Aqua amine reactive fluorescent dye (Invitrogen) for viability and with anti-CD3 APC-eFluor 780 (eBioscience) and anti-CD4 PE (BD Biosciences) to detect CD4 T cells. Cells were fixed and permeabilized for HIV Gag p24 using the mAb KC57 (Beckman-Coulter). Between 50,000 and 200,000 events were acquired on a BD FACSCanto II flow cytometer (BD Biosciences) and data was analyzed with Flowjo software Mac 9.4 (Treestar).

To ascertain the requirement for NK-CD4 cell contact for NK cell–mediated inhibition of HIV replication, autologous NK cells were physically separated from iCD4 cells in transwell plates (Corning). iCD4 cells (10^5/well) were cultured in the upper chamber with either 10^6 NK cells alone or 10^6 NK cells with 10^5 iCD4 T cells in the lower chamber. Cells in wells containing iCD4 cells were collected on days 3, 7 and 10 to quantitate the frequency of HIV Gag p24 positive CD4 cells.

NK cell stimulation

Purified NK cells, isolated as described above were plated at a 10:1 ratio with either iCD4 or CD4 cells overnight in R10 with 100 IU/ml IL-2. Brefeldin (6 µg/ml, Sigma-Aldrich) and monensin (5 µg/ml, Golgi Stop, BD Biosciences) were added at the last 5 hrs before of the culture period. After stimulation, viability was assessed using the UV Live/Dead fixable dead cell stain kit (Invitrogen) and non-specific interactions were minimized using TruStain FcX reagent (BioLegend), as per the manufacturer's instructions. Cells were then stained for surface markers with CD3-BV605 (OKT3), CD56-BV711 (NCAM), 3DL1-BV421 (DX9) (all from BioLegend) and anti-CD107a-

PECF594 (BD), for 30 min. Samples were washed with phosphate buffered saline (PBS) containing 1% FBS (Wisent), fixed and permeabilized using a cell fixation and permeabilization kit (Invitrogen) and stained for intracellular cytokines/chemokines using IFN-c-Alexa700 (B27) (BD Biosciences), CCL3-APC (93342) and CCL4-FITC (24006; both from R&D Systems). Samples were washed, fixed with a solution of 1% paraformaldehyde (Fisher Scientific) and acquired within 24hrs.

Between $4x10^5$ and $1.5x10^6$ total events were acquired for each sample on an LSRFortessa flow cytometer (BD Biosciences). The gating strategy used to analyze the functional profile of NK cells stimulated with iCD4 or uninfected CD4 cells is shown in Fig. 4. For all analyses, NK cells were defined as $CD3^-CD56^+$. The percent of CCL3, CCL4, IFN-γ and CD107a positive total, $3DL1^+$ and $3DL1^-$ NK cells was determined. Flow cytometry analysis for NK cell activation following stimulation was performed using FlowJo software version 9.5. The results for the percent of functional NK cells stimulated with iCD4 reported were background corrected for NK cells stimulated with uninfected CD4 cells. Statistical analyses were performed using background corrected results.

HIV p24 ELISA

An Ab-sandwich ELISA was used to detect HIV Gag p24 in culture supernatants as described previously [30]. Briefly, 96-well ELISA plates were coated with anti-p24 Ab clone 183 H12-5C at 2.5 ug/ml overnight. Culture supernatants (100 µl/well) were added for 1 hr at 37uC. The following sequential additions were made with 3 washes between

steps using PBS; 0.05% Tween 20 (Sigma-Aldrich): 1) biotinylated anti-p24 Ab clone 31-90-25 at 0.5 µg/ml for 1 hr at 37C, 2) 0.067 µg/ml horseradish peroxidase conjugated-steptavidin (Fitzgerald Industries International) for 30 min at RT and 3) TMB-substrate (Sigma Aldrich) for 20 min at RT. Color development was stopped with 50 µl of 1M H_2SO_4. ELISA plates were read at OD450 on an ELISA plate reader (PerkinElmer). Percent viral inhibition was calculated using the equation [(p24 of iCD4 wells – p24 of NK+iCD4 wells)/(p24 of iCD4 wells) x 100].

CC-chemokine ELISA

Culture supernatant levels of CC-chemokines was measured using Ab sandwich ELISAs detecting CCL3, CCL4 and CCL5 (R&D Systems) according to directions provided by the manufacturer.

Statistical analysis

Statistical analyses and graphical presentations were performed using GraphPad InStat 3.05 and GraphPad Prism 5. Mann-Whitney and Kruskal-Wallis tests with Dunn's post tests were used to compare the significance of mean differences between 2 and more than 2 groups, respectively. Wilcoxon matched pairs and Friedman tests were used to compare the effect of a condition on 2 or more than 2 matched groups, respectively. $P<0.05$ were considered significant.

Table 1. Characteristics of study subjects.

ID	Age	Gender	Category	3DL/S1 Genotype	HLA Genotype					
					A		B		C	
1001	50	M	*h/*y+B57	$3DL1_{HMZ\ *h/*y}$	A*01:01	A*26:01	B*38:01	B*57:01	C*06:02	C*12:03
1002	54	M	*h/*y+B57	$3DL1_{HMZ\ *h/*y}$	A*02:22	A*03:01	B*44:02	B*57:01	C*05	C*06:02
1003	25	M	*h/*y+B57	$3DL1_{HMZ\ *h/*y}$	A*01:01	A*02:01	B*38:01	B*57:01	C*06:02	C*12:03
1004	49	F	*h/*y+B57	$3DL1_{HMZ\ *h/*y}$	A*02:01	A*02:01	B*57:01	B*57:01	C*03:03	C*07:01
1005	58	M	*h/*y+B57	$3DL1_{HMZ\ *h/*y}$	A*24:02	A*25:01	B*37:01	B*57:01	C*06:02	C*06
1006	35	M	*h/*y+B57	$3DL1_{HMZ\ *h/*y}$	A*01:01	A*03:01	B*14:02	B*57:01	C*06:02	C*08:02
1007	30	M	*h/*y+B57	$3DL1_{HMZ\ *h/*y}$	A*01:01	A*02:01	B*15:01	B*57:01	C*05	C*06:02
2008	45	M	3DS1+80I	HTZ_{high}	A*02:01	A*26:01	B*38:01	B*44:02	C*05	C*16:04
2009	47	M	3DS1+80I	HTZ_{high}	A*01:01	A*23:01	B*44:03	B*57:01	C*04:01	C*06:02
2010	52	M	3DS1+80I	$HTZ_{null\ (004)}$	A*02:01	A*02:01	B*44:02	B*49:01	C*05	C*07:01
2011	60	M	3DS1+80I	HTZ_{high}	A*02:01	A*03:01	B*14:02	B*51:01	C*02:02	C*08:02
2012	35	F	3DS1+80I	HTZ_{high}	A*11:01	A*26:01	B*07:02	B*57:01	C*06:02	C*07:02
2013	45	F	3DS1+80I	HTZ_{high}	A*01:01	A*02:01	B*38:01	B*57:01	C*06:02	C*12
2014	44	M	3DS1+80I	HTZ_{high}	A*01:01	A*24:02	B*35:01	B*52:01	C*04:01	C*12:02
2015	28	M	3DS1+80I	HTZ_{high}	A*02:01	A*02:01	B*44	B*51	C*05:01/02	C*15:02
2016	51	M	3DS1+80I	HTZ_{high}	A*03:01	A*11:01	B*40:02	B*57:01	C*02:02	C*04:01
2017	31	M	3DS1+80I	HTZ_{high}	A*03:01	A*32:01	B*13:02	B*53:01	C*04:01	C*06:02
2018	42	M	3DS1+80I	$HTZ_{null\ (004)}$	A*01:01	A*26:01	B*52:01	B*55:01	C*03:03	C*12:02
2019	44	F	3DS1+80I	$3DS1_{HMZ}$	A*01:01	A*01:01	B*08:01	B*57:01	C*07:01	C*07:01
3020	44	M	Bw6	$3DL1_{HMZ\ *h/*y}$	A*02:01	A*25:01	B*18:01	B*55:01	C*03:03	C*12:03
3021	38	M	Bw6	$3DL1_{HMZ\ *h/*y}$	A*03:01	A*11:01	B*07:02	B*35:01	C*04:01	C*07
3022	55	M	Bw6	$3DL1_{HMZ\ *l/*x}$	A*01:01	A*02:01	B*08:01	B*08:01	C*07	C*07
3023	42	F	Bw6	HTZ_{high}	A*02:01	A*03:01	B*15:01	B*07:02	C*03	C*07
3024	49	M	Bw6	$3DL1_{HMZ\ *h/*y}$	A*02:01	A*32:01	B*18:01	B*50:01	C*06:02	C*07:01
3025	48	M	Bw6	$3DL1_{HMZ\ *h/*y}$	A*02:01	A*03:01	B*07:02	B*40:01	C*03:02	C*07
3026	48	M	Bw6	$3DL1_{HMZ\ *h/*y}$	A*01:01	A*24:03	B*07:02	B*07:02	C*07	C*07
3027	49	M	Bw6	HTZ_{high}	A*31:01	A*68:01	B*40:01	B*40:01	C*03:04	C*07:01
3028	44	M	Bw6	HTZ_{high}	A*02:01	A*02:01	B*35:01	B*35:01	C*05	C*05
3029	45	M	Bw6	$3DL1_{HMZ\ *l/*x}$	A*02:01	A*33:03	B*15:01	B*35:08	C*03:03	C*04:01
3030	45	M	Bw6	$3DL1_{HMZ\ *h/*y}$	A*02:01	A*02:01	B*07:02	B*08:01	C*07	C*07
4031	36	F	*l/*x+B57	$3DL1_{HMZ\ *l/*x}$	A*02:01	A*02:01	B*07:02	B*57:01	C*05	C*06:02
4032	68	M	*l/*x+B57	$3DL1_{HMZ\ *l/*x}$	A*24:02	A*26:01	B*15:01	B*57:01	C*05	C*06:02
4033	44	M	*l/*x+B57	$3DL1_{HMZ\ *l/*x}$	A*02:01	A*02:01	B*15:01	B*57:01	C*03:04	C*06:02
4034	33	F	*l/*x+B57	$3DL1_{HMZ\ *l/*x}$	A*02:02	A*30:02	B*53:01	B*57:03	C*04:01	C*18
5035	59	F	$3DS1_{HMZ}+_{non}80I$	$3DS1_{HMZ}$	A*01:01	A*02:01	B*08:01	B27:05	C*02:02	C*07:01
5036	36	M	$3DS1_{HMZ}+_{non}80I$	$3DS1_{HMZ}$	A*02:01	A*03:01	B*07:02	B*56:01	C*01:02	C*07
5037	42	M	$3DS1_{HMZ}+_{non}80I$	$3DS1_{HMZ}$	A*03:01	A*30:01	B*27	B*35:01	C*01	C*04:01
5038	49	M	$3DS1_{HMZ}+_{non}80I$	$3DS1_{HMZ}$	A*02:01	A*23:01	B*07:02	B*35:03	C*12:03	C*12:03
5039	22	M	$3DS1_{HMZ}+_{non}80I$	$3DS1_{HMZ}$	A*01:01	A*02:03	B*37:01	B*46:01	C*01:02	C*06:02
5040	52	M	$3DS1_{HMZ}+_{non}80I$	$3DS1_{HMZ}$	A*02	A*03	B*08	B*44		
5041	53	M	*80T	$3DL1_{HMZ\ *l/*x}$	A*02:01	A*03:01	B*07:02	B*27:05	C*01:02	C*07:02
5042	46	M	*80I not B*57	$3DL1_{HMZ\ *h/*y}$	A*01:01	A*31:01	B*49:01	B*49:01	C*07:01	C*07:01
5043	45	M	*80I not B*57	$3DL1_{HMZ\ *h/*y}$	A*31:01	A*68:01	B*27	B*51:01	C*02:02	C*12:03
5044	43	M	*80I not B*57	$3DL1_{HMZ\ *l/*x}$	A*11:01	A*11:01	B*27:05	B*53:01	C*02:02	C*04:01
5045	45	F	*80I not B*57	$3DS1_{HMZ}$	A*02:01	A*24:02	B*38:01	B*78:01	C*07:02	C*07:02
5046	49	M	*80I not B*57	$3DL1_{HMZ\ *l/*x}$	A*24:02	A*29:02	B*07:02	B*35:01	C*04:04	C*07:02
5047	29	M	*80I not B*57	$3DL1_{HMZ\ *h/*y}$	A*01:01	A*03:01	B*44:03	B*49:01	C*07:01	C*16:01

2.5 RESULTS

NK cells from individuals carrying protective KIR/HLA genotypes inhibit HIV replication more potently than those from Bw6hmz

NK cells from carriers of *h/*y+B*57 are associated with protection from HIV disease progression, VL control and lowered infection risk, however it is unknown whether this combined genotypes is advantageous at the level of inhibition of viral replication. Fig. 1 shows results for inhibition of HIV replication by NK cells from subjects positive for *h/*y+B*57 (n= 7), 3DS1+*80I (n= 9), *l/*x+B*57 (n= 4) and Bw6hmz (n= 11). In this experiment NK cells from 3DS1+*80I carriers are used as a positive control since Alter *et al.* had previously shown their capacity to inhibit HIV replication in autologous iCD4 cells [18]. No significant differences in inhibition of viral replication were measured between this positive control and *h/*y+B*57. NK cells from *h/*y+B*57 carriers inhibited HIV replication better than those from Bw6hmz and this was significant at all times tested (p=0.01, 0.007, and 0.05 for days 3, 7, and 10, respectively, Mann-Whitney test). They also inhibited HIV replication better than those from *l/*x+B*57 carriers ($p<0.05$ for days 7 and 10). We confirmed that NK cells from 3DS1+*80I carriers inhibit HIV

Table 2. p-values analysis of CC-Chemokines secretion of various genotypes tested.

Genotypes compared	CCL3	CCL4	CCL5
**h/*y+B*57* vs *3DS1+*80I*	0.77	0.12	0.73
**h/*y+B*57* vs **l/*x+B*57*	0.38	0.92	0.15
**h/*y+B*57* vs *Bw6hmz*	0.02	0.05	0.05
*3DS1+*80I* vs **l/*x+B*57*	0.23	0.06	0.001
*3DS1+*80I* vs *Bw6hmz*	<0.01	<0.01	<0.01
**l/*x+B*57* vs *Bw6hmz*	0.61	0.03	0.15

replication better than those from Bw6hmz and *l/*x+B*57 carriers ($p<0.05$ for all comparisons at days 7 and 10). There were no significant differences in the ability of HIV to replicate in CD4 cells from subjects carrying these 4 genotypes as measured longitudinally by p24 ELISA (data not shown, $p>0.05$, Kruskal-Wallis test). Together these results show that NK cells from carriers of the educated *h/*y+B*57 genotype inhibit HIV replication in autologous CD4 cells better than those from uneducated Bw6hmz or carriers of the 'less educated' *l*x+B*57 genotype.

NK-iCD4 cell contact contributes to NK cell mediated inhibition of HIV replication

NK cell mediated inhibition of viral replication was assessed by measuring the frequency of intracellular HIV-Gag-p24 positive CD4 cells using anti-p24 specific KC57 monoclonal antibody (mAb). Fig. 2A depicts flow cytometry plots showing the frequency of p24 positive CD4 cells at day 7 for several culture conditions for a single individual. In this study HIV positivity of infected *in vitro* cultured was defined as live $CD3^+$ cells that had a positive signal for p24. The CD4 expression status during *in vitro* HIV infection can reflect the stage of infection, where earlier infected cells can be double positive ($CD4^+p24^+$) and later infected cells will downregulate CD4 from their surface ($CD4^-p24^+$). This approach to quantify and monitor *in vitro* HIV infection was utilized by other groups [453, 454], and appears to depend on the nature of the intracellular p24 antibody used [455]. Fig. 2B shows longitudinal results for up to 12 subjects, 5 on day 3, 10 on day 7 and 12 on day 10. In the presence of NK cells (NK+iCD4) the frequency of $p24^+$CD4 cells was lower than that in cultures of iCD4 cells alone ($p=0.18$, $p=0.002$ and $p<0.001$ for day 3, 7 and 10, respectively, Wilcoxon tests). When NK and iCD4 cells

were cultured in different trans-well chambers (NK/iCD4 TW), which prevents NK and iCD4 cell contact, the frequency of $p24^+CD4$ cells was significantly higher than in conditions where NK and iCD4 were cultured together either in regular wells or the same chamber of a trans-well (NK+iCD4 or NK+iCD4 TW versus NK/iCD4 TW, $p<0.05$ for all comparisons at days 7 and 10, Wilcoxon). However, the frequency of $p24^+CD4$ cells in the NK/iCD4 TW condition remained below that observed in iCD4 cells ($p\leq0.002$ for comparisons at days 7 and 10). These results implicate contact between NK and iCD4 cells as a contributing factor in suppression of infection propagation. However, since abrogating NK and iCD4 contact does not return the percent of $p24^+CD4$ cells to levels seen in iCD4 cells cultured alone, non-contact dependent mechanisms are also likely involved in NK cell mediated inhibition of HIV replication.

If iCD4 cells and co-cultures of NK and iCD4 cells are incubated in upper and lower trans-well chambers, respectively, the frequency of $p24^+CD4$ cells in the upper chamber (iCD4 TW) is lower than that seen when only NK cells are present in the lower chamber (NK/iCD4 TW) ($p=0.007$ and $p=0.08$ for days 7 and 10, Wilcoxon). These results suggest that contact between NK and autologous iCD4 cells produces soluble factors that can then suppress HIV spread in the same well or cross a trans-well membrane to suppress the spread of HIV in iCD4 cells physically separated from NK cells.

NK cells produce CC-chemokines in response to stimulation with autologous HIV iCD4 cells

We questioned whether autologous iCD4 cells could activate NK cells to secrete CC-

chemokines. We reasoned that if this were the case, these soluble factors could be responsible for inhibiting HIV replication under conditions where iCD4 are either co-cultured with NK cells or in a separate trans-well chamber from NK+iCD4 cultures. We assessed CC-chemokine secretion under several conditions at days 1, 2 and 3 of culture. Fig. 3A-C show that iCD4 cells co-cultured with NK cells in the presence of IL-2 (NK+iCD4+IL-2) produced CCL3, CCL4 and CCL5 at higher levels than do either NK cells with IL-2 (NK+IL-2), NK cells cultured with uninfected CD4 cells and IL-2 (NK+CD4+IL-2) or iCD4 cells with IL-2 (iCD4+IL-2) ($p<0.05$ for all CC-chemokines on each day tested, Kruskal-Wallis test). All pair-wise comparisons between CC-chemokine levels secreted in the NK+iCD4+IL-2 condition and those in each of the other 3 conditions were statistically significant, except for those between NK+iCD4+IL-2 and NK+CD4+IL-2 for CCL5 at days 1, 2 and 3 ($p=0.07$, 0.25 and 0.34, respectively, Dunn's post-test comparisons). NK cells cultured without IL-2 and CD4 cells, whether PHA stimulated or not, HIV infected or not and cultured with or without IL-2 produced low levels of these CC-chemokines in the range of 200 pg/ml or lower (data not shown). Thus, NK cells stimulated by autologous iCD4 cells and IL-2 are a source of secreted CC-chemokines and produce more CC-chemokines than NK cells or iCD4 cells alone culture media containing IL-2.

Inhibition of HIV replication by NK cells can be reversed by neutralizing anti-CCL3, CCL4 and CCL5 antibodies (Abs).

To confirm that CC-chemokines contribute to inhibition of HIV replication, neutralizing Abs to each of the CC-chemokine were added to the NK-iCD4 co-cultures. As seen in

Fig. 3D for percent inhibition of viral replication compared to iCD4 cells alone, the addition of neutralizing Abs to individual CC-chemokines had no effect on percent inhibition of HIV replication mediated by NK cells ($p>0.05$ for all comparisons, Wilcoxon test). Addition of Abs to all 3 chemokines reduced NK-mediated HIV suppression. Comparisons of percent inhibition of HIV replication between NK+iCD4+neutralizing Abs to all 3 CC-chemokines and NK+iCD4 with either no Abs or antibodies to single CC-chemokines were significant for all comparisons except one at days 3 and 7 ($p<0.05$, Wilcoxon). The exception was the comparison of percent inhibition between NK+iCD4+neutralizing Abs to the 3 CC-chemokines and NK+iCD4 with no Abs ($p= 0.23$, Wilcoxon). None of the comparisons for percent inhibition at day 10 achieved statistical significance. These results indicate that iCD4 stimulated NK cell secretion of CC-chemokines contributes to inhibition on HIV replication.

NK cells from individuals carrying protective KIR/HLA genotypes secrete higher levels of CC-chemokines than those from Bw6hmz

We next asked whether NK cells from individuals carrying protective KIR/HLA genotype combinations and Bw6hmz differed from each other in the amount of CC-chemokines they secreted upon stimulation with autologous iCD4 cells. We assessed the amount of CC-chemokines secreted over 3 days by NK cells from 7 *h/*y+B*57, 12 3DS1+*80I and 5 *l/*x+B*57 carriers, and 10 Bw6hmz. Stimulated NK cells from *h/*y+B*57 and 3DS1 +*80I carriers secreted similar levels of CCL3, CCL4 and CCL5 to each other and more than those from Bw6hmz (Table 2). CC-chemokine secretion by stimulated NK cells from *l/ *x+B*57 carriers was similar to that from Bw6hmz for CCL3 and CCL5 and

higher than that from Bw6hmz for CCL4 (Table 2). In general, iCD4 stimulated NK cells from *h/*y+B*57 and 3DS1+*80I carriers secreted higher CC-chemokine levels compared to those from *l/*x+B*57 carriers, though several of these comparisons did not achieve statistical significance.

We also stimulated NK cells overnight with autologous 7 day iCD4 and assessed intracellular levels of CCL3 and CCL4 by total NK cells and $3DL1^{+/-}$ NK cell populations using the gating strategy shown in Fig. 4. Higher frequency of NK cells from *h/*y+B*57 carriers secrete CCL3 and CCL4 chemokines upon stimulations with autologous iCD4 than those from Bw6hmz (Fig. 5A and D, respectively). A similar but non-significant trend is when $3DL1^{+}$ NK cells are gated on that is absent in the $3DL1^{-}$ population (Fig. 5B and E versus 5C and F, respectively). We also compared the frequency of $3DL1^{+}$ and $3DL1^{-}$ cells within individuals secreting CCL3 and CCL4. In general, a higher frequency of functional $3DL1^{+}$ than $3DL1^{-}$ NK cells was observed in *h/*y+B*57 carriers ($p=0.15$ and 0.02, for CCL3 and CCL4 secretion, respectively), but not in *l/*x+B*57 carriers and Bw6hmz. It would have been desirable to compare the frequency of intracellular CCL3 and CCL4 positive cell in $3DL1^{+}$ *h versus *l allele expressing NK cell subsets following iCD4 stimulation of *l/*x+B*57 NK cells. Unfortunately, only 2 *l/*x+B*57 subjects carried both an *h and *l allele. The others were either homozygous for *l alleles or carried an *l and an *004 allele. The composition of the *l/*x+B*57 group precluded making firm conclusions regarding CC-chemokine secretion in these $3DL1^{+}$ NK populations. Together, the intracellular cytokine staining results show that KIR/HLA genotype is a determinant of iCD4 stimulated NK cell functionality with regard to CC-chemokine secretion. The higher functionality of $3DL1^{+}$ NK cells in *h/*y+B*57

compared to *I/*x+B*57 carriers and Bw6hmz implicates this KIR/HLA combination in potent NK cell licensing for functional potential

.

2.6 DISCUSSION

In this report we showed that NK cells cultured with autologous iCD4 cells limit the spread of HIV resulting in a lower frequency of iCD4 cells and lower levels of viral replication compared to iCD4 cells cultured alone. Contact between NK and iCD4 cells stimulates NK cells to produce soluble factors, which suppress HIV replication in a non-contact dependent fashion. NK cells activated by autologous iCD4 cells in the presence of IL-2 secrete CC-chemokines at higher levels than when only IL-2 is present. CC-chemokine secretion is responsible, at least in part, for the inhibitory effect of NK cells on viral replication. KIR/HLA genotype influences the potency of inhibition of viral replication. We showed that NK cells from *h/*y+B*57 and 3DS1+*80I carriers, genotypes associated slower time to AIDS and VL control, inhibited HIV replication more potently than did those from Bw6hmz and carriers of the *l/*x+B*57genotype. NK cells, and in particular the $3DL1^{+}$ population of NK cells, from carriers of the *h/*y+B*57 genotype secrete higher levels of CC-chemokines than those from Bw6hmz and *l/*x+B*57 subjects.

The superior control of HIV replication in autologous iCD4 cells by NK cells from carriers of *h/*y+B*57 versus those from *l/*x+B*57 and Bw6hmz subjects implicates NK cell education as a determinant of this anti-viral NK function. NK cell education is important for the development of self-tolerant NK cells and for endowing NK cells with the capacity to mediate cytokine/chemokine secretion and cytolysis upon encountering cells with reduced MHC-I cell surface expression, such as occurs in the context of HIV infected targets [11, 21, 22]. The ligation of inhibitory NK receptors, such as 3DL1, is required for

NK education but the process is tuned by the set of signals received from all the NK cell surface activating and inhibitory receptors interacting with their ligands on neighboring target cells [23–25]. The stronger the inhibitory signals received during NK cell education the broader and more potent the effector functions that NK cells will have against appropriate targets [23]. The *h/*y+B*57 KIR/HLA combination appears to be a particularly potent one for NK cell education, since NK cells from *h/*y+B*57 carriers showed higher functionality when stimulated with HLA-null cells than those from carriers of 3DL1*h/*y genotypes co-carried with other Bw4 or *80I alleles, 3DL1*l/*x genotypes cocarried with B*57 or those from Bw6hmz [10,15]. The difference in functional potential between NK cells from carriers of *h/*y+B*57 versus those from 3DL1hmz who carry other Bw4 alleles may reflect differences in the impact of HLA-B*57 versus other Bw4 antigens in providing educational signals to NK cells during development. Transgenic mice expressing single MHC-I alleles have been used to show that MHC-I antigens differ in their impact on NK cell education [24]. The strength of the inhibitory input during education, as determined by the strength of the interaction between inhibitory NK receptors and their ligands, is directly related to the functional responsiveness of individual NK cells [23, 24]. Thus, it appears that B*57 differs from most other Bw4 molecules in the strength with which it interacts with 3DL1 to educate NK cells. NK cells from 3DL1*h/*y positive subjects express higher levels of 3DL1 inhibitory receptors than those from 3DL1*l/*x positive individuals [6]. The observation that NK cells from *l/*x+B*57 carriers secrete less CC-chemokines and inhibit HIV replication more poorly than those from *h/*y+B*57 carriers may be related to less potent NK education due to lower levels of cell surface 3DL1 mediating lower inhibitory signals for NK cell

education, even in the presence of the potent B*57 3DL1 ligand. A caveat to this interpretation is that while there is experimental evidence that B*57 binds 3DL1 it has not been demonstrated that the affinity of the interaction between these 2 molecules is greater than that between 3DL1 and other Bw4 molecules because different peptides influence 3DL1 Bw4 binding [26, 27]. In the presence of the same epitope and 3DL1 receptor HLA-Bw4*80T variants bind with about 60% of the affinity of B*57 [27]. The impact of *h/*y+B*57 on NK cell education and the relationship between NK education and NK cell responsiveness may underlie epidemiological findings that carriers of this genotype have a lower risk of HIV infection and in those who become infected have a slower time to AIDS and lower VL than carriers of other 3DL1hmz Bw4 genotypes, including *l/*x+B*57 carriers [7, 9]. The influence of *h/*y+B*57 on NK cell education may also play a role in the superior ability of NK cells from carriers of this KIR/HLA genotype to inhibit viral replication in autologous HIV infected cells compared to those from Bw6hmz.

It is notable that the frequency of $p24^{+}CD4$ cells in conditions where NK and iCD4 cells are in separate trans-wells is lower than that of iCD4 cells cultured alone but higher than that of iCD4 cells and NK cells cultured together. This implies that NK-CD4 cell contact contributes to NK cell activation and secretion of soluble factors that can inhibit HIV replication in a non-contact dependent manner. IL-2 by itself can also activate NK cells to secrete soluble factors such as CC-chemokines, though at lower levels than when iCD4 cells are also present. This may be why the percent of $p24^{+}CD4$ cells in conditions where iCD4 and NK cells are in separate trans-well chambers is not as high as when

iCD4 are cultured alone. It is not known whether these soluble factors are limited to CC-chemokines. Simultaneous neutralization of the CCL3, CCL4 and CCL5 restored HIV replication measured at 3 and 7 days of culture to levels that were significantly higher than when NK and iCD4 cells were co-cultured in the absence of CC-chemokine neutralization. Neutralization of all 3 CC-chemokines was not sufficient to reduce NK cell mediated inhibition of HIV replication at day 10 of culture. The reason for this is unclear but may be due to the continued production of chemokines over and above the amounts that anti-CC-chemokine Abs are able to neutralize. High inter-subject variability precludes making a clear determination as to whether CC-chemokine neutralization is sufficient to reverse NK cell mediated inhibition. It is possible that iCD4 stimulate NK cells to inhibit HIV replication by other mechanisms in addition to CC-chemokine secretion. These activities could target other stages of the HIV replication cycle and may or may not be dependent on contact between NK and iCD4 cells.

Previous studies have shown that NK cells secrete CC-chemokines following stimulation through CD16 cross-linking and co-culture with iCD4 cells in the presence of IL-2 [20]. Here we report for the first time that a KIR/HLA genotype combination that influences the potency of NK cell education also determines the level of CC-chemokines that NK cells secrete in response to autologous iCD4 cells. CC-chemokines can bind CCR5, the HIV co-receptor, and prevent HIV from interacting with this receptor thus reducing HIV entry [19, 20]. Trans-well experiments implicate cell contact as a factor in NK cell stimulation leading to CC-chemokine secretion.

Pelak et al. reported that in carriers of 3DS1htz+*80I, the copy number of 3DL1 alleles influenced NK cell mediated inhibition of HIV replication in autologous iCD4 T cells [28]. Copy number variation (CNV) is common at the 3DL1/S1 locus. Screening for CNV at this locus revealed no duplications or deletions at this locus among subjects having the 4 genotypes focused on in this study. Therefore, CNV at the 3DL1/S1 locus can be excluded as a factor influencing the experimental findings reported here.

In summary, we show that NK cells from carriers of *h/*y+B*57 inhibit HIV viral replication in autologous iCD4 cells more effectively than those from *l/*x+B*57 carriers and Bw6hmz. The level of anti-viral function of NK cells from carriers of this genotype is likely related to NK cell education arising from B*57 interactions with high expression inhibitory 3DL1 receptors. Antiviral function is mediated at least in part by CC-chemokine secretion levels able to block HIV entry into CD4 cell targets. The higher level of CC-chemokine secretion by NK cells from carriers of protective versus non-protective KIR/HLA genotypes may underlie their superior ability to inhibit HIV replication in infected targets.

2.7 FIGURES AND LEGENDS

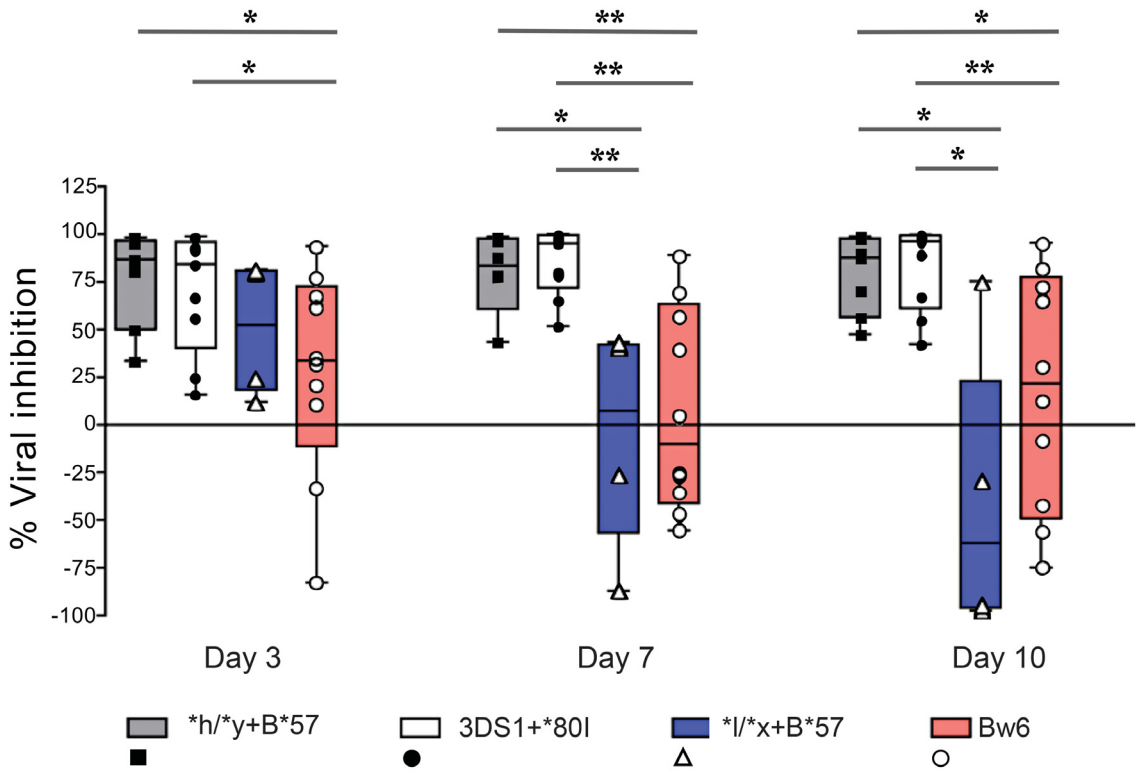


Figure 1. NK cells from subjects carrying *h/*y+B*57 and 3DS1+*80I suppress viral replication better than those from Bw6hmz and *l/*x+B*57 carriers. The box and whisker plots show the percent viral inhibition observed when NK cells from subjects positive for *h/*y+B*57 (n = 7), 3DS1+*80I (n = 9), Bw6hmz (n = 10) and *l/*x+B*57 (n = 4) are cultured with autologous HIV infected CD4 (iCD4) cells at a ratio of 10:1 for up to 10 days. The line in each box represents the median value, the lower and upper limits of the boxes the 25% and 75% quartiles and the whiskers the minimum and maximum values for each group; each point is the percent viral inhibition value for a single individual. Lines linking groups indicate comparisons where medians were significantly different. ‘‘*’’ = $p<0.05$, ‘‘**’’ = $p<0.01$.

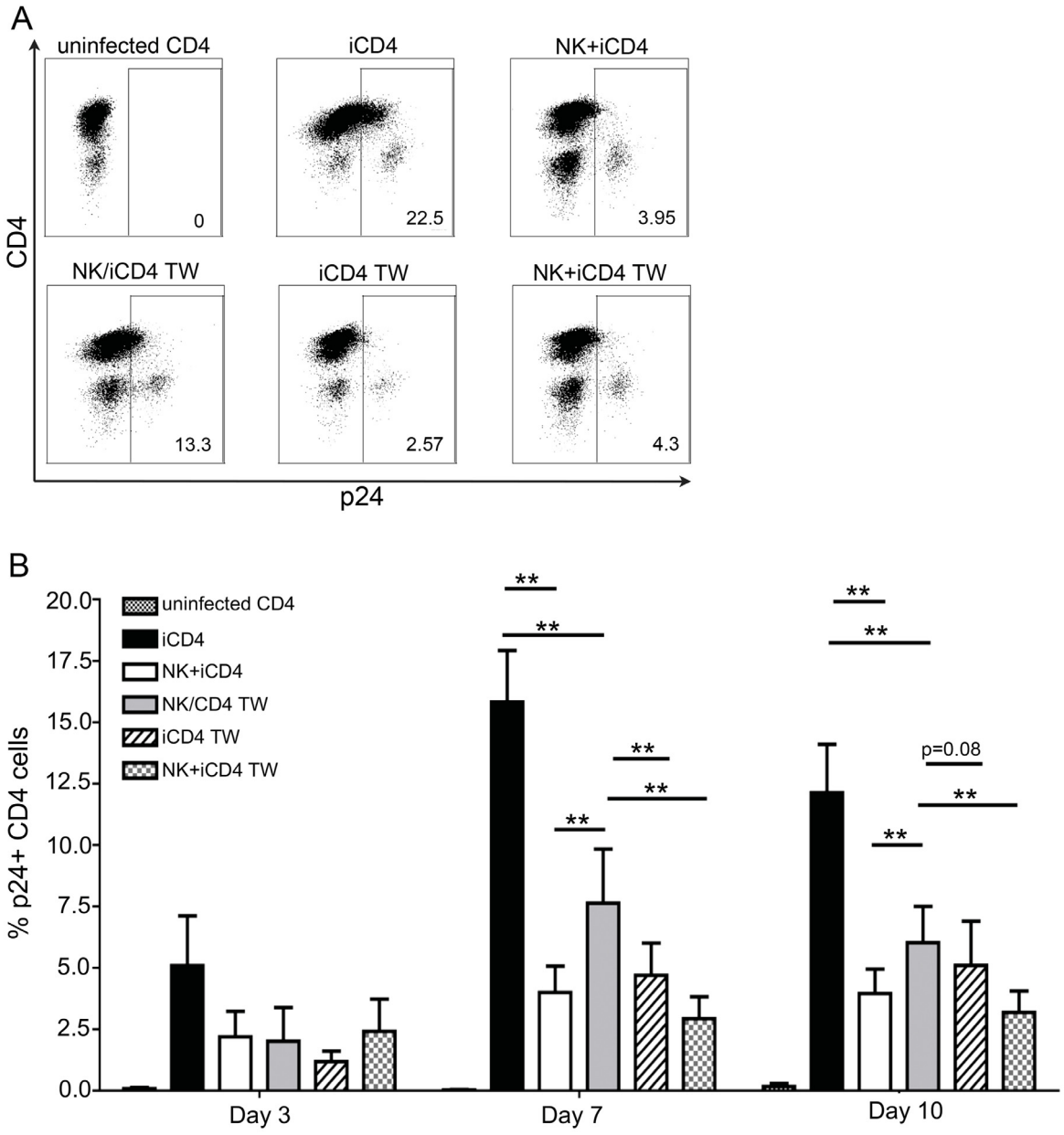


Figure 2. NK cells inhibit HIV replication in autologous HIV infected CD4 T cells in a contact dependent manner. Legend on the following page.

Figure 2. NK cells inhibit HIV replication in autologous HIV infected CD4 T cells in a contact dependent manner. (A) Flow plots show the frequency of p24 positive CD4 cells from a single individual cultured for 7 days under the following conditions: uninfected CD4 T cells cultured alone, infected CD4 (iCD4) cells cultured alone, iCD4 cells cultured with autologous NK cells in the same well at a 10:1 NK:iCD4 ratio (NK +iCD4), iCD4 cells and NK cells cultured in separate transwell chambers at a 10:1 NK:iCD4 ratio (NK iCD4 TW), iCD4 cells cultured alone in the upper chamber of a transwell with NK cells and iCD4 cells cultured together in the lower transwell chamber at a 10:1 NK:iCD4 ratio (iCD4 TW), iCD4 cells cultured with NK cells in the same transwell chamber at a 10:1 NK:iCD4 cell ratio (NK+iCD4 TW). (B) Bar graphs show the frequency of HIV infected cells on days 3, 7 and 10 under the same culture conditions as described in (A) for up to 12 individuals. One subject was positive for *h/*y+B*57, 7 were 3DS1+*80I, 2 were Bw6hmz, 1 was 3DS1+Bw4 not *80I and 1 was 3DL1hmz+*80I (not B*57). Bar height and error bars represent the mean and the standard error of the mean for each group. Lines linking bars indicate comparisons where means are significantly different. ‘‘*’’ = $p<0.05$, ‘‘**’’ = $p<0.01$

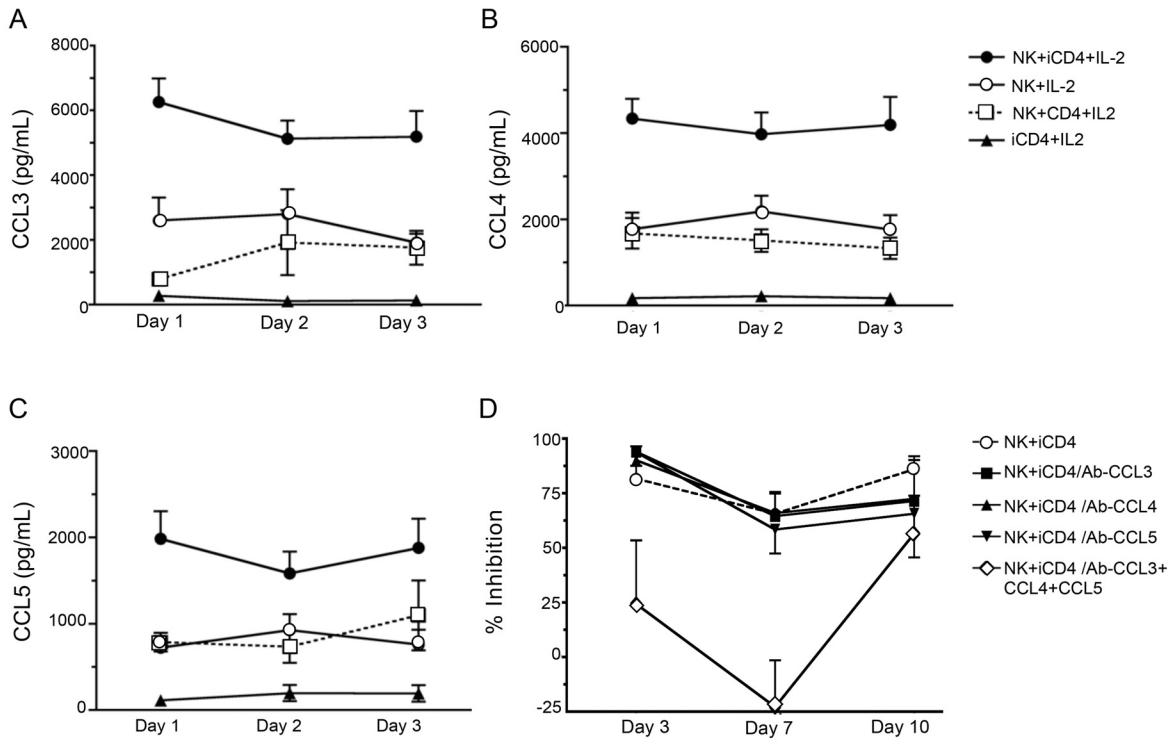


Figure 3. Infected CD4 T cells stimulate autologous NK cell to produce CC-chemokines. Supernatants levels of CCL3 (A), CCL4 (B) and CCL5 (C) were assessed by ELISA. Shown are results for NK cells cultured with infected CD4 cells (iCD4) at a 10:1 ratio with IL-2 (NK+iCD4+IL-2, n = 33), NK cells cultured with IL-2 (NK+IL-2, n = 24), NK cells cultured with uninfected CD4 and IL-2 (NK+CD4+IL-2, n = 9) and iCD4 with IL-2 (iCD4+IL-2, n = 11). The following KIR/HLA genotypes were tested; *h/*y+B*57 (n = 4), 3DS1+*80I (n = 5), Bw6hmz (n = 4), *l/*x+B*57 (n = 4) and other KIR/HLA (n = 5). Samples were tested on up to 4 occasions in separate experiments. (D) Percent inhibition of viral replication of an NK cell autologous iCD4 cell co-culture in the absence of anti-CC-chemokine nAbs, in the presence of anti-CCL nAbs, individually, or together. Results were generated using subjects with the following KIR/HLA gentotypes; *h/*y+B*57 (n = 2) and 3DS1+*80I (n = 5). All data was plotted as mean+SEM.

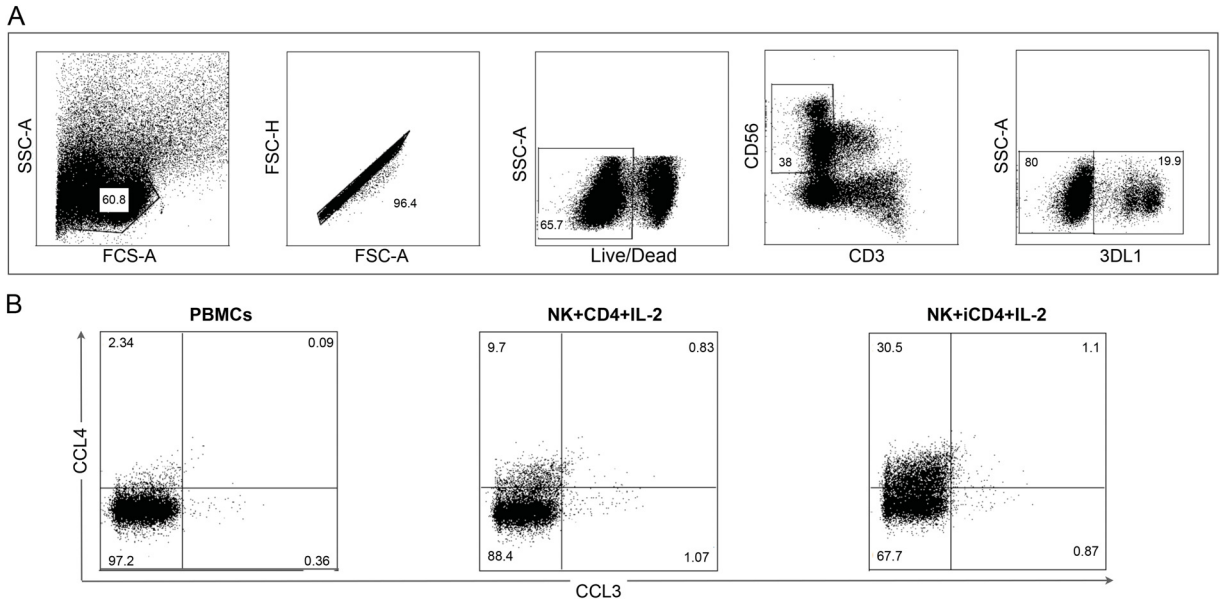


Figure 4. Gating strategy used to assess the percent of functional NK cells and NK cell subsets stimulated with autologous infected CD4 (iCD4) cells. (A) We used FSC-A and SSC-A to gate on lymphocytes and FSC-A and FSC-H to gate on single cell events from co-cultures of NK cells with autologous CD4 or iCD4 cells. Only live $CD3^{-}CD56^{+}$ NK cells were included in this analysis. The representative subject shown in this figure carries a KIR3DL1 *l/*x genotype with 1 high and 1 low KIR3DL1 allele. (B) Functional gates were set using unstimulated PBMCs that were gated on the $KIR3DL1^{+}$ NK cell population using the gating strategy shown in panel A. The percent of CCL3, and CCL4 positive cells was determined for conditions in which NK cells were cultured in IL-2 media and stimulated with uninfected CD4 (background) and iCD4 cells. All data presented was background subtracted. FCS-A= forward scatter area; SSC-A= side scatter area; FSC-H = forward scatter height.

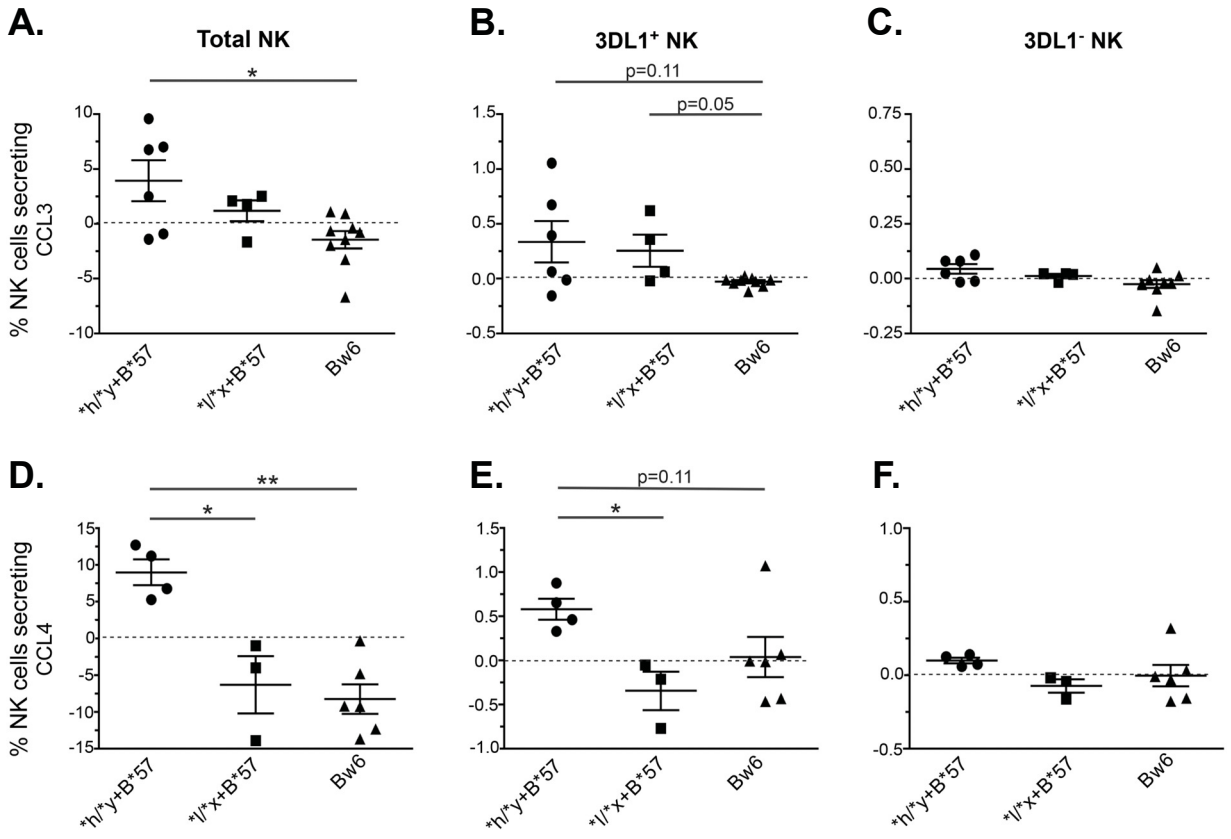


Figure 5. Percent of CCL3$^+$ and CCL4$^+$ NK cells and NK cell subsets following stimulation with autologous HIV infected CD4 (iCD4) cells. CD4 cells infected with HIV and cultured for 7 days were used to stimulate autologous NK cells overnight. Intracellular cytokine staining was used to determine the percent of CCL3$^+$ (A-C) and CCL4$^+$ (D-F) in total NK cells (A, D), KIR3DL1$^+$ (B, E) and KIR3DL1$^-$ (C, F) NK cell populations in subjects positive for *h/*y+B*57 (n = 7), *l/*x+B*57 (n = 4) and Bw6hmz (n = 9). Each point represents the value for a single individual, the line and error bars through each group show the mean+/-SEM for each data set. Lines linking groups indicate between group comparisons. "*" = a $p<0.05$, "**" = a $p<0.01$.

2.8 REFERENCES

1. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, et al. (2003) Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J Exp Med 198: 1069–1076.

2. Lanier LL (2005) NK cell recognition. Annu Rev Immunol 23:225–74.: 225–274.

3. Bashirova AA, Thomas R, Carrington M (2011) HLA/KIR restraint of HIV: surviving the fittest. Annu Rev Immunol 29: 295–317.

4. Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P (2003) 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 171: 6640–6649.

5. Taner SB, Pando MJ, Roberts A, Schellekens J, Marsh SG, et al. (2011) 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 186: 62–72.

6. Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, et al. (2006) Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J Exp Med 203: 633–645.

7. 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.

8. Wan AM, Ennis P, Parham P, Holmes N (1986) The primary structure of HLAA32 suggests a region involved in formation of the Bw4/Bw6 epitopes. J Immunol 137: 3671–3674.

9. 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.

10. Kamya P, Boulet S, Tsoukas CM, Routy JP, Thomas R, et al. (2011) Receptor-ligand requirements for increased NK cell poly-functional potential in *h/*y+B57 HIV-1 infected Slow progressors. J Virol . JVI.02652-10.

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

12. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P (1995) 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 181: 1133–1144.

13. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, et al. (2005) Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 436: 709–713.

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. 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.

16. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, et al. (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet 31: 429–434.

17. Qi Y, Martin MP, Gao X, Jacobson L, Goedert JJ, et al. (2006) KIR/HLA

Pleiotropism: Protection against Both HIV and Opportunistic Infections. PLoS Pathog 2: e79.

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

19. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, et al. (1995) Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 270: 1811–1815.

20. Oliva A, Kinter AL, VaccarezzaM, Rubbert A, Catanzaro A, et al. (1998) Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. J Clin Invest 102: 223–231.

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

22. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, et al. (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.

23. 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.

24. Johansson S, Johansson M, Rosmaraki E, Vahlne G, Mehr R, et al. (2005) Natural killer cell education in mice with single or multiple major histocompatibility complex class

I molecules. J Exp Med 201: 1145–1155.

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

26. Fadda L, O'Connor GM, Kumar S, Piechocka-Trocha A, Gardiner CM, et al. (2011) Common HIV-1 peptide variants mediate differential binding of KIR3DL1 to HLA-Bw4 molecules. J Virol 85: 5970–5974. JVI.00412-11

27. Vivian JP, Duncan RC, Berry R, O'Connor GM, Reid HH, et al. (2011) Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. Nature 479: 401–405.

28. Pelak K, Need AC, Fellay J, Shianna KV, Feng S, et al. (2011) Copy number variation of KIR genes influences HIV-1 control. PLoS Biol 9 (11).

29. Boulet S, Sharafi S, Simic N, Bruneau J, Routy JP, et al. (2008) Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. AIDS 22: 595–599.

30. Bounou S, Leclerc JE, Tremblay MJ (2002) Presence of host ICAM-1 in laboratory and clinical strains of human immunodeficiency virus type 1 increases virus infectivity and CD4(+)-T-cell depletion in human lymphoid tissue, a major site of replication in vivo. J Virol 76: 1004–1014.

CHAPTER 3

A Higher Frequency of NKG2A$^+$ than NKG2A- NK Cells Respond to Autologous HIV-Infected CD4 Cells, Irrespective of Whether they Co-express 3DL1

Lisovsky I, Isitman G, Song R, DaFonseca S, Tremblay-McLean A, Lebouché B, Routy JP, Bruneau J and Bernard NF

This chapter was adapted from an article published in Journal of Virology, 2015 October. 89(19): 9909-19.

Contribution of authors: IL designed research, conducted experiments, analyzed data, interpreted and prepared the manuscript. IG provided assistance with data interpretation and manuscript preparation. SR and DS provided assistance with conducting experiments. TMA provided assistance with manuscript preparation. LB, RJP and BJ provided clinical samples used in this study. NFB designed research, supervised the research and prepared the manuscript.

3.1 RATIONALE AND RESEARCH OBJECTIVES

NK cells, and particularly NK populations expressing iKIR that developed in subjects co-expressing respective HLA antigens to generate strong educational signals, are important in controlling HIV replication and infection. In Chapter 2 we showed that KIR3DL1^{+} NK cells from donors with highly expressed variants who also co-expressed the B*57 ligand had superior inhibition of HIV replication via secretion of CC-chemokines. NK cells acquire their functional competence via engagement of iNKRs with their respective ligands. HIV infection selectively dowregulates HLA-A and -B, but not -E, which are the ligands for KIR3DL1 and NKG2A, respectively. In this chapter we sought to study the impact of KIR3DL1 and NKG2A expression on NK cell functionality at the NK cell population level. This was done by analyzing the functional responses of NK cell populations from uninfected individuals defined by these iNKRs in response to autologous HIV infected cells, and thus models the role played by NK cells at the level of protection from infection.

3.2 ABSTRACT

Epidemiological and functional studies implicate NK cells in HIV control. However, there is little information available on what NK cell populations, as defined by inhibitory NK cell receptors (iNKRs) they express, respond to autologous HIV-infected $CD4^+$ T cells (iCD4). NK cells acquire anti-viral functions through education, which requires signals received from iNKRs, such as NKG2A and KIR3DL1 (3DL1), engaging their ligands. NKG2A interacts with HLA-E and 3DL1 interacts with HLA-A/B antigens expressing the Bw4 epitope. HIV infected cells downregulate HLA-A/B, which should interrupt negative signaling through 3DL1, leading to NK cell activation, provided there is sufficient engagement of activating NKRs. We examined the functionality of NK cells expressing or not NKG2A and 3DL1 stimulated by HLA null and autologous iCD4 cells. Flow cytometry was used to gate on each $NKG2A^{+/-}3DL1^{+/-}$ population and to measure the frequency of all possible combinations of CD107a expression and IFN-γ, and CCL4 secretion. The highest frequency of functional NK cells responding to HLA-null cell stimulation was the $NKG2A^+3DL1^+$ NK cell population. The highest frequencies of functional NK cells responding to autologous iCD4 were those expressing NKG2A; co-expression of 3DL1 did not further modulate responsiveness. This was the case for the functional subsets characterized by the sum of all functions tested (total responsiveness), as well as, by the tri-functional, $CD107a^+IFN\text{-}\gamma^+$, total $CD107a^+$ and total $IFN\text{-}\gamma^+$ functional subsets. These results indicate the NKG2A receptor has a role in NK cell mediated anti-HIV responses.

3.3 INTRODUCTION

Natural Killer (NK) cells are a subset of lymphocytes that mediate immune responses against virally-infected and transformed cells [1]. They contribute to innate immune defenses directly by eliciting functions such as cytotoxicity and secretion of cytokines and chemokines. They also contribute to shaping adaptive immune responses through their interactions with dendritic cells [2]. NK cell activation can occur without prior sensitization, before T cell mediated immune responses can be induced [3]. The timing of NK cell responses suggests that they may have a role in initial viral control. This is supported by studies that implicate NK cells in resistance to human immunodeficiency virus (HIV) [4-6]. NK cells also appear to play a role in several viral infections (HIV, HCMV, HBV, HCV, influenza) [7-11]. The importance of NK cell function in the context of HIV infection is highlighted by the development of HIV sequence polymorphisms that allow the virus to evade NK cell anti-viral pressure [12].

The state of activation of NK cells is determined by the integration of signals received from stochastically expressed germ-line encoded cell-surface receptors upon interaction with ligands on target cells. NK cells acquire functional competence through an ontogenic process known as education which requires the interaction of inhibitory NK receptors (iNKRs) with their cognate HLA ligands on neighbouring cells [13, 14]. Education is not an on/off switch as functionality can be tuned by the number of iNKRs engaged, the strength of interactions between NKRs and their ligands and whether activating NK cell receptors (aNKRs) are also engaged [15, 16]. NK cells lacking iNKRs for self-HLA ligands remain unlicensed and hyporesponsive [14]. Licensed NK cells are

tolerant to normal healthy cells but have the potential to respond to target cells that upregulate ligands for aNKR and have reduced levels of cell surface HLA ligands for iNKR, as often occurs in viral infection and tumor transformation.

The Killer Immunoglobulin-like Receptor (KIR) 3DL1 and NKG2A surface receptors are both examples iNKRs. NKG2A, a C-type lectin receptor expressed in all people, forms a heterodimer with CD94 and interacts with non-classical major histocompatibility complex (MHC) class I HLA-E molecules that present leader peptides from many HLA class I (HLA-I) proteins [17, 18]. Thus, NKG2A$^+$ NK cells can survey autologous cells for overall HLA levels. KIR3DL1 (henceforth 3DL1) interacts with a subset of HLA-A and –B antigens that belong to the Bw4 subset [19, 20]. HLA variants belonging to the Bw4 group differ from the remaining Bw6 HLA-B variants at amino acids 77-83 of the HLA heavy chain [21]. Bw6 isoforms do not interact with any 3DL1 receptors. Unlike 3DL1$^+$ NK cells from individuals homozygous for Bw6 (with no *Bw4* alleles at the *HLA-A* locus), those from Bw4$^+$ subjects are licensed through 3DL1. NK cell activation can occur when inhibitory signals through iNKR are interrupted due to loss of ligands for iNKR on target cells, or if signaling through aNKR overcomes negative signals originating through iNKR engagement [13, 22].

At present there is little known about the impact of co-expression of NKG2A and 3DL1 iNKRs on NK functionality in the context of HIV infection. We hypothesized that NK cells expressing both iNKRs would have higher functional potential compared to NK cells expressing one or none of these iNKRs, as assessed by responsiveness to HLA-null

cells [23], and that this principle would also apply to NK responses to stimulation by autologous HIV infected CD4 T cells (iCD4). To test this hypothesis we first characterized the functional potential of NK cell populations expressing the four possible combinations of NKG2A and 3DL1 following stimulation with the HLA-null cell lines, K562 and 721.221 (henceforth 721), which are commonly used to assess NK functionality. We found that while NK cells co-expressing NKG2A and 3DL1 had a higher functional potential in response to HLA-null cells, $NKG2A^+$ NK cells responded more robustly than $NKG2A^-$ NK cells to autologous iCD4 cells irrespective of whether they co-expressed the 3DL1 iNKR. This is the first time a study has examined the impact of these iNKRs on multi-functional responses to iCD4.

3.4 MATERIALS AND METHODS

Ethics statement

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. It was approved by the Institutional Review Boards of the Comité d'Éthique de la Recheche du Centre Hospitalier de l'Université de Montréal and the Research Ethics Committee of the McGill University Health Centre - Montreal General Hospital. All subjects provided written informed consent for the collection of samples and subsequent analysis.

Study population

26 HIV-1 uninfected 3DL1 homozygotes were studied, including 16 with at least 1 *Bw4* allele and 10 who were *Bw6* homozygotes and had no *Bw4* alleles at the *HLA-A* locus. The HLA and 3DL1 allotypes of each of the study subjects are listed in Table I.

Genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) or EBV-transformed cells using a QIAamp DNA blood kit (QIAGEN). MHC class-I alleles were typed by sequencing using commercial reagents (Atria Genetics Inc.). Assign 3.5+ software was used to interpret sequence information for allele assignment (Conexio Genetics). *3DL1* genotyping was performed by PCR as previously described [5]. *3DL1* allotypes were identified as described [6].

Cells

PBMCs were isolated by density gradient centrifugation (Lymphocyte separation medium; Wisent) and cryopreserved in 10% dimethyl sulfoxide (DMSO; Sigma Aldrich); 90% fetal bovine serum (FBS; Wisent). 721 cells were a kind gift from Dr. Galit Alter (Ragon Institute, Harvard University). The K562 cell line was obtained from the American Type Culture Collection, (Manassas, VA). 721 and K562 cells were cultured in complete RPMI: RPMI 1640; 10% FBS; 2 mM L-glutamine; 50 IU/ml penicillin; 50 mg/ml streptomycin (R10) (all from Wisent). CD4 and NK cells were isolated from PBMCs by positive and negative selection kits, respectively (STEMCELL Technologies, Inc.). Purity was verified by flow cytometry and was an average of 95.3% and 97.2% for CD4 and NK cells, respectively.

Viral infection

Autologous CD4 cells were infected with the HIV-$1_{JR\text{-}CSF}$ clone as previously described [24]. In brief, purified CD4 cells (10^6/ml) were stimulated overnight with 1 µg/ml phytohemagglutinin (PHA-P; MP Biomedicals) and 100 IU/ml of recombinant human IL-2 (rhIL-2 Chiron Corp.) in R10 at 37°C in a 5% CO2 humidified incubator. Stimulated CD4 cells were then washed and cultured in R10 with 100 IU/mL rhIL-2 for 3 days. On day 4, CD4 cells were infected at a multiplicity of infection of 0.01 with HIV-$1_{JR\text{-}CSF}$ for 4 hrs. Cells were washed and cultured for 7 days in R10 with rhIL-2. Uninfected CD4 (CD4) cells were cultured in parallel to iCD4 cells for 7 days in R10 with rhIL-2. On day 7, iCD4 and CD4 cells were used to stimulate NK cells. Day 7 iCD4 and CD4 cells were stained with UV Live/Dead fixable dead cell stain kit (Invitrogen), CD3-BV785 (OKT3)

and CD4-BV421 (OTK4) (both from Biolegend). Percent of HIV infected cells was measured intracellularly (ICS) with anti-p24-FITC (KC57) (Beckman Coulter). ICS background for p24 was set on uninfected CD4 controls.

NK cell stimulation and staining

Cryopreserved PBMCs were thawed and co-cultured with HLA-null cells at a 5:1 ratio in a 96-well plate for 6hrs at 37^{o}C in a humidified 5% CO_2 incubator. CD107a-PE-CF594 (BD) was added at the start of the stimulation; Brefeldin A (5 mg/ml; Sigma Aldrich) and monensin (6 mg/ml, Golgi Stop; BD) were added 30 min after the initiation of the co-culture. PBMCs were also stimulated with 1.25 ug/ml of PMA–0.25g/ml ionomycin (Sigma Aldrich) and all cells used in this study were responsive to this positive control. Viability was assessed using the UV Live/Dead fixable dead cell stain kit (Invitrogen). Non-specific interactions of Fc receptors (FcR) on effectors with the antibody panel were minimized using TruStain FcX reagent (BioLegend), all as per manufacturer's instructions. Cells were stained with antibodies to the following cell surface markers: CD3-BV785 (OKT3), CD56-BV711 (NCAM), 3DL1-BV421 (DX9; all from BioLegend) and NKG2A-PECy7 (Beckman Coulter). After washing cells were fixed and permeabilized using the Fix and Perm kit (Invitrogen), and stained by ICS with CCL4-APC (24006; R&D) and IFN-γ-BV605 (B27; BD). After staining, cells were washed twice, fixed with 1% paraformaldehyde (Santa Cruz) and acquired within 24hrs.

Stimulation of purified NK cells with autologous HIV iCD4 cells was done as previously described [24]. In brief, 1x10^{6} NK cells were co-cultured with iCD4 or CD4 cells at a ratio

of 10:1 in a 96-well plate in R10 with 100 IU/ml rhIL-2 for 24 hrs. Brefeldin A (6 ug/ml) and monensin (5 ug/ml, Golgi Stop) were added 5 hrs before the end of the culture period. Viability, FcR blocking and surface staining was done as described above for PBMCs stimulated with HLA-null cells. Samples were washed, fixed and permeabilized and stained ICS using CCL4-FITC (24006; R&D) and IFN-γ-Alexa700 (B27; BD). Samples were washed, fixed with 1% paraformaldehyde and acquired within 24hrs.

For certain experiments purified NK cells were co-cultured with iCD4 or uninfected CD4 controls for 24hrs in the presence or absence of blocking antibodies to NKG2A (clone 131411 from R&D), HLA-E (clone MEM-E02 from Abcam) or both. Antibodies were added 10μg/mL at initiation of the co-culture.

Flow cytometry analysis

Between $4x10^5$ and $1.5x10^6$ total events were acquired for each sample using an LSRFortessa flow cytometer (BD) calibrated using 8-peak color rainbow beads (Spherotech, Inc). The Ab panel was standardized, validated and single stained control beads (CompBead Plus; BD) were used in every experiment to calculate compensation. Boolean gating was used to identify the frequency of NK cells with each of the 7 possible functional profiles, i.e. tri-functional ($CD107a^+IFN\text{-}\gamma^+CCL4^+$), bi-functional (any combination of two functions) and mono-functional profiles, within the $NKG2A^-3DL1^-$, $NKG2A^-3DL1^+$, $NKG2A^+3DL1^-$ and $NKG2A^+3DL1^+$ NK cell populations. Total responsiveness was defined as the sum of the frequencies of tri-, bi- and mono-functional NK cell populations. Flow cytometry analysis for NK cell activation after stimulation was performed using FlowJo software version 9.8 (TreeStar). The data

obtained were background corrected by subtracting values for unstimulated cells in the case of HLA-null cell stimulation or uninfected CD4 cells in the case of iCD4 stimulation.

Statistical analysis

Analysis was performed using GraphPad Prism6. Friedman tests were used to determine significant differences between the four NKG2A$^{+/-}$3DL1$^{+/-}$ populations. Where multiple comparisons were performed, such as for the 7 possible functional subsets, Bonferonni corrections were applied. Wilcoxon test was used to assess the significance of comparisons for 2 within-subject matched data sets for functional NKG2A$^{+/-}$3DL1$^{+/-}$ populations. Spearman's correlation tests were used to assess the relationship between the frequencies of NK responses to iCD4 and the frequency of intracellular p24^{+} cells in the iCD4 stimuli. $P<0.05$ were considered significant.

3.5 RESULTS

Surface expression analysis of NKG2A and 3DL1 following stimulation with HLA-null cell lines

Figure 1A shows the gating strategy used to identify the expression of NKG2A and 3DL1 on $CD3^{-}CD56^{+}$ NK cells. Boolean gating was then used to identify the frequency of NK cells expressing the four possible combinations of NKG2A and 3DL1. Figure 1B shows the frequency of $NKG2A^{+/-}3DL1^{+/-}$ populations defined by the surface expression of these iNKRs from 13 HIV uninfected individuals following K562, 721 and no stimulation (PBMC alone). For unstimulated PBMCs, 6.6% (median) (3.8, 9.9) (IQR) of NK cells expressed both iNKRs, 51.7% (35.4, 63.7) expressed NKG2A alone, 9.2% (6.3, 20.1) expressed 3DL1 alone and 22.6% (20.2, 33.2) lacked expression of both iNKR. Neither K562 nor 721 stimulation had a significant effect, compared to no stimulation, on the frequency of NKG2A and 3DL1 expression on these NK cell phenotypic populations ($p>0.05$, Friedman).

721 cells induced a higher frequency of double $iNKR^{+}$ NK cells characterized by total, tri-functional, $CD107a^{+}IFN\text{-}\gamma^{+}$, $IFN\text{-}\gamma^{+}CCL4^{+}$ and $IFN\text{-}\gamma^{+}$ response profiles, while $NKG2A^{+}3DL1^{-}$ cells had the highest CD107a response

The functional potential of each $NKG2A^{+/-}3DL1^{+/-}$ NK cell population was measured following stimulation with 721 cells (Fig. 2A) and K562 (Fig. S1). Both 721 and K562 cell lines stimulated different frequencies of total responsiveness among $NKG2A^{+/-}3DL1^{+/-}$ NK cell populations ($p<0.0001$, Friedman, adjusted p-value<0.0007). The frequency of functional $NKG2A^{+}3DL1^{+}$ (double $iNKR^{+}$) NK cells was significantly higher than those of

the other three populations (Fig. 2A and Fig. S1B-D, p≤0.003 for all comparisons). The frequency of functional NK cells stimulated with PMA-ionomycin was higher than that seen for NK cells triggered by HLA-null cells, though these frequencies did not differ significantly among the populations identified by NKG2A and/or 3DL1 expression. This suggests that differential levels of stimulation by HLA null cells is not an innate property of these NK cell populations since they are activated to similar levels by PMA-ionomycin (Fig. S2). Given that K562 and 721 induced similar patterns of functional responsiveness among the four phenotypic NK cell populations, subsequent results will only show results for 721 stimulation.

Next, we examined the individual 721 induced functional subsets that comprised the total responsiveness of NKG2A$^{+/-}$3DL1$^{+/-}$ NK cell populations. 721 induced differential levels of tri-functional (Fig. 2B), bi-functional CD107a^{+}IFN-γ^{+} and IFN-γ^{+}CCL4^{+} (Fig. 2C and 2D, respectively), and mono-functional IFN-γ^{+} (Fig. 2E) responses in the four NKG2A$^{+/-}$3DL1$^{+/-}$ populations (p≤0.0002, Friedman, adjusted p-value≤0.0014). The frequencies of the 721 stimulated double iNKR^{+} population with above mentioned functions were higher than those of the other three populations. Between-population comparisons were significant for all but the IFN-γ^{+}CCL4^{+} functional subset, for which the differences between the double iNKR^{+} and single 3DL1^{+} populations did not achieve significance (Fig. 2D, p=0.07, Wilcoxon).

The four NKG2A$^{+/-}$3DL1$^{+/-}$ populations also differed from each other in terms of the frequency of 721 triggered CD107a only expression (Fig. 2F, Friedman, adjusted

p<0.0007). Paired comparisons showed that 721 stimulated a higher frequency of CD107a$^+$ double iNKR$^+$ than double iNKR$^-$ and single 3DL1$^+$ NK cells (Fig. 2F, p≤0.001, Wilcoxon). In contrast to what was observed for several other functional subsets, 721 induced a higher frequency of CD107a expression in the single NKG2A$^+$ than in the double iNKR$^+$ NK cell population (Fig. 2G, p=0.003, Wilcoxon). Therefore, while the double iNKR$^+$ population had the highest functional potential of the four NKG2A$^{+/-}$3DL1$^{+/-}$ groups for total, tri-functional, CD107a$^+$IFN-γ$^+$, IFN-γ$^+$CCL4$^+$ and IFN-γ$^+$ responses, the single NKG2A$^+$ population had the highest functional potential for degranulation.

The higher functional potential of NKG2A$^+$3DL1$^+$ cells was limited to NK cells originating from individuals carrying a *Bw4* allele

To address whether education via the 3DL1 receptor contributed to the observed results pattern we extended our analysis based to NK cells originated from carriers of a *Bw4* allele or not (Bw6 controls). The total responsiveness of 3DL1$^+$ cells following stimulation with 721 cells was significantly greater in Bw4 than Bw6 individuals that co-expressed NKG2A (Fig. 3A, p=0.03, Mann-Whitney). Furthermore, the double iNKR$^+$ population from *Bw4* carriers was significantly more responsive to HLA null cells than the other three NKG2A$^{+/-}$3DL1$^{+/-}$ populations (Fig. 3B, p=0.007 for all comparisons). In contrast, for NK cells from *Bw6* homozygotes this trend was not observed, and the single NKG2A$^+$ population had the same level of total responsiveness as the double iNKR cells (Fig. 3C, p=0.8). Together these results suggest that having 2 iNKRs provides a functional advantage to NK cells responding to 721 cells only for NK cells

from Bw4$^+$ individuals able to educate their NK cells though both 3DL1 and NKG2A.

Higher total responsiveness of the NKG2A$^+$ than NKG2A$^-$ populations following stimulation with autologous iCD4

Presently, little is known regarding whether iCD4 stimulate NK cell populations differentially and if so, which populations and functional subsets they activate. Autologous iCD4 cell stimulation of NK cells for 24 hrs had no effect on the frequency of the four NKG2A$^{+/-}$3DL1$^{+/-}$ populations compared to stimulation with uninfected CD4 controls (Fig. 4A, $p>0.05$, Wilcoxon). As seen for HLA-null cell stimulation, iCD4 cells induced differential levels of total responsiveness among the four NK cell populations studied ($p=0.0001$, Friedman, adjusted $p=0.0007$). Infected CD4 cells triggered frequencies of functional NKG2A$^+$3DL1$^+$ populations that were significantly higher than that of NKG2A$^-$3DL1$^+$ and NKG2A$^-$3DL1$^-$ populations, but that were not higher than that of NKG2A$^+$3DL1$^-$ NK cells (Fig. 4B, $p=0.03$ and $p=0.004$, respectively). Furthermore, iCD4 cells also induced a higher frequency of single NKG2A$^+$ cells than single 3DL1$^+$ and double iNKR$^-$ NK cells (Fig. 4B, $p=0.005$ and $p=0.001$, respectively). To assess whether the frequency of p24$^+$ cells within the iCD4 cell stimuli correlated differentially with the frequency of functional responses among the four NK cell populations we performed a correlation analysis between these parameters. We observed a near significant correlation between the frequency of p24$^+$iCD4 cells used for stimulation and the total responsiveness of the single NKG2A$^+$ NK population (Fig. 4C, $p=0.056$). The trend observed for the correlation of p24$^+$iCD4 cells and the total responsiveness of the other three NK cell populations was not significant. In summary, unlike HLA-null

stimulation, autologous iCD4 cells induced NKG2A^{+} NK cells more robustly than NKG2A^{-} NK cells, irrespective of 3DL1 co-expression.

iCD4 stimulation induced a higher frequency of functional subsets in the NKG2A^{+} populations, particularly in CD56^{Bright} cells

The emergence of NKG2A^{+} NK cells as mediators of anti-viral responses prompted us to examine separately the contribution of CD56^{Bright} and CD56^{Dim} NK cell compartments to the iCD4 induced responses of the NKG2A$^{+/-}$3DL1$^{+/-}$ populations (using the modified gating strategy shown in Fig. S3A). First, we measured the frequency of each NKG2A$^{+/-}$3DL1$^{+/-}$ populations that were CD56^{Bright} and CD56^{Dim} following a 24hr stimulation with either iCD4 or uninfected CD4 cells. Previous studies reported that CD56^{Bright} NK cells are largely negative for KIRs, including 3DL1 [25, 26]; thus, we excluded 3DL1 from the analysis of the cell surface staining results shown in Figure 5A. We observed a significantly lower percentage of NKG2A^{+} cells in the CD56^{Bright} compartment following stimulation with iCD4 cells compared to uninfected CD4 controls ($p=0.007$, Wilcoxon). No other significant differences were seen for the frequency of the other NK cell populations following stimulation with iCD4 versus uninfected CD4 cells.

Autologous iCD4 cells induced differential frequencies of total, tri-, CD107a^{+}IFN-γ^{+}, total CD107a^{+} and total IFN-γ^{+} functional subsets among the NKG2A$^{+/-}$3DL1$^{+/-}$ populations (Fig. 5B-F, $p=0.0003$, $p=0.001$ and $p<0.0001$, $p=0.0003$ and $p<0.0001$, respectively, Friedman). For the CD56^{Bright} compartment, iCD4 induced higher frequencies of functional NKG2A^{+} than NKG2A^{-} NK cells among the tri, CD107a^{+}IFN-γ^{+} and total

CD107a^{+} functional subsets (Fig. 5C-E, p≤0.02, Wilcoxon). Analysis of the CD56^{Dim} compartment revealed a trend similar to the one observed for the CD56^{+} analysis of total responsiveness (Fig. 4B), where no significant differences were observed in the frequency of iCD4 induced functional cells between the NKG2A^{+} populations that expressed 3DL1 or not, with the exception of CD107a^{+}IFN-γ^{+} (Fig. 5D, p=0.04, Wilcoxon). In addition, CD56^{Dim} NKG2A^{+}3DL1^{-} cells were more responsive to iCD4 cell than double iNKR^{-} and NKG2A^{-}3DL1^{+} populations (Fig. 5C-F, p≤0.04, Wilcoxon). Comparisons of iCD4 induced CD56^{Bright} versus CD56^{Dim} single NKG2A^{+} functional cells revealed that CD56^{Bright} NK cells were consistently more responsive to autologous iCD4 that CD56^{Dim} NK cells (p<0.04 for all comparisons, Wilcoxon). Infected CD4 cells did not induce significant differences in the frequencies of bi-functional CD107a^{+}CCL4^{+}, CCL4^{+}IFN-γ^{+} or total CCL4^{+} responses between the CD56^{Bright} and CD56^{Dim}NKG2A$^{+/-}$3DL1$^{+/-}$ populations (p=0.1, p=0.1 and p=0.5, respectively, unadjusted Friedman, data not shown). Together, these results indicate that NKG2A^{+} NK cell populations, particularly CD56^{Bright}NKG2A^{+}, were activated by iCD4 cells to higher levels than NKG2A^{-} populations and that 3DL1 co-expression did not have an impact on modulating functionality to iCD4 cells.

Infected CD4 cells maintain expression of HLA-E [27], which should inhibit NK cells expressing the iNKR NKG2A. The enhanced activity of NKG2A^{+} cells in response to iCD4 prompted us to verify whether NKG2A was behaving as an iNKR. We addressed this by including blocking antibodies specific for NKG2A or HLA-E or both in the 24hr co-culture of NK cells with iCD4. The gating strategy used to analyze the results of this

experiment is shown in Fig S3A. The presence of blocking antibodies enhanced the total responsiveness of $CD56^{Dim}$ NK cells to iCD4 (Fig. 6). Blocking both NKG2A and HLA-E together resulted in an even higher percentage of functional $CD56^{Dim}NKG2A^{+}$ NK cells than blocking either the receptor or ligand separately (Fig. 6). Similar results were observed for NK cells secreting IFN-γ and CCL4 (Fig. S3B). Blocking the interaction of HLA-E and NKG2A produced a more modest increase in the frequency of NK cells expressing CD107a (Fig. S3C). Overall these results confirm that NKG2A maintains its behavior as an inhibitory NK receptor.

NK cells from $Bw4^{+}$ individuals have higher frequencies of tri- and total $CCL4^{+}$ functional $CD56^{Dim}NKG2A^{+}3DL1^{+}$ cells than *Bw6* homozygotes

Epidemiological studies have shown that certain 3DL1-Bw4 combinations are associated with slow time to AIDS and protection from HIV infection. For example, carriage of the high expression *3DL1* genotype *(*h/*y*) with its ligand *HLA-B*57* (**h/*y+B*57*) has the strongest effect on slow time to AIDS and HIV viral load control compared to Bw6 homozygotes and it carriage is associated with protection from HIV infection [5, 28, 29]. The impact of carriage of **h/*y+B*57* on NK cell education and function has been documented [24, 30, 31]. NK cells from **h/*y+B*57* carriers, compared to those from *Bw6* homozygotes, have a superior ability to inhibit HIV viral replication through mechanisms that involve secretion of CC-chemokines [24]. Furthermore, $3DL1^{+}$, compared to $3DL1^{-}$, NK cells from **h/*y+B*57* carriers secrete more CC-chemokines [24]. We questioned whether comparing iCD4 stimulated NK cells functional profiles from carriers of a *Bw4* allele versus *Bw6* homozygotes would reveal

evidence of 3DL1-Bw4 interactions on anti-viral responses to autologous iCD4 cells. Although the total responsiveness of $CD56^{Dim}3DL1^{+}$ cells following stimulation with iCD4 cells was higher among NK cells from *Bw4* carriers than from *Bw6* homozygotes, differences did not achieve statistical significance (Fig. 7A, p=0.2, Mann-Whitney). Analysis of the individual functional subsets that contributed to the total responsiveness revealed significant differences between Bw4 and Bw6 groups for tri-functional and total $CCL4^{+}$ responses, where $CD56^{Dim}3DL1^{+}$ cells that co-expressed NKG2A from Bw4 individuals were more functional than those cells from Bw6 individuals (Fig. 7B-C, p=0.03 and p=0.02, respectively). Collectively, our data suggest that responses to iCD4 cells of NK cell populations co-expressing 3DL1 and NKG2A were comparable to those of single $CD56^{Dim}NKG2A^{+}$ expressing cells. However, $CD56^{Dim}3DL1^{+}$ NK cells from individuals educated via 3DL1-Bw4 interactions that also co-express NKG2A respond more robustly to iCD4 cells than do these cells from *Bw6* homozygotes by secreting CCL4, either alone or in a tri-functional combination with IFN-γ and CD107a.

3.6 DISCUSSION

In this report we investigated the functional responses of four NK cell populations defined by expression of the iNKRs, NKG2A and/or 3DL1, to stimulation with HLA-null and autologous HIV iCD4 cells. The frequency of HLA-null stimulated functional cells was significantly higher for the $NKG2A^{+}3DL1^{+}$ population compared to the two single $iNKR^{+}$ and double $iNKR^{-}$ populations. This observation was limited to individuals carrying the *3DL1-Bw4* genotype combination. In contrast, iCD4 cell stimulation induced a higher frequency of $NKG2A^{+}$ than $NKG2A^{-}$ NK cells irrespective of whether 3DL1 was co-expressed. The functional subsets that contributed to the higher functionality of iCD4 stimulated $CD56^{Bright/Dim}NKG2A^{+}$ NK cells included the tri-functional, $CD107a^{+}IFN\text{-}\gamma^{+}$, total $CD107a^{+}$ and total $IFN\text{-}\gamma^{+}$ subsets. Higher frequencies of these functional subsets were seen in the $CD56^{Bright}$ than in the $CD56^{Dim}$ compartments. Given that NK cells have been implicated in HIV control [7, 24, 28, 32], these results further elucidate the NK cell populations and anti-viral functions induced by autologous iCD4 cells and that may play a role in HIV pathogenesis.

The term “functional potential” refers to NK cell responsiveness induced by HLA-null cell stimulation. The HLA-null 721 and K562 cell lines are commonly used to activate NK cells educated through the interaction of iNKRs with HLA antigens. Their lack of cell surface HLA abrogates negative signaling through iNKRs, which inhibits NK cell function. NK cell functional potential depends on how potently NK cells were educated, which in turn depends on the iNKRs expressed by an NK cell, the HLA type of the NK cell donor, the number of iNKRs to self ligands an NK cell expresses and the potency of

particular iNKR-HLA ligand combinations [30, 31, 33, 34]. NK cell activation also requires the engagement of aNKRs with their ligands present on HLA null cells. We originally hypothesized that NK cells expressing both iNKRs would demonstrate a greater functional potential than NK cells expressing one or neither of the iNKRs investigated here [23]. The higher response frequency of NKG2A^{+}3DL1^{+} cells to HLA-null cells, than any of the other NKG2A^{-}3DL1$^{+/-}$ populations, is in line with this hypothesis. However, this higher functionality should be limited to NK cells from Bw4^{+} individuals with the ligand for 3DL1 (i.e. with at least two iNKRs to self HLA). Most NKG2A^{+} cells should be educated through this iNKR, since its ligand, HLA-E, is expressed on most human cells [35-37]. In response to stimulation with 721, NKG2A^{+}3DL1^{+} cells from Bw4^{+} individuals (educated via 3DL1) were significantly more functional than double iNKR^{+} cells from Bw6 controls (not educated via 3DL1). Furthermore, these educated cells were significantly more functional than the other 3 NKG2A$^{+/-}$3DL1$^{+/-}$ populations in Bw4^{+} group, which was not observed for the Bw6 group (Fig. 3B-C). Thus, the higher frequency of HLA-null induced NKG2A^{+}3DL1^{+} NK cells compared to the other NK populations studied is limited to NK cells from individuals expressing the HLA ligand for 3DL1. It is likely that the NK cells in these NKG2A$^{+/-}$3DL1$^{+/-}$ populations were also educated through other iNKRs depending on the KIR/HLA genotype of the individual they originated from. However, any influence of education through iNKRs other than NKG2A and 3DL1 is likely to be similar among the NK cell populations defined by NKG2A and 3DL1 expression (i.e. that developed in the same iNKR-self HLA environment).

NK cell functionality can also vary according to the state of NK cell maturation/ differentiation and which stimuli were used to induce function [25, 38]. The expression of CD56, NKG2A and KIRs can distinguish 5 subsets of peripheral blood NK cells that range from least to most differentiated. As the NK cell matures, NKG2A is lost and inhibitory KIRs (iKIRs) are sequentially acquired and can be phenotypically categorized from the least to most differentiated as follows; $CD56^{Bright}NKG2A^{+}KIR^{-}$ < $CD56^{Dim}NKG2A^{+}KIR^{-}$ < $CD56^{Dim}NKG2A^{+}KIR^{+}$ < $CD56^{Dim}NKG2A^{-}KIR^{+}$ and $CD56^{Dim}NKG2A^{-}KIR^{-}$. The latter population is considered to be hyporesponsive due to failure to acquire iKIRs before the loss of NKG2A [25, 39-42]. Consistent with this scheme we found that the $CD56^{+/Dim}NKG2A^{-}3DL1^{-}$ population, which would include hyporesponsive NK cells negative for both iKIRs to self HLA was poorly responsive to HLA-null and iCD4 cells. 721 cells stimulated the highest functionality for most of the functional subsets examined, except degranulation, in the less mature $NKG2A^{+}3DL1^{+}$ (i.e. double $iNKR^{+}$) population, rather than in the more mature $NKG2A^{-}3DL1^{+}$ (i.e. single $iNKR^{+}$) population (Fig. 2). In a previous report Fauriat *et al* show that $CD56^{Bright}$ NK cells were poorly stimulated by HLA-null cells; thus, results for total $CD56^{+}$ cells would be expected to reflect what is occurring in the $CD56^{Dim}$ subset [43]. In an extended analysis of functional results for the 721 stimulation we reached similar findings (not shown). Therefore, for stimulation with HLA-null cells we show functional results for $CD56^{+}$ NK cells, that encompass the functional contributions of both the low responding $CD56^{Bright}$ and high responding $CD56^{Dim}$ subsets (Fig. 2). Others have shown no significant differences in the frequency of cells responding to HLA-null stimuli (CD107a or IFN-γ responses) in the $CD56^{Dim}$ compartment between the more mature $CD57^{+}$

versus the less mature $CD57^-$ subset [25, 38]. This implies that within the $CD56^{Dim}$ compartment differentiation stage does not play a primary role in determining responses to HLA-null cells. On the other hand education via iNKR-self HLA was found to be important in acquisition of functional potential to HLA null cell stimuli as the double $iNKR^+$ NK cells had higher total responses compared to the other 3 $NKG2A^{+/-}3DL1^{+/-}$ populations in individuals who were carriers of a *Bw4* allele compared to Bw6 controls (Fig. 3B-C).

With respect to iCD4 cell stimulation, we also observed that the less mature $CD56^{Bright}NKG2A^+3DL1^-$, $CD56^{Dim}NKG2A^+3DL1^-$ and $CD56^{Dim}NKG2A^+3DL1^+$ populations exhibit stronger functional responses than the more mature $CD56^{Dim}NKG2A^-3DL1^+$ counterparts. The $CD56^{Bright}NKG2A^+3DL1^-$ NK population responded to iCD4 stimulation with the highest frequency of functional cells (Fig. 5). It is notable that we observed a modest but significantly lower frequency of $CD56^{Bright}NKG2A^+$ NK cells following a 24hr co-culture with iCD4 compared to co-culture with uninfected CD4 cells (Fig. 5A). Despite the short co-culture period, the downregulation of NKG2A expression may be related to the potent stimulation of the $CD56^{Bright}NKG2A^+$ population by autologous iCD4. We also observed a modest impact of education via 3DL1-Bw4 on responses to iCD4 cells, since we only measured significantly higher tri-functional and total CCL4 responses for $CD56^{Bright}NKG2A^+3DL1^+$ cells in $Bw4^+$ subjects compared to Bw6 controls (Fig. 7). These results further highlight the impact that education via 3DL1-Bw4 pair has on NK cell functionality in response to HIV infected targets.

Our results suggest that interactions between NKG2A and HLA-E may influence NK cell responses to iCD4 cells. HIV Nef does not downmodulate HLA-E expression on infected cells. On the contrary, its expression is increased in CD4 cells *in vitro* infected with R5- and X4-tropic clinical isolates [44]. Blocking HLA-E or NKG2A with antibodies increases lysis of *in vitro* HIV-infected cells [44-46]. These findings are consistent with our observation that iCD4 induced NK cell functionality is increased by blocking the interaction of HLA-E and NKG2A (Fig. 6 and S3B-C). This suggests that NKG2A is acting as an iNKR but that inhibition is incomplete. The aNKRs NKG2C and NKG2E also bind HLA-E, though with a lower affinity than NKG2A [36, 47]. However, NKG2C and NKG2A are usually expressed on non-overlapping NK cell populations [46], making it unlikely that co-expression of NKG2C accounts for the triggering of the $NKG2A^+$ NK cells by iCD4. Thus, it remains unclear how $NKG2A^+$ NK cells are being activated by iCD4. A possible explanation for this finding may be HIV infection-driven changes in the proteomic environment that $NKG2A^+$ cells detect through interactions with leader peptides expressed by HLA-E. The cell surface expression of HLA-E depends on binding of highly conserved leader peptides, mostly from HLA-A, B, C and G [36, 47]. The downregulation of HLA-A and B by HIV may affect the pool of leader peptides available to bind HLA-E, which could have an impact on interactions between HLA-E and NKG2A [49, 50]. Viruses have also developed other mechanisms to evade immune recognition by encoding peptide homologues to stabilize expression of MHC-I. HIV derived Gag peptide p24 aa14-22 has been shown to stabilize and increase HLA-E surface expression [44]. Further investigations are needed to understand how autologous iCD4 cells are activating $NKG2A^+$ NK cells.

In conclusion, we investigated the responses of four distinct iNKR bearing NK cell populations ($NKG2A^{+/-}3DL1^{+/-}$) to HLA-null and HIV iCD4 cells and characterized the patterns of their functional subsets. To our knowledge, this is the first time a study has examined the impact of these iNKRs on multi-functional responses to HIV-1. Infected CD4 cells stimulated a significantly higher frequency of $NKG2A^{+}$ cells with several functional profiles and in both the $CD56^{Bright}$ and $CD56^{Dim}$compartments and co-expression of 3DL1 did not further modulate the functionality of $NKG2A^{+}$ NK cells. Further studies are required to elucidate the mechanism of $NKG2A^{+}$ NK cell recognition of HIV iCD4 cells and identify other factors that may enhance the potency of these functional responses. It important to understand how these parameters are modulated by NK cells in response to iCD4 cells due to the diverse role that NK cells have in shaping immune responses [2, 51]. Our results suggest that $NKG2A^{+}$ NK cells may have an important role in NK cell responses to HIV infected cells. Clarifying these mechanisms may shed light on correlates of protection and aid in developing strategies that harness NK cells to prevent or control HIV infection.

3.7 FIGURES AND LEGENDS

Table 1. Study population HLA and KIR3DL1 allotypes

	Category	HLA-allotypes						3DL1		Stimuli	
		A		B		C				CL	HIV
1	Bw4	01:01	03:01	14:02	57:01	06:02	08:02	004	015		✓
2	Bw4	01:01	02:01	15:01	57:01	05	06:02	001	001		✓
3	Bw4	02:02	30:02	53:01	57:03	04:01	18	001	001		✓
4	Bw4	02:01	24:02	44:02	51:01	05	08	002	005		✓
5	Bw4	02:01	25:01	18:01	55:01	03:03	12:03	004	005	✓	✓
6	Bw4	01:01	02:01	38:01	57:01	06:02	12:03	004	002	✓	✓
7	Bw4	01:01	26:01	38:01	57:01	06:02	12:03	001	001	✓	✓
8	Bw4	02:01	02:01	07:02	57:01	05	06:02	004	005		✓
9	Bw4	24:02	26:01	15:01	57:01	05	06:02	005	005		✓
10	Bw4	02:01	03:01	07:02	27:05	01:02	07:02	005	005		✓
11	Bw6	02:01	02:01	07:02	08:01	07	07	001	015		✓
12	Bw6	02:01	33:03	15:01	35:08	03:03	04:01	002	005	✓	✓
13	Bw6	03:01	11:01	07:02	35:01	04:01	07:02	001	001	✓	✓
14	Bw6	02:01	03:01	35:01	40:01	03:04	04:01	002	004		✓
15	Bw4	01:01	03:01	44:03	49:01	07:01	16:01	001	002		✓
16	Bw4	29:01	36:01	07:02	53:01	04:01	07:02	015	015	✓	✓
17	Bw6	01:01	11:01	18:01	55:01	03:04	12:03	004	005		✓
18	Bw4	01:01	23:01	14:01	38:05	08:02	12:03	001	008	✓	✓
19	Bw6	02:01	30:02	07:02	35:01	04:01	07:02	002	005	✓	✓
20	Bw4	01:01	31:01	49:01	49:01	07:01	07:01	004	001	✓	✓
21	Bw4	02:01	31:01	27:01	40:01	03:02	15:02	004	008	✓	✓
22	Bw4	01:01	24:02	18:01	35:03	04:01	07:01	001	001	✓	✓
23	Bw6	02:01	03:01	07:02	40:01	03:02	07:10	004	001	✓	
24	Bw6	02:01	01:01	08:01	40:01	03:02	07:01	007	007	✓	
25	Bw6	02:01	11:01	07:02	35:01	04:01	07:02	004	008		✓
26	Bw6	02:01	03:01	07:02	08:01	07:01	16:01	004	015		✓

CL: Cell lines (721, K562)

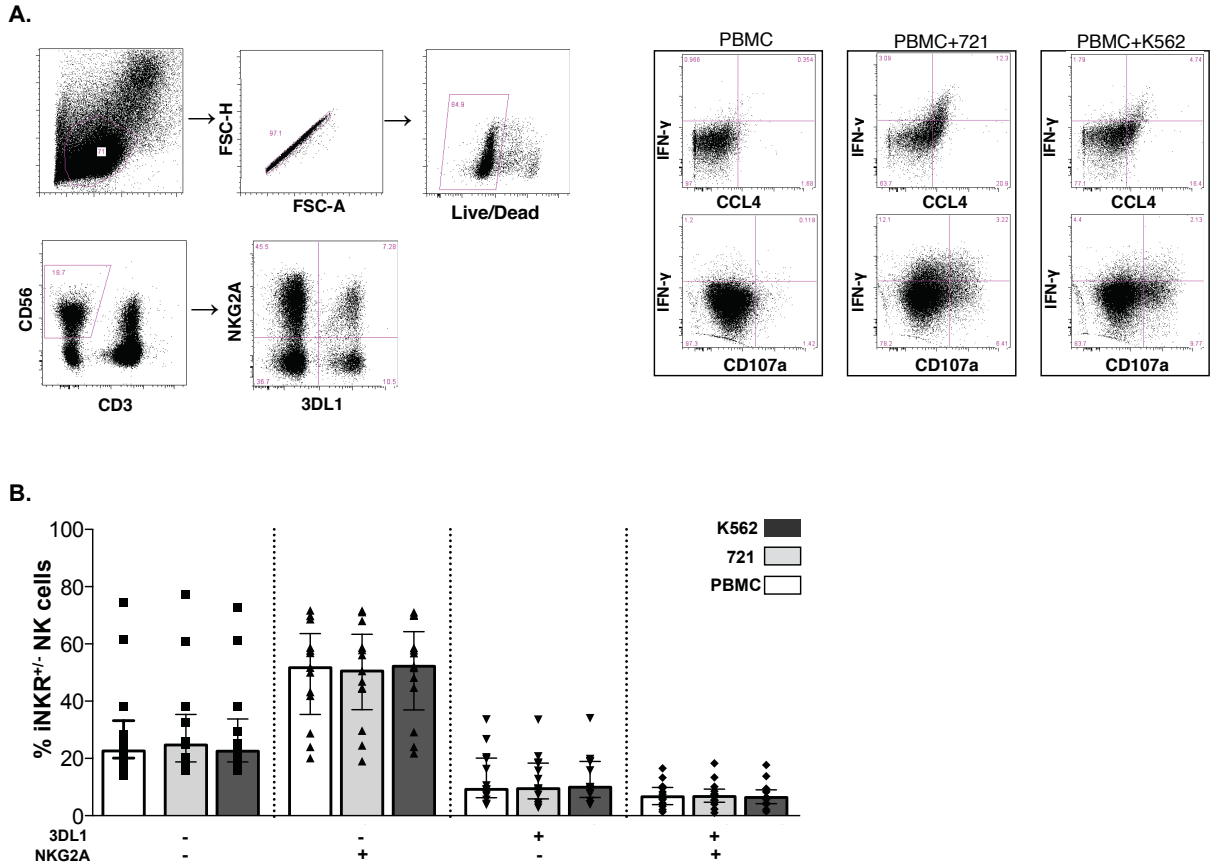


Figure 1. Gating strategy for identification of NK cell subsets and functional responses. (A) The live lymphocytic singlet population was used to gate on NK cells, which were defined as CD3-CD56+. The NKG2A+/-3DL1+/- populations were derived from the NK cell gate. Functional gates for CD107, IFN-γ, and CCL4 were set on gated NK cells in unstimulated PBMCs. (B) The frequency of NKG2A+/-3DL1+/- populations, as defined by surface expression of these two inhibitory NK receptors, following stimulation with HLA-null cells (K562 or 721) compared to background stimulation with PBMC alone (PBMC). Data from 13 individuals tested in duplicate were included in this analysis. Bar height represents the median and error bars the IQR for the each group. A Friedman test was used determine significance between group comparisons (pFriedman > 0.05, data not shown).

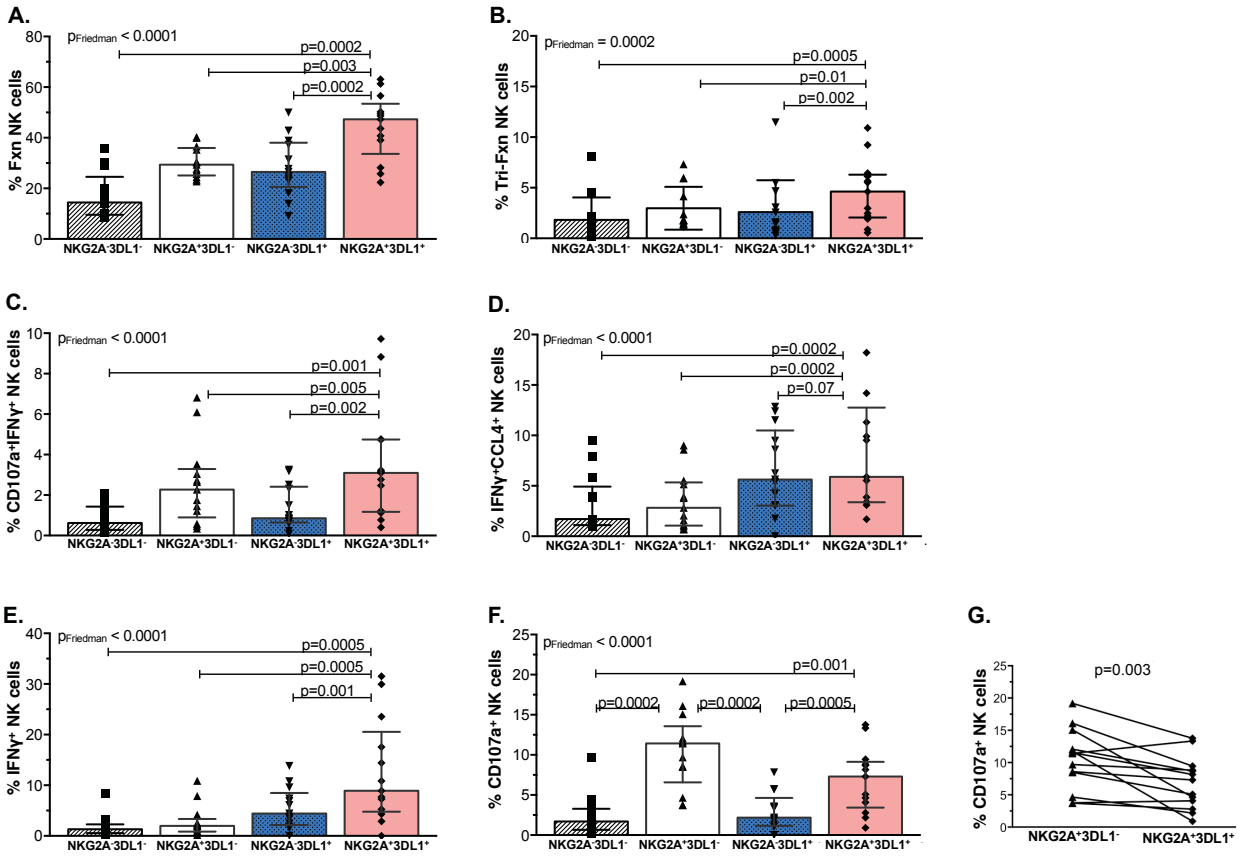


Figure 2. The NKG2A+3DL1+ NK cell population was significantly more responsive to stimulation by HLA-null cell lines in all examined functions except degranulation. Shown on the y-axes are the frequency of the four NKG2A+/-3DL1+/- NK cell populations that responded to 721 HLA-null cell stimulation with responses characterized by total responsiveness (A), tri-functional CD107a+IFN-γ+CCL4+ (B), bi-functional CD107a+IFN-γ+ (C) and IFN-γ+CCL4+ (D), mono-functional IFN-γ+ (E) and CD107a (F, G) functional profiles. Bar height represents the median and error bars the IQR for the dataset. 13 individuals were analyzed in duplicate. Friedman (pFriedman) and Wilcoxon (p) tests were used determine significance between group differences. P-values for between group comparisons are shown over lines linking the two groups being compared. Fxn= functional.

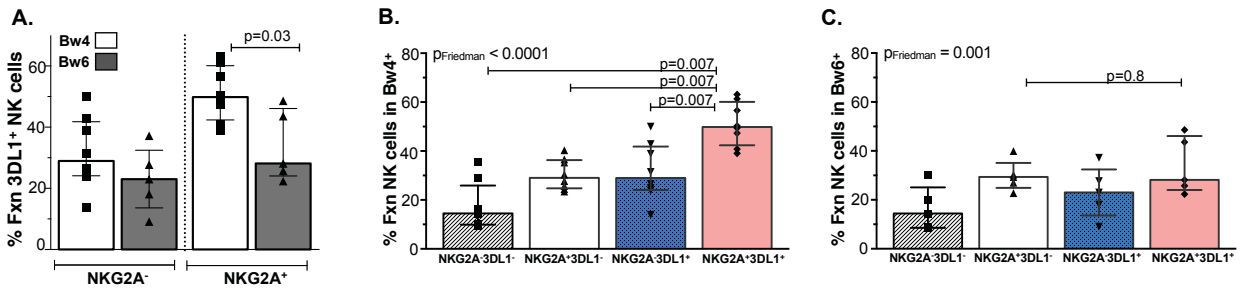


Figure 3: A higher frequency of NKG2A^{+}3DL1^{+} NK cells from carriers of a *Bw4* allele respond to 721 cells than those from Bw6 individuals. The frequency of all functional 721 stimulated NKG2A$^{+/-}$3DL1^{+} NK cells from individuals belonging to the Bw4 and Bw6 groups was plotted on the y-axis (A). Mann-Whitney test was used to determine significance between-group difference. The frequency of all functional 721 stimulated NKG2A$^{+/-}$3DL1$^{+/-}$ populations from Bw4 (B) and Bw6 (C) individuals was plotted on the y-axis. Friedman ($p_{Friedman}$) and Wilcoxon (p) tests were used determine significance between group differences. Bar height represents the median and error bars the IQR for 13 individuals analyzed in duplicate.

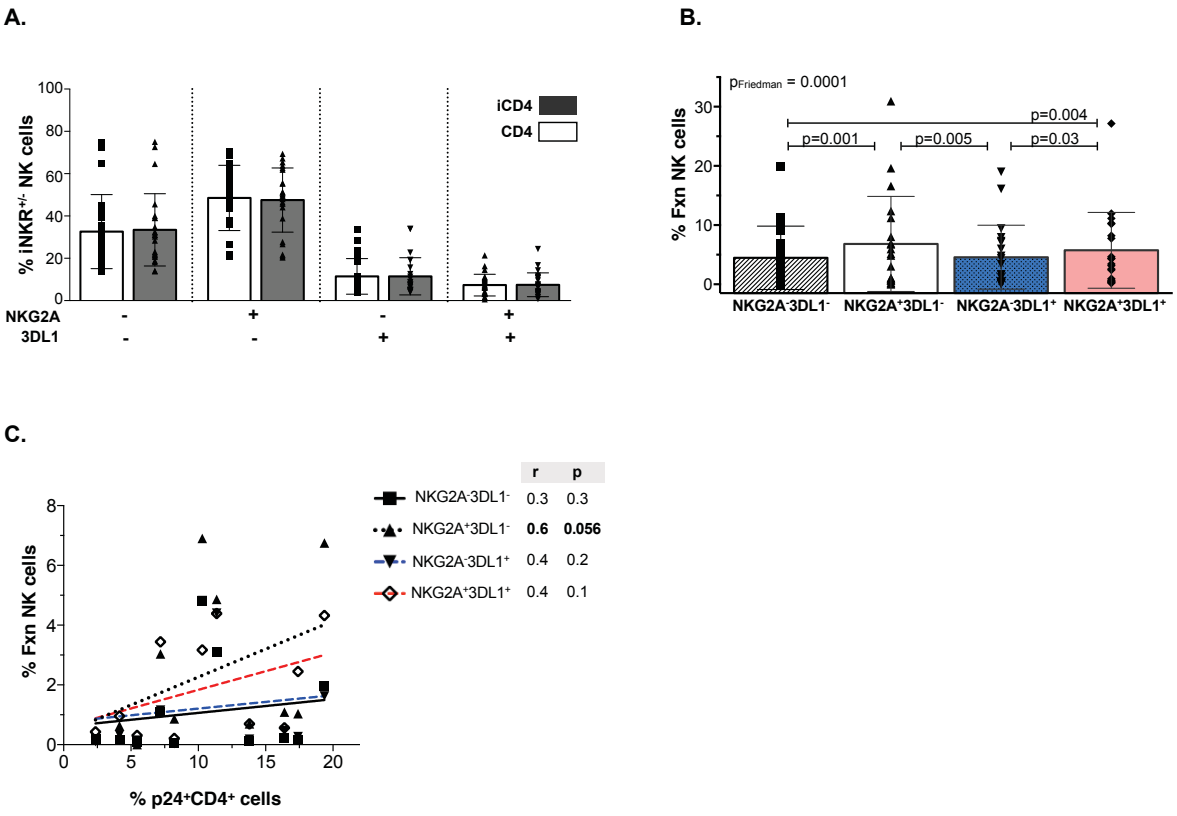


Figure 4: Total responsiveness of NK populations to autologous HIV-iCD4 cells. (A) Frequency of iNKR bearing $CD56^{+}NKG2A^{+/-}3DL1^{+/-}$ populations following stimulation with autologous iCD4 cells and uninfected controls. (B) Frequency of total responsiveness (y-axis) of $NKG2A^{+/-}3DL1^{+/-}$ populations following stimulation with iCD4 cells. Bar height represents the mean+/-SD for the group and Friedman (pFriedman) and Wilcoxon tests (p) were used to determine significance between group differences. Data from 24 individuals analyzed in duplicate were used to generate results. (C) Correlation between percent of $p24^{+}iCD4$ cells used for stimulation (x-axis) and total responsiveness (y-axis) of each $NKG2A^{+/-}3DL1^{+/-}$ population. Data from 12 individuals analyzed in duplicate were used to generate these plots. Spearman's correlation test (r and p) were used to assess the significance of the association between these two measures.

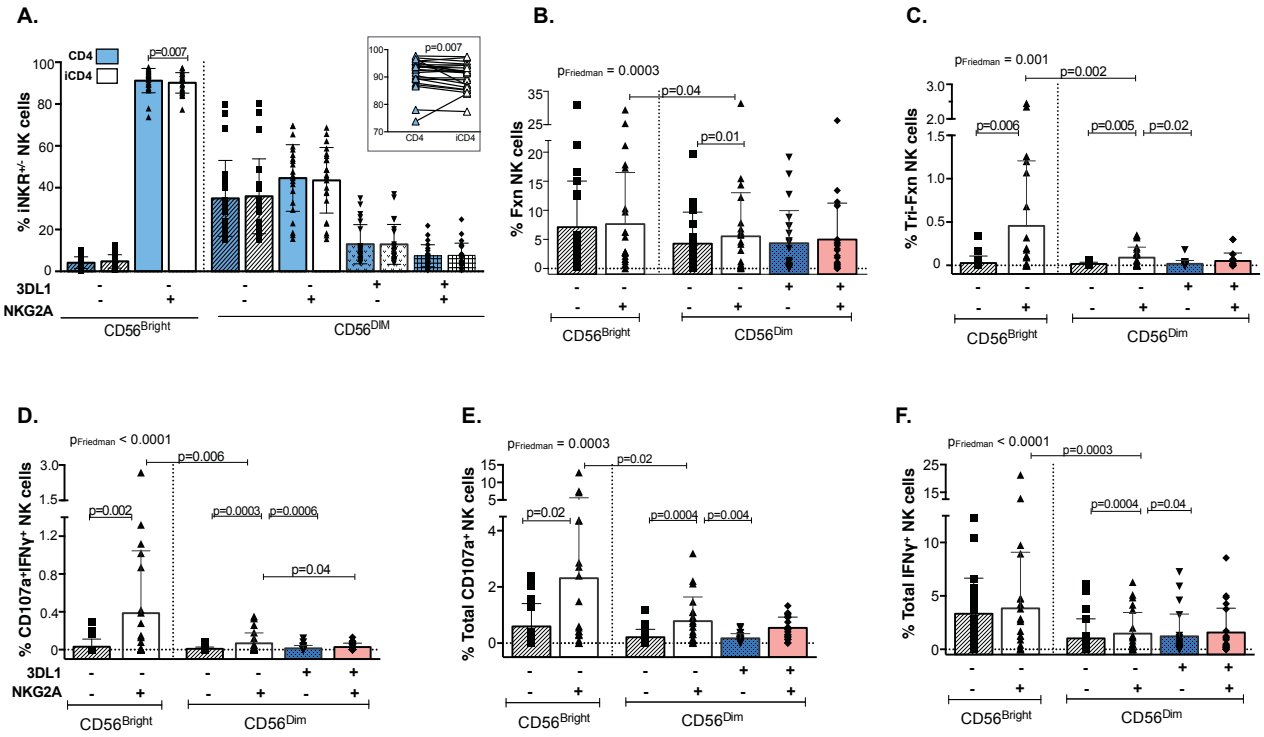


Figure 5. iCD4 stimulation of functional subsets contributing to the total responsiveness of 3DL1$^{+/-}$NKG2A$^{+/-}$ NK cell populations. (A) The frequency of CD56^{Bright} and CD56^{Dim} iNKR bearing NKG2A$^{+/-}$3DL1$^{+/-}$ populations following stimulation with autologous iCD4 and uninfected CD4 controls. Inset at the top right is an expanded view of the frequency CD56^{Bright}NKG2A^{+} population in iCD4 versus uninfected CD4 conditions. The frequency of iCD4 stimulated NK cell populations characterized by total-responsiveness (B), tri-functional (C), bi-functional CD107a^{+}IFN-γ^{+} (D), and total CD107a (E) and total IFN-γ (F) response profiles are shown in the y-axis for each CD56^{Bright}NKG2A$^{+/-}$ and CD56^{Dim}NKG2A$^{+/-}$3DL1$^{+/-}$ population. Bar height represents the mean+/-SD for each group. Data from 24 individuals analyzed in duplicate were used to generate these results. Friedman ($p_{Friedman}$) and Wilcoxon (p) tests were used to determine significance between data sets. P-values for between group comparisons are shown over lines linking the two groups being compared.

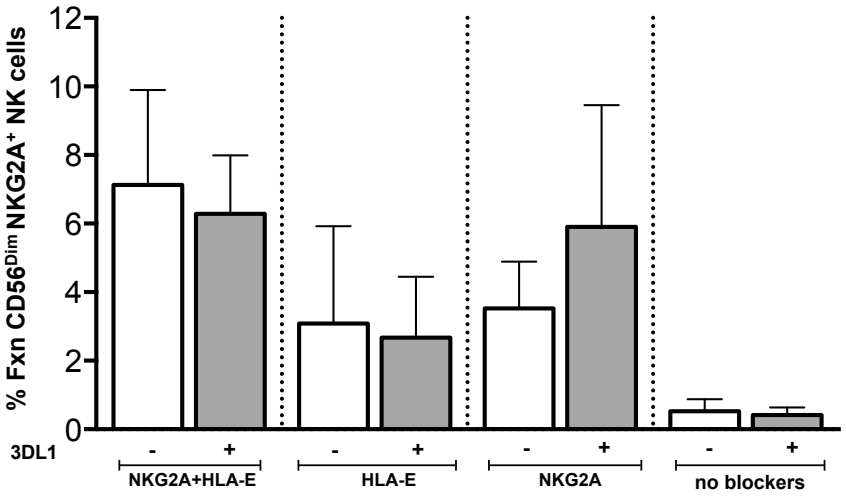


Figure 6: Blocking NKG2A and HLA-E enhances anti-viral functional responses of CD56^{Dim}NKG2A^{+} cells. Purified NK cells were co-cultured with autologous iCD4 or uninfected CD4 controls for 24hrs. Blocking antibodies to NKG2A, HLA-E or both were added to the corresponding co-cultures overnight. These co-cultures were processed as per NK cell stimulation and staining section detailed in the Materials and Methods. We focused on CD56^{Dim}NKG2A^{+} cells and measured total-responsiveness (y-axis). Data for four individuals is shown where bars represent the mean+/-SD.

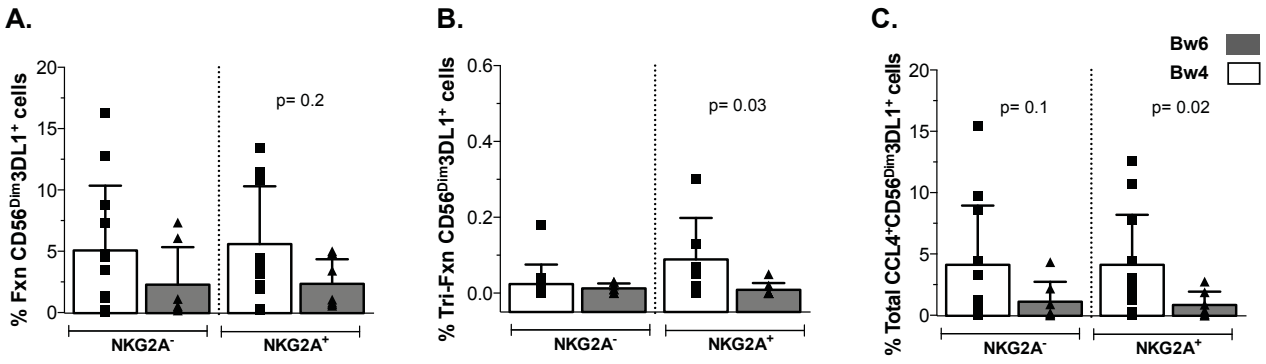
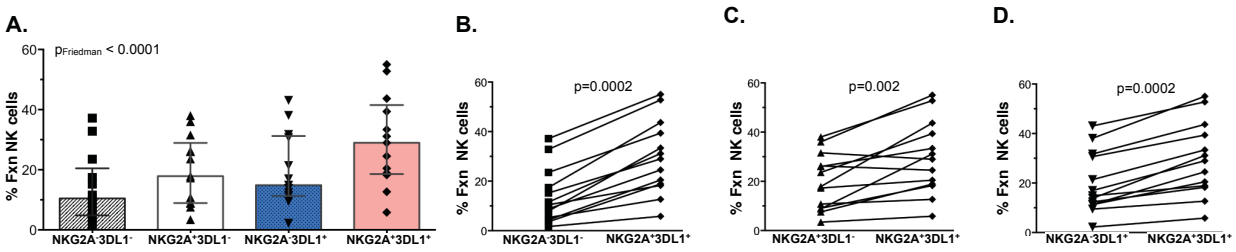
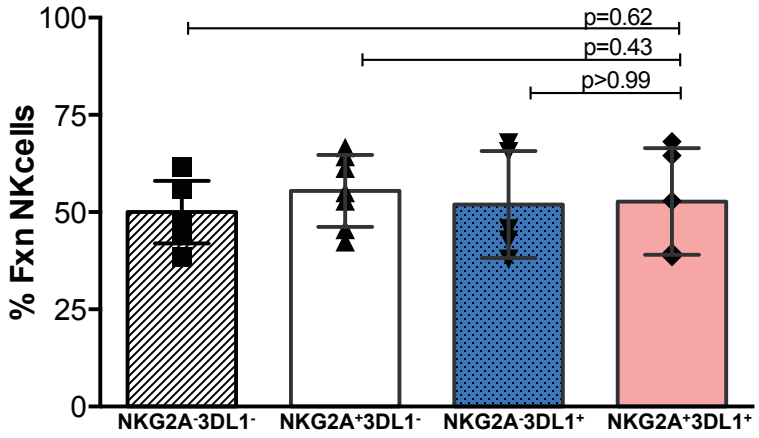


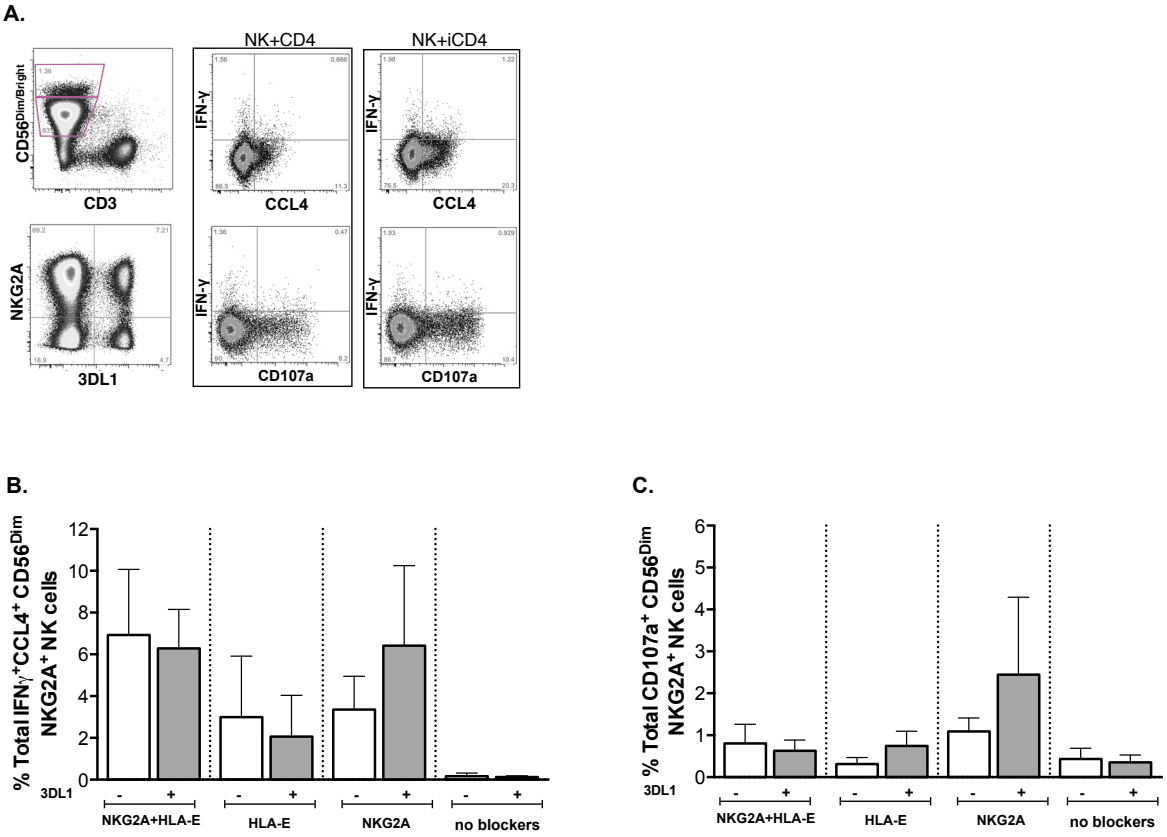
Figure 7. iCD4 stimulated CD56^{Dim}NKG2A^{+}3DL1^{+} cells from Bw4^{+} individuals have greater tri-functional and total CCL4 responses than those from *Bw6* homozygotes. The frequency of iCD4 stimulated CD56^{Dim}NKG2A$^{+/-}$3DL1^{+} NK cells from carriers of a *Bw4* allele versus *Bw6* homozygotes characterized by total responsiveness (A), tri-functional (B) and total CCL4 responses profiles are shown on the y-axis. Bar height represents the mean+/- SD for each group. Data from 24 individuals analyzed in duplicate is plotted. Mann-Whitney test was used to determine significance between group difference.



Supplementary Figure 1: The frequency of NKG2A$^{+/-}$3DL1$^{+/-}$ populations responding to stimulation with K562 HLA-null cells. Shown on the y-axes are the frequency of the four NKG2A$^{+/-}$3DL1$^{+/-}$ NK cell populations that responded to K562 HLA-null cell stimulation with responses characterized by total responsiveness (A), and matched responses of NKG2A^{+}3DL1^{+} cells with NKG2A^{-}3DL1^{-}, NKG2A^{+}3DL1^{-} and NKG2A^{-}3DL1^{+} NK cell populations (B-D). Bar height represents the median and error bars the IQR for the dataset. 13 individuals were analyzed in duplicate. Friedman (pFriedman) and Wilcoxon (p) tests were used determine significance between group differences.



Supplementary Figure 2: The frequency of NKG2A$^{+/-}$3DL1$^{+/-}$ populations responding to stimulation with PMA/Ionomycin. Shown on the y-axes is the frequency of the four NKG2A$^{+/-}$3DL1$^{+/-}$ NK cell populations responding to stimulation with PMA/Ionomycin with total functions measured. PBMCs were stimulated with 1.25 ug/ml of PMA–0.25g/ml ionomycin (both from Sigma Aldrich) for 2.5 hours. Data for seven individuals is shown for the NKG2A^{-}3DL1^{-} and NKG2A^{+}3DL1^{-} populations and for 5 subjects for the NKG2A^{-}3DL1^{+} and NKG2A^{+}3DL1^{+} populations. Bar heights and error bars represent the mean+/-SD.



Supplementary Figure 3: Blocking NKG2A and HLA-E enhances anti-viral functional responses of CD56^{Dim}NKG2A^{+} cells. (A) As in Figure1A, the live singlet lymphocytic population was used to gate on CD3^{-}CD56^{+}, CD56^{Dim} and CD56^{Bright} NK cells. Functional gates for CD107, IFN-γ, and CCL4 were set on NK cells in unstimulated PBMCs, and NK cells cultured with uninfected CD4 (NK+CD4) served as background controls. (B-C) Purified NK cells were co-cultured with iCD4 or uninfected CD4 controls in the presence of blocking antibodies to NKG2A, HLA-E or both (each at 10μg/mL) for 24hrs. Shown in (B) are the frequencies of iCD4 and uninfected CD4 stimulated CD56^{Dim}NKG2A^{+} NK cells positive for total IFN-γ and total CCL4 secretion (IFN-γ^{+}CCL4^{+}) and in (C) positive for total CD107a expression (total CD107a^{+}. Data for four individuals is shown where bars represent the mean+/-SD.

3.8 REFERENCES

1. Trinchieri G. 1989. Biology of natural killer cells. Advances in immunology 47:187-376.
2. Munz C, Steinman RM, Fujii S. 2005. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. The Journal of experimental medicine 202:203-207.
3. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, Kronenberg M, Locksley RM. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. The Journal of experimental medicine 198:1069-1076.
4. Scott-Algara D, Truong LX, Versmisse P, David A, Luong TT, Nguyen NV, Theodorou I, Barre-Sinoussi F, Pancino G. 2003. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. Journal of immunology 171:5663-5667.
5. Boulet S, Kleyman M, Kim JY, Kamya P, Sharafi S, Simic N, Bruneau J, Routy JP, Tsoukas CM, Bernard NF. 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, Sharafi S, Simic N, Bruneau J, Routy JP, Tsoukas CM, Bernard NF. 2008. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. Aids 22:595-599.
7. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ, Buchbinder S, Hoots K, Vlahov D, Trowsdale J, Wilson M, O'Brien SJ, Carrington M. 2002.

Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nature genetics 31:429-434.

8. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, Cheng J, Goedert JJ, Vlahov D, Hilgartner M, Cox S, Little AM, Alexander GJ, Cramp ME, O'Brien SJ, Rosenberg WM, Thomas DL, Carrington M. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 305:872-874.
9. Ahlenstiel G, Martin MP, Gao X, Carrington M, Rehermann B. 2008. Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. The Journal of clinical investigation 118:1017-1026.
10. Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, De Filippi F, Bruno S, Mondelli MU. 2009. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology 137:1151-1160, 1160 e1151-1157.
11. Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, Ghany MG, Hoofnagle JH, Liang TJ, Heller T, Rehermann B. 2010. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner. Gastroenterology 138:325-335 e321-322.
12. Alter G, Heckerman D, Schneidewind A, Fadda L, Kadie CM, Carlson JM, Oniangue-Ndza C, Martin M, Li B, Khakoo SI, Carrington M, Allen TM, Altfeld M. 2011. HIV-1 adaptation to NK-cell-mediated immune pressure. Nature 476:96-100.
13. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, French AR,

Sunwoo JB, Lemieux S, Hansen TH, Yokoyama WM. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 436:709-713.

14. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA, Breso V, Frassati C, Reviron D, Middleton D, Romagne F, Ugolini S, Vivier E. 2006. Human NK cell education by inhibitory receptors for MHC class I. Immunity 25:331-342.
15. Brodin P, Karre K, Hoglund P. 2009. NK cell education: not an on-off switch but a tunable rheostat. Trends in immunology 30:143-149.
16. Joncker NT, Fernandez NC, Treiner E, Vivier E, Raulet DH. 2009. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. Journal of immunology 182:4572-4580.
17. Braud V, Jones EY, McMichael A. 1997. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. European journal of immunology 27:1164-1169.
18. Llano M, Lee N, Navarro F, Garcia P, Albar JP, Geraghty DE, Lopez-Botet M. 1998. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/ NKG2 receptors: preferential response to an HLA-G-derived nonamer. European journal of immunology 28:2854-2863.
19. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. 1994. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. The Journal of experimental medicine 180:1235-1242.
20. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. 1995. The Bw4 public

epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. The Journal of experimental medicine 181:1133-1144.

21. Wan AM, Ennis P, Parham P, Holmes N. 1986. The primary structure of HLA-A32 suggests a region involved in formation of the Bw4/Bw6 epitopes. Journal of immunology 137:3671-3674.

22. Sunwoo JB, Kim S, Yang L, Naik T, Higuchi DA, Rubenstein JL, Yokoyama WM. 2008. Distal-less homeobox transcription factors regulate development and maturation of natural killer cells. Proceedings of the National Academy of Sciences of the United States of America 105:10877-10882.

23. Yu J, Heller G, Chewning J, Kim S, Yokoyama WM, Hsu KC. 2007. 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. Journal of immunology 179:5977-5989.

24. Song R, Lisovsky I, Lebouche B, Routy JP, Bruneau J, Bernard NF. 2014. HIV protective KIR3DL1/S1-HLA-B genotypes influence NK cell-mediated inhibition of HIV replication in autologous CD4 targets. PLoS pathogens 10:e1003867.

25. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, Bjorklund AT, Flodstrom-Tullberg M, Michaelsson J, Rottenberg ME, Guzman CA, Ljunggren HG, Malmberg KJ. 2010. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood 116:3853-3864.

26. Bashirova AA, Thomas R, Carrington M. 2011. HLA/KIR restraint of HIV: surviving

the fittest. Annual review of immunology 29:295-317.

27. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, Baltimore D. 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.

28. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, Pereyra F, Colombo S, Brown EE, Shupert WL, Phair J, Goedert JJ, Buchbinder S, Kirk GD, Telenti A, Connors M, O'Brien SJ, Walker BD, Parham P, Deeks SG, McVicar DW, Carrington M. 2007. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nature genetics 39:733-740.

29. Tallon BJ, Bruneau J, Tsoukas CM, Routy JP, Kiani Z, Tan X, Bernard NF. 2014. Time to seroconversion in HIV-exposed subjects carrying protective versus non protective KIR3DS1/L1 and HLA-B genotypes. PloS one 9:e110480.

30. Boulet S, Song R, Kamya P, Bruneau J, Shoukry NH, Tsoukas CM, Bernard NF. 2010. HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. J. Immunol 184:2057-2064.

31. Kamya P, Boulet S, Tsoukas CM, Routy JP, Thomas R, Cote P, Boulassel MR, Baril JG, Kovacs C, Migueles SA, Connors M, Suscovich TJ, Brander C, Tremblay CL, Bernard N, Canadian Cohort of HIVISP. 2011. Receptor-ligand requirements for increased NK cell polyfunctional potential in slow progressors infected with HIV-1 coexpressing KIR3DL1*h/*y and HLA-B*57. Journal of virology 85:5949-5960.

32. Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A, Streeck H,

Waring M, Meier A, Brander C, Lifson JD, Allen TM, Carrington M, Altfeld M. 2007. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. The Journal of experimental medicine 204:3027-3036.

33. Johansson S, Johansson M, Rosmaraki E, Vahlne G, Mehr R, Salmon-Divon M, Lemonnier F, Karre K, Hoglund P. 2005. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. The Journal of experimental medicine 201:1145-1155.

34. 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.

35. Houlihan JM, Biro PA, Harper HM, Jenkinson HJ, Holmes CH. 1995. The human amnion is a site of MHC class Ib expression: evidence for the expression of HLA-E and HLA-G. Journal of immunology 154:5665-5674.

36. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. The Journal of experimental medicine 187:813-818.

37. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. 2011. Innate or adaptive immunity? The example of natural killer cells. Science 331:44-49.

38. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, Norris PJ, Nixon DF, Lanier LL. 2010. CD57 defines a functionally distinct population of

mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood 116:3865-3874.

39. Ouyang Q, Baerlocher G, Vulto I, Lansdorp PM. 2007. Telomere length in human natural killer cell subsets. Annals of the New York Academy of Sciences 1106:240-252.

40. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, Ratto G, Forte G, Carrega P, Lui G, Conte R, Strowig T, Moretta A, Munz C, Thiel A, Moretta L, Ferlazzo G. 2007. CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. Journal of immunology 178:4947-4955.

41. Beziat V, Descours B, Parizot C, Debre P, Vieillard V. 2010. NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. PloS one 5:e11966.

42. Yu J, Mao HC, Wei M, Hughes T, Zhang J, Park IK, Liu S, McClory S, Marcucci G, Trotta R, Caligiuri MA. 2010. CD94 surface density identifies a functional intermediary between the CD56bright and CD56dim human NK-cell subsets. Blood 115:274-281.

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

44. Nattermann J, Nischalke HD, Hofmeister V, Kupfer B, Ahlenstiel G, Feldmann G, Rockstroh J, Weiss EH, Sauerbruch T, Spengler U. 2005. HIV-1 infection leads to increased HLA-E expression resulting in impaired function of natural killer cells.

Antiviral therapy 10:95-107.

45. Bonaparte MI, Barker E. 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.

46. Ward JP, Bonaparte MI, Barker E. 2004. HLA-C and HLA-E reduce antibody-dependent natural killer cell-mediated cytotoxicity of HIV-infected primary T cell blasts. Aids 18:1769-1779.

47. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 391:795-799.

48. Beziat V, Dalgard O, Asselah T, Halfon P, Bedossa P, Boudifa A, Hervier B, Theodorou I, Martinot M, Debre P, Bjorkstrom NK, Malmberg KJ, Marcellin P, Vieillard V. 2012. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. European journal of immunology 42:447-457.

49. Thananchai H, Gillespie G, Martin MP, Bashirova A, Yawata N, Yawata M, Easterbrook P, McVicar DW, Maenaka K, Parham P, Carrington M, Dong T, Rowland-Jones S. 2007. Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. Journal of immunology 178:33-37.

50. Fadda L, Borhis G, Ahmed P, Cheent K, Pageon SV, Cazaly A, Stathopoulos S, Middleton D, Mulder A, Claas FH, Elliott T, Davis DM, Purbhoo MA, Khakoo SI.

2010. Peptide antagonism as a mechanism for NK cell activation. Proceedings of the National Academy of Sciences of the United States of America 107:10160-10165.

51. Altfeld M, Fadda L, Frleta D, Bhardwaj N. 2011. DCs and NK cells: critical effectors in the immune response to HIV-1. Nature reviews. Immunology 11:176-186.

CHAPTER 4

Differential Effect of Education on NK cells Responding to HIV Infected Autologous CD4 cells

Lisovsky I, Isitman G, Song R, DaFonseca S, Tremblay-McLean A, Lebouché B, Routy JP, Bruneau J and Bernard NF

This chapter was adapted from an article currently in revisions for publication in Clinical Experimental Immunology.

Contribution of authors: LI designed research, conducted experiments, analyzed data, interpreted and prepared the manuscript. IG provided assistance with data interpretation and manuscript preparation. SR and DS provided assistance with conducting experiments. LB, RJP and BJ provided clinical samples used in this study. NFB designed research, supervised the research and prepared the manuscript.

4.1 RATIONALE AND RESEARCH OBJECTIVES

Previously, our group and others showed that KIR/HLA genotype combinations are important in determining NK cell functional potential. In Chapters 2 and 3 we show the importance of the KIR3DL1/Bw4*80I pair on NK cell mediated responses to autologous HIV-infected cells. In addition, in Chapter 3 we ascertained the importance of NKG2A$^+$ NK cells in mediating responses to HIV-infected cells. NK cells can gain function through other iKIRs co-expressed on KIR3DL1$^+$ and NKG2A$^+$ NK cells; such as KIR2DL3, whose HLA-C ligand is not downregulated by HIV. In Chapter 4 our objective was to investigate the impact of KIR2DL3, KIR3DL1 and NKG2A expression on responses to autologous HIV infected cells in NK cell populations defined by these iNKRs. We also analysed the impact of NK cell education via the KIR2DL3 and HLA-C1 combination on KIR2DL3$^+$ NK cells in response to HIV infected cells.

4.2 ABSTRACT

Carriage of certain inhibitory NK cell receptor (iNKR)/HLA ligand pairs is associated with protection from infection and slow time to AIDS implicating NK cells in HIV control. NK cells acquire functional potential through education, which requires the engagement of iNKRs by their HLA ligands. HIV infection downregulates cell surface HLA-A/B, but not HLA-C/E. We investigated how NK cell populations expressing combinations of the iNKRs NKG2A, KIR2DL3 (2DL3) and KIR3DL1 (3DL1) responded to autologous HIV infected CD4 (iCD4) cells. Purified NK cells from HIV-uninfected individuals were stimulated with autologous HIV iCD4 or uninfected CD4 T cells. Using flow cytometry we gated on each of the 8 $NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ populations and analyzed all possible combinations of IFN-γ, CCL4 and CD107a functional subsets responding to iCD4 cells. Infected CD4 cells induced differential frequencies of $NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ populations with total IFN-γ^+, $CCL4^+$ and $CD107a^+$ functional profiles. $2DL3^+NKG2A^+$ NK cells had a higher frequency of responses to iCD4 than other populations studied. A higher frequency of $3DL1^+$ NK cells responded to iCD4 by secreting CCL4 when from individuals positive, versus not, for the HLA-Bw4 ligand for 3DL1. In contrast, a higher frequency of $2DL3^+$ NK cells responded to iCD4 from individuals that were not HLA-C1 homozygotes. These results show that $2DL3^+$ NK cells are mediators of HIV-specific responses. Furthermore, responses of NK cell populations to iCD4 are influenced not only by NK cell education through specific KIR/HLA pairs, but also by differential HIV mediated changes in HLA expression.

4.3 INTRODUCTION

Natural Killer (NK) cells are a subset of lymphocytes that contribute to both innate and adaptive immune responses [1-3]. They are important in anti-viral defenses and may have a role in initial viral control since their activation occurs without prior sensitization and before T cell responses are elicited [4, 5]. The importance of NK cell function in the context of HIV infection is highlighted by the ability of NK cells to inhibit HIV replication and the development of HIV sequence polymorphisms that allow the virus to evade NK cell anti-viral pressure [6-8].

The state of activation of NK cells is determined by an ontogenic process known as education which requires the interaction of inhibitory NK receptors (iNKRs) with their cognate human leukocyte antigen (HLA) ligands on neighboring cells [9]. Education is not an on/off switch as functionality can be tuned by the number of iNKRs engaged, the strength of interactions between iNKRs and their ligands and whether activating NK cell receptors (aNKRs) are also engaged [10, 11]. NK cells lacking iNKRs for self-HLA ligands remain uneducated and hyporesponsive [12]. NKG2A and Killer Immunoglobulin-like Receptor (KIR)3DL1 (hereafter 3DL1) and KIR2DL3 (2DL3), are examples of iNKRs. NKG2A, a C-type lectin receptor, forms a heterodimer with CD94 and interacts with non-classical major histocompatibility complex (MHC) class I HLA-E molecules that present leader peptides from many MHC class I proteins [13]. Both NKG2A and its ligand are highly conserved and their effect on NK cell education is similar from one person to another [14]. 3DL1 interacts with a subset of HLA-A and –B antigens that belong to the Bw4 group [15]. Bw4 antigens differ from the remaining Bw6

variants encoded by the HLA-B locus at amino acids 77-83 of the HLA heavy chain [16]. Bw6 isoforms do not interact with 3DL1 receptors such that $3DL1^+$ NK cells from individuals carrying no *Bw4* alleles are not educated through this receptor. 2DL3 interacts with HLA-C group 1 (C1) variants that have an asparagine at position 80 [17-19]. The remaining HLA-C variants have a lysine at this position and belong to the C2 group, which are ligands for KIR2DL1 (2DL1) receptors on NK cells. 2DL3 can also bind certain allelic variants of C2, though with lower affinity than 2DL1 [20]. Therefore, $2DL3^+$ NK cells from individuals expressing the C1 ligand are educated, while those from carriers of this iNKR without its ligand remain uneducated through this receptor. Furthermore, such cells would be hyporesponsive if they express no other iNKR able to interact with a co-expressed HLA ligand.

Genome-wide association studies (GWAS) revealed that genes influencing HIV viral load set point mapped to the MHC class I region on chromosome 6 [21-24]. While the impact of HLA antigens on HIV control is well known to be mediated through the recognition of HIV epitope MHC class I complexes by $CD8^+$ T cells, these complexes are also recognized by iKIR on NK cells. Epidemiological and functional studies have implicated iNKRs, particularly 3DL1, in combination with certain HLA-B variants in protection from HIV infection and slow disease progression, in those infected [25, 26]. For example, the high expression 3DL1 homozygous genotype **h/*y* co-carried with *HLA-B*57* (**h/*y+B*57*) encodes a receptor ligand combination that has the strongest effect on slow time to AIDS and HIV viral load control compared to Bw6 homozygotes [25]. Its carriage is also associated with protection from HIV infection [26]. NK cells from

**h/*y+B*57* carriers, compared to those from *Bw6* homozygotes, have a superior functional potential and ability to inhibit HIV replication through mechanisms that involve secretion of CC-chemokines [8, 27, 28]. A region upstream of HLA-C, which plays a role in determining HLA-C expression levels was also associated with HIV control in individuals of European Americans origin based on results from GWAS studies [21, 23, 29]. While it is assumed that the mechanism underlying this association is related to the potency of $CD8^+$ T cell recognition of HLA-C-HIV peptide complexes, whether NK cells also play a role has not been excluded [23].

HIV infected CD4 (iCD4) cells downmodulate HLA-A and B, but not HLA-C and –E [30, 31]. Therefore, iCD4 may interact with NK cell subsets expressing and educated through 2DL3 differently from those expressing and educated through 3DL1 and/or NKG2A. Here, using a standardized and validated multi-parametric flow cytometry panel, we examined the functional profiles of the eight possible $NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ NK cell populations responding to autologous iCD4 cells. We also investigated how KIR/HLA educating combinations of 2DL3-C1 and 3DL1-Bw4 impacted NK cell functional responses to autologous iCD4 cells. Collectively, our results show that NK cell education is a process that is influenced not only by a subject's KIR/HLA combinations, but also by environmental changes to HLA surface expression driven by HIV infection.

4.4 MATERIALS AND METHODS

Ethics statement and Study population

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. It was approved by the Institutional Review Boards of the Comité d'Éthique du Centre de Recheche du Centre Hospitalier de l'Université de Montréal and the Research Ethics Committee of the McGill University Health Centre - Montreal General Hospital. All individuals provided written informed consent for the collection of samples and subsequent analysis.

We studied 20 HIV-1–uninfected 3DL1 homozygous donors. Results on the stimulation of NK cell populations positive for NKG2A and 3DL1 with autologous iCD4 using cells from these donors were previously reported [32]. All were positive for the KIR*2DL1* (*2DL1*) locus. The HLA type, *3DL1* allotype and *KIR2DL2/2DL3* (*2DL2/2DL3*) locus generic genotype of each of the study participants is shown in Table I.

Genotyping

MHC class-I alleles were typed by sequencing using commercial reagents (Atria Genetics Inc., South San Francisco, CA). Genotyping and allotyping of *3DL1* was performed as previously described [26, 33]. Presence of a *2DL2/2DL3* locus and alleles belonging to the *2DL2* and *2DL3* allele groups was determined by KIR region typing (One Lambda) and verified by PCR using specific primers and conditions described by Kulkarni et al. [34].

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated as previously described [8]. CD4 and NK cells were isolated from PBMCs by positive and negative selection kits, respectively (STEMCELL Technologies, Inc., Vancouver, BC). The purity of isolated CD4 and NK cells was verified by flow cytometry and averaged 95.3% and 97.2%, respectively.

Viral infection

Autologous CD4 cells were infected *in vitro* with HIV as previously described [8]. In brief, purified CD4 cells were stimulated overnight with 1 μg/ml phytohemagglutinin (PHA-P; MP Biomedicals, Santa Ana, CA) and 100 IU/ml of recombinant human IL-2 (rhIL-2 Chiron Corp., Emeryville, CA) in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin and 50 mg/ml streptomycin (R10) (all from Wisent). On day 4, CD4 cells were infected at an MOI of 0.01 with HIV-$1_{JR\text{-}CSF}$ for 4hrs and cultured for 7 days in R10 with 100 IU rhIL-2 (R10-100). Uninfected CD4 (CD4) cells were cultured in parallel to iCD4 for 7 days in R10-100 media. On day 7, iCD4 and CD4 cells were used to stimulate NK cells.

Day 7 iCD4 were used to measure HLA-A and –E surface levels. Infected (HIV $p24^+$) and uninfected CD4 cell were stained for viability with the LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen) and with the surface antibody mix: CD3-BV785 (OKT3, BioLegend), CD4-BUV737 (SK3, BD BioSciences) and either HLA-A2-APC (BB7.2, eBioscience) or HLA-E-PECy7 (3D12, BioLegend). After fixation and permeabilization

(FIX & PERM® kit, Invitrogen) intracellular HIV Gag p24 positive cells were detected with anti-p24-FITC (K57, Beckman-Coulter). Between 400,000 and 500,000 events were acquired per sample on an LSRFortessa X20 flow cytometer (BD Biosciences). We measured and analyzed the mean fluorescence intensity (MFI) of expression of HLA-A2 and -E and percentage of cells positive for these antigens in $p24^+$ HIV-infected CD4 cultures (HIV^+) compared to matched uninfected controls (HIV^-).

NK cell stimulation and staining

NK cells were stimulated by iCD4 cells as previously described [8]. Briefly, $1x10^6$ NK cells were co-cultured with iCD4 or CD4 cells at a ratio of 10:1 in R10-100 for 24 hrs. Brefeldin A (6 ug/ml; Sigma Aldrich, St. Louis, MO) and Golgi Stop (5 ug/ml; BD Bioscience [BD], San Jose, CA) were added 5hrs before the end of the culture period. Cells were stained for viability using LIVE/DEAD® Cell Stain Kit (Invitrogen) and surface markers with the following antibodies: CD107a-PE-CF594 (H4A4; BD), CD3-BV785 (OKT3), CD56-BV711 (NCAM), 3DL1-BV421 (DX9; all from BioLegend, San Diego, CA), 2DL3-PE (180701; R&D Systems, Minneapolis, MN) and NKG2A-PECy7 (Z199; Beckman Coulter, Mississauga, ON). Samples were washed, fixed, permeabilized and stained for intracellular cytokines/chemokines using CCL4-FITC (24006; R&D) and IFNγ-Alexa700 (B27; BioLegend).

F*low cytometry analysis*

Between $4x10^5$ and $1x10^6$ total events were acquired for each sample using a calibrated LSRFortessa flow cytometer (BD). Single stained control beads (CompBead; BD) were

used in every experiment to calculate compensation. Boolean gating was used to identify the frequency of NK cells with each of the 7 possible functional profiles: tri-functional ($CD107a^{+}IFN\text{-}\gamma^{+}CCL4^{+}$), bi-functional (any combination of two functions) and mono-functional. Total responsiveness was defined as the sum of the frequencies of tri-, bi- and mono-functional NK cells. Total CD107a, total IFN-γ, and total CCL4 were defined as the sum of all functional subsets that included these functions. Analysis was performed using FlowJo software V9.8 (TreeStar, Ashland, OR).

Statistical analysis

Analysis was performed using GraphPad Prism6 (La Jolla, CA). Friedman and Kruskal-Wallis tests with Dunn's post tests were used to assess the significance of differences between the eight $NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ NK cell populations. Where multiple comparisons were performed Bonferonni corrections were applied. A Wilcoxon test was used to assess the significance of comparisons for 2 matched data sets. Spearman's tests were used to test the significance of correlation analyses. P-values < 0.05 were considered significant.

4.5 RESULTS

Among $CD56^{Dim}$ NK cells, the populations most responsive to autologous iCD4 cells were $NKG2A^{+}2DL3$

The gating strategy used to detect NK cell populations expressing various combinations of NKG2A, 2DL3 and 3DL1 is shown in Figure S1A. Figure S1B shows the gating strategy for analyzing the functional responses of these NK cell populations using Boolean gating. $CD3^{-}CD56^{+}$ NK cells include the less mature $CD56^{Bright}$ and more differentiated $CD56^{Dim}$ populations [35-37]. $CD56^{bright}$ NK cells express low levels of iKIRs and therefore for the analyses presented here we gated on the $CD56^{Dim}$ compartment [28, 38]. iCD4 cells induced differential frequencies of $NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ NK cell populations characterized by total responsiveness, total IFN-γ and CCL4 secretion, and total CD107a expression (Fig. 1A-D, p≤0.004 for all, Friedman tests, adjusted p-value≤0.02). iCD4 also induced differential frequencies of $NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ NK cell populations characterized by tri-, and bi-functional $CD107a^{+}IFN\text{-}\gamma^{+}$ and $IFN\text{-}\gamma^{+}CCL4^{+}$ subsets (Fig. S2 A-C, adjusted p-value≤0.01 for all). Between-population comparisons revealed that iCD4 stimulated a higher frequency of $NKG2A^{+}2DL3^{+}3DL1^{+/-}$ compared to $NKG2A^{-}2DL3^{+}$ cells. This was the case for the NK cell populations characterized by total responsiveness, total $IFN\text{-}\gamma^{+}$ and $CCL4^{+}$ secretion, and total $CD107a^{+}$ expression (Fig. 1A-D). The differences were statistically significant for all comparisons (p≤0.03), except for those between $NKG2A^{+}$ and $NKG2A^{-}$ $2DL3^{+}3DL1^{+}$ cells secreting total CCL4, and $NKG2A^{+}$ and $NKG2A^{-}$ $2DL3^{+}3DL1^{-}$ cells expressing total CD107a (Fig. 1C and D).

Within the NKG2A$^+$ populations, iCD4 stimulated a higher frequency of 2DL3$^+$3DL1$^+$ than 2DL3$^-$3DL1$^+$ cells with the following functional profiles: total responsiveness, total IFN-γ and CCL4 secretion and total CD107a$^+$ expression (Fig. 1A-D, p≤0.01 for all). In addition, iCD4 induced a higher frequency of 2DL3$^+$3DL1$^-$ than 2DL3$^-$3DL1$^+$ cells characterized by total IFN-γ secretion (Fig. 1B, p=0.001). Within the NKG2A$^-$ populations, those co-expressing 2DL3 and 3DL1 did not respond to iCD4 with a higher frequency of functional cells than did those that were single positive for 2DL3 or 3DL1 or double negative for these iNKR. The only comparison that achieved statistical significance was that for total IFN-γ secretion, where the NKG2A$^-$2DL3$^-$3DL1$^+$ population was more responsive to iCD4 than the triple-iNKR$^-$ NK cells (Fig. 1B, p=0.04). Together, these results highlight the importance of NKG2A for NK cell responsiveness to autologous HIV iCD4. The higher proportion of NKG2A$^+$2DL3$^+$ than NKG2A$^+$2DL3$^-$ populations responding to iCD4 suggests that 2DL3$^+$ cells also contribute to responses to HIV iCD4 stimuli. On the other hand, NK cells expressing 3DL1, without NKG2A or 2DL3, do not respond any better to iCD4 than NK cell populations negative for these 3 iNKR. In general, expression of 3DL1 did not significantly modulate the frequency of NKG2A$^+$2DL3$^+$ NK cells able to respond to iCD4 when the presence/ absence of co-carried ligands for 3DL1 were not considered.

Autologous HIV iCD4 cells from HLA-C1 carriers inhibit the responsiveness of educated 2DL3$^+$ NK cells

HIV iCD4 cells retain cell surface HLA-C, thereby permitting interactions between HLA-C1 (on iCD4s) and 2DL3$^+$ NKs from individuals carrying alleles encoding this KIR/HLA

pair. To test whether iCD4 cells from carriers of an *HLA-C1* allele inhibited 2DL3^{+} NK cells responsiveness, we stratified the study participants based on homo- and heretozygosity for *HLA-C1* and –*C2* and compared the frequency of educated 2DL3^{+} NK cells (i.e. originating from carriers of a *C1* allele) and uneducated 2DL3^{+} cells (i.e. from *C2* homozygotes) that responded to autologous iCD4. The total responsiveness of CD56^{Dim}2DL3^{+} cells that co-expressed all possible combinations of NKG2A^{-}3DL1$^{+/-}$ to iCD4 was lower in NK cells from *C1* than *C2* homozygotes (Fig. 2A, p≤0.02 for all comparisons, Kruskal-Wallis with Dunn's post tests). Analysis of the individual functional subsets that contributed to total responsiveness revealed significant differences only for the total CCL4^{+} functional response (Fig. 2B), and not in the other functional subsets (data not shown). A lower frequency of CD56^{Dim}2DL3^{+} that were NKG2A^{-}3DL1 and NKG2A^{-}3DL1^{+} from *C1* than *C2* homozygotes responded to iCD4 by secreting CCL4 (Fig. 2B, p=0.07 and p=0.03, respectively). No significant differences in responses to iCD4 were measured between CD56^{Dim}2DL3^{+} NK cells from *C2* homozygotes and *C1/C2* heterozygotes (Fig. 2, p>0.05 for all, Kruskal-Wallis with Dunn's post tests). CD56^{Dim}2DL3^{+} NK cells from *C1/C2* heterozygotes had greater responses to iCD4 cells than *C1* homozygotes in a number of NK sub-populations, though differences did not reach significance.

Collectively, these results show that the function of educated CD56^{Dim}2DL3^{+} NK cells from *C1* carriers was inhibited by 2DL3 interacting with HLA-C1 on iCD4. On the other hand, CD56^{Dim}2DL3^{+} NK cells from *C2* homozygotes were not educated by interactions between this receptor ligand pair and not inhibited by the presence of HLA-C2 on their

autologous iCD4. Furthermore, iCD4 from *C1/C2* heterozygotes compared to *C1* homozygotes, showed a trend towards being less potent at inhibiting CCL4 secretion in $CD56^{dim}2DL3^{+}$ NK cells.

$CD56^{Dim}3DL1^{+}$ NK cells from *Bw4/Bw6* heterozygotes exhibit greater responsiveness to iCD4 cells compared to *Bw6* homozygotes

The downregulation of HLA-A and B by HIV Nef reduces cell surface levels of HLA-Bw4, the ligand for 3DL1 on iCD4 [15, 30, 31]. To assess changes in cell surface expression driven by HIV Nef we measured the frequency and MFI of HLA-A*02, as an example of an MHC-I antigen downmodulated by HIV Nef, and HLA-E as an example of an MHC-I antigen not downmodulated on HIV infected cells. We studied eight carriers of one of the most frequent alleles in Caucasians, HLA-A*02 [31]; six donors were tested for HLA-E levels. We excluded subjects who carried HLA-B and –C alleles that cross-react strongly with the 3D12 HLA-E specific antibody used to detect HLA-E levels [39]. The MFI of HLA-A2 measured on HIV iCD4 cells was 16928 [11634, 20363] (median, range) and was significantly lower than the levels on uninfected CD4 cells, 36386 [27279, 41188] (Fig. S3A, p=0.004). These values correspond to a downregulation of 46.5% in HLA-A2 surface levels. In contrast, the MFI of HLA-E detected by 3D12 was 770 [637, 939] and 770 [681, 1187] on iCD4 and uninfected CD4 cells, respectively (Fig. S3B, p=0.15). The percentage of $HLA\text{-}A2^{+}$ uninfected CD4 T cells was also significantly higher than that of $p24^{+}$ CD4 T cells, while the frequency of $HLA\text{-}E^{+}$ cells in infected and uninfected CD4 T cells did not differ significantly (not shown). These results confirm that in our *in vitro* cultures HIV downmodulates HLA-A but not HLA-E on CD4 cells.

We next questioned whether iCD4 from *Bw4* homozygotes (Bw4) versus those who were Bw4/Bw6 heterozygotes or *Bw6* homozygotes, with no *Bw4* alleles at the HLA-A or B loci (Bw6), stimulated $CD56^{Dim}3DL1^{+}$ cells differentially. The frequency of $CD56^{Dim}3DL1^{+}$ cells exhibiting total responsiveness and total CCL4 secretion to autologous iCD4 stimulation was higher when NK cells originated from *Bw4/Bw6* heterozygotes than from *Bw4* or *Bw6* homozygotes, though these differences did not achieve statistical significance ($p>0.05$, Kruskal-Wallis tests) (Fig. 3). In an exploratory sub-analysis and in order to determine whether between group difference were close to the threshold of significance we compared the difference between *Bw4/Bw6* heterozygotes and *Bw6* homozygotes using Mann-Whitney tests. When this statistical test was applied differences in the total responsiveness of the $CD56^{Dim}3DL1^{+}$ populations that were $NKG2A^{-}2DL3^{+}$ and $NKG2A^{+}2DL3^{-}$ significant ($p\leq0.03$). Furthermore, in these two NK sub-populations iCD4 from *Bw4/Bw6* heterozygotes stimulated a higher frequency of responsive $CD56^{Dim}3DL1^{+}$ NK cells than did those from *Bw4* homozygotes ($p=0.05$, for both, Mann-Whitney). Analysis of the individual functional subsets that contributed to total responsiveness also revealed significant differences for the total $CCL4^{+}$ functional response, where $CD56^{Dim}3DL1^{+}$ NK cells from *Bw4/Bw6* heterozygotes were more responsive to iCD4 stimulation than NK cells from *Bw6* homozygotes (Fig. 3B, $p\leq0.03$, Mann-Whitney). Although responses characterized by the functional subsets other than total CCL4 secretion, that contributed to total responsiveness, were also analyzed, no significant between NK population differences were observed in the frequency of NK cells responding to autologous iCD4 (not shown).

There were no significant differences measured in total responsiveness or CCL4 secretion between *Bw4* and *Bw6* homozygotes (Fig. 3, $p>0.05$ for all, Kruskal-Wallis). We also examined the correlation between the frequency of $p24^{+}$ iCD4 used to stimulate NK cells and total responsiveness of $iKIR^{+/-}$ NK populations (Fig. S4). Examination of single $KIR^{+/-}$ populations stratified based on expression of NKG2A (Fig. S4A-C) or single $KIR^{+}NKG2A^{+}$ populations stratified by presence or absence of the cognate HLA ligand (Fig. S4D and E) revealed no significant trends between these measures. In summary, these results showed that $CD56^{Dim}3DL1^{+}$ NK cells from *Bw4/Bw6* heterozygotes, that also co-express either NKG2A and/or 2DL3, showed a non significant trends towards responding more robustly to iCD4 cells by secreting CCL4 than those from *Bw6* homozygotes, expressing no ligands for 3DL1. In some cases $CD56^{Dim}3DL1^{+}$ NK cells from *Bw4/Bw6* heterozygotes that co-express either NKG2A and/or 2DL3, also trended towards responding more robustly to iCD4 cells by secreting CCL4 than *Bw4* homozygotes with educated $3DL1^{+}$ NK cells.

4.6 DISCUSSION

In this study we examined the functional profiles elicited by autologous HIV iCD4 stimulation in the eight NK cell populations defined by all permutations of NKG2A, 2DL3 and 3DL1 expression. We found that a higher frequency of NKG2A$^+$ than their NKG2A$^-$ NK cell counterparts responded to this stimulus. Within the NKG2A$^+$ populations, those co-expressing 2DL3 were more responsive to iCD4 than their 2DL3$^-$ counterparts. Expression of 3DL1 did not significantly modulate the frequency of iCD4 responsive NKG2A$^+$2DL3$^{+/-}$ NK cells when all subjects were considered together. When these results were categorized into groups based on whether NK cells originated from individuals carrying or not iKIR/HLA educating pairs, we found that 2DL3$^+$ NK cells from *C2* carriers had higher responses to autologous iCD4 than educated 2DL3$^+$ NK cells from *C1* homozygotes, while 3DL1$^+$ cells from carriers of a *Bw4* allele, particularly *Bw4/ Bw6* heterozygotes, were more responsive to iCD4 than uneducated 3DL1$^+$ NK cells from Bw6 individuals.

When results from all study subjects were considered, iNKR bearing 2DL3$^+$ and NKG2A$^+$ NK cells were more responsive to iCD4 cell stimulation than their respective iNKR$^-$ counterparts. This was unexpected as HLA-C and -E levels are maintained on iCD4 cells and should transmit inhibitory signals through these iNKR engaging their receptors. We previously showed that blocking the interactions between NKG2A and HLA-E increased the frequency of functional NKG2A$^+$ NK cells responding to iCD4 [32]. In addition, blocking interactions between 2DL3 and HLA-C enhanced responses of 2DL3$^+$ NK cells to iCD4 targets [40]. Though it remains unclear how HIV iCD4 activate

some NKG2A^{+} and 2DL3^{+} NK cells better than their iNKR^{-} counterparts, these findings confirm the functionality of these iNKRs as inhibitory receptors and may also have the potential of contributing to protective responses to HIV. This should be investigated in future studies.

NK cell functional potential depends on how potently NK cells are educated, which in turn depends on which iNKRs they express, which HLA allele they co-carry, the number of iNKRs to self HLA ligands an NK cell expresses and the potency of particular iNKR/ HLA ligand combinations [11, 20, 27, 28]. NK cell activation can occur when inhibitory signals through iNKRs are interrupted due to loss, or reduced levels of ligand for iNKRs on target cells, or if signaling through aNKRs overcomes negative signals originating through iNKR engagement [11, 14, 41, 42]. In this study we examined the consequences of interactions between certain iNKRs and their ligands on NK cell responses to autologous iCD4 cells. HIV infection selectively downregulates cell surface HLA-A/B, but not HLA-C/E, on *in vitro* HIV iCD4 cells [30, 31]. Cohen *et al.* measured this using an HIV infection model of a cell line that was engineered to express a single HLA antigen. It is difficult to evaluate surface HLA levels in healthy and HIV infected primary cells, due to the co-dominant expression of up to 6 HLA antigens and the lack of specific antibodies for HLA variants encoded by a single locus that do not cross-react with molecules encoded by other HLA loci. Recent work by Apps *et al*., measured the levels of specific allelic variants of HLA-A*02, -B*44 and -C*05 on *ex vivo* uninfected cells and *in vitro* HIV infected primary cells using non-commercial antibodies. Surface levels of HLA-A and HLA-B were 15 and 18 times higher than HLA-C levels on *ex vivo*

uninfected $CD3^+$ cells by flow cytometry; HIV Nef downregulated both HLA-A and B relative to HLA-C, with HLA-A being reduced marginally more than HLA-B [31]. Since our investigation of HLA-A*02 levels on HIV infected $CD3^+$ cells found that this antigen was reduced to a median of 46% of its level on uninfected cells we expect that HLA-B variants will be reduced by approximately this levels on iCD4 cells as well. (Fig. S3).

NK cells expressing the 3DL1 receptor are educated in Bw4 donors, while those from Bw6 individuals remain uneducated through this iNKR. We measured a non significant trend towards higher frequencies of iCD4 triggered $3DL1^+$ NK cells from *Bw4/Bw6* heterozygotes than *Bw6* homozygous donors, whose $3DL1^+$ NK cells are not educated through this receptor. Thus, the frequency of functional $3DL1^+$ NK cells from *Bw6* homozygotes would represent a background level of activation dependent on education though other iNKR and loss of ligands for these iNKR on autologous iCD4. We also observed a higher frequency of iCD4 triggered $3DL1^+$ NK cells *Bw4/Bw6* heterozygotes than *Bw4* homozygous donors that trended towards statistical significance for some of the $3DL1^+$ populations. This observation may reflect an HLA dosage effect and the level to which HIV Nef downmodulates HLA-A and -B. We measured a decrease in HLA-A*02 levels following *in vitro* HIV infection and would expect a similar reduction in the surface levels of HLA-B on iCD4 cells [30, 31]. HLA antigens are co-dominantly expressed on the cell surface. *Bw4* homozygotes would be expected to express twice the number of cell surface HLA-Bw4 antigens compared to *Bw4/Bw6* heterozygotes. Following HIV Nef mediated down-modulation it is expected that Bw4 levels on cells from *Bw4/Bw6* heterozygotes would be lower than those of *Bw4* homozygotes. In a murine system

Brodin *et al.,* provided evidence that MHC class I ligands for iNKR need to fall below a threshold of 20% of maximal expression to interrupt negative iNKR signaling [41]. Thus, the higher frequency of iCD4 induced functional NK cells in *Bw4/Bw6* heterozygotes may be due to the level of Bw4 antigens being reduced sufficiently to abrogate the inhibition of NK cell activity through 3DL1 in educated $3DL1^+$ NK cells. On the other hand, even if HIV Nef down-modulates HLA-B to similar levels in *Bw4/Bw6* heterozygotes as in *Bw4* homozygotes, twice as many Bw4 antigens would remain on the surface of iCD4 from *Bw4* homozygotes, which may be enough to mediate inhibition of $3DL1^+$ NK cells. Although autologous iCD4 activated $3DL1^+$ NK cells for total functionality, the secretion of CCL4 appeared to be the main contributor to total responsiveness. CCL4 secretion can exert an anti-viral effect by competing with HIV for binding to the CCR5 co-receptor required for viral entry [43]. Therefore, the response of the $3DL1^+$ NK cell population from *Bw4/Bw6* heterozygotes, have the potential to limit HIV infection through secretion of CCL4.

The frequency of $2DL3^+$ NK cells from *C1* homozygotes responding to autologous iCD4 was lower than that of *C2* homozygotes. As HLA-C1 is retained on iCD4 it would be available to interact with 2DL3 on NK cells educated through this receptor to inhibit their activation. In contrast, $2DL3^+$ NK cells from *C2* homozygotes would largely not be educated through this iNKR, nor would HLA-C2 on autologous iCD4 interact with 2DL3 to inhibit the function of $2DL3^+$ NK cells. Thus, the frequency of functional $2DL3^+$ NK cells from C2/C2 homozygotes would represent a background level of activation dependent on education though other iNKR and loss of ligands for these iNKR on

autologous iCD4. 2DL3$^+$ NK cells from *C1/C2* donors are educated, though possibly not as potently as 2DL3$^+$ NK cells from *C1* homozygotes. Infected CD4 from *C1/C2* donors activate 2DL3$^+$ NK cells to lower levels than do iCD4 from C2/C2 homozygotes, however, the difference does not achieve statistical significance. On the other hand, expression of HLA-C1 on iCD4 from *C1/C2* heterozygotes, which is half that on iCD4 from *C1* homozygotes, appears to be insufficient to suppress 2DL3$^+$ NK cell functionality to the low level of responsiveness observed in 2DL3$^+$ NK cells from *C1* homozygotes. HLA-C has been reported to be expressed on the cell surface at lower levels than HLA-A and –B antigens [22, 31, 44]. This may explain why iCD4 from *C1/C2* carriers are not as robust as those from *C1* homozygotes at inhibiting 2DL3$^+$ NK cell activation. In other words, C1 levels on iCD4 from *C1/C2* heterozygotes may fall below the threshold required to inhibit 2DL3$^+$ NK cells. Together, these results show that the functionality of NK cell populations to HIV is not only dependent on the educating iKIR/HLA combinations carried by the donor, but also on environmental changes in HLA expression levels driven by HIV infection.

We did not investigate the frequency of 2DL1 and KIR2DL2 (2DL2) positive NK cells stimulated by autologous iCD4 in this report. The reason for this is that the commercially available antibodies that bind 2DL2 also bind 2DL3 with or without KIR2DS2 compromising the detection of NK cells expressing 2DL2. Furthermore, the antibody previously thought to detect 2DL1 only, cross-reacts with other inhibitory and activating KIRs [45]. A subset of each of the iNKR$^+$ NK cells studied here would be expected to express 2DL1 and be educated through this receptor in persons who co-carried a C2

allele. As well, a subset of all of the subjects who are 2DL2$^+$ would be expected to express this iKIR and be educated through this receptor to varying degrees depending on which C1 or C2 alleles they carry. However, as iKIR are stochastically expressed NK cells expressing these receptors would be distributed among the NK cell populations expressing 2DL3, 3DL1 and NKG2A as well as none of these iNKR.

In conclusion, our results highlight a role for NKG2A$^+$ and 2DL3$^+$ NK cell populations in anti-HIV responses. We also observed a differential impact of education on the response of NK cell populations to HIV iCD4. A higher frequency of educated, compared to uneducated, 3DL1$^+$ NK cells secreted CCL4 in response to iCD4 cells, while a lower frequency of educated versus uneducated 2DL3$^+$ NK cells, responded to iCD4 by secreting CCL4. The higher functionality, in terms of CCL4 secretion, of 3DL1$^+$ NK cell populations from Bw4/Bw6 individuals may be a mechanism underlying the superior viral control and slower time to AIDS observed in epidemiological studies for some carriers of *3DL1* homozygous genotypes co-carrying certain Bw4 ligands compared to *Bw6* homozygous individuals [25].

4.7 FIGURES AND LEGENDS

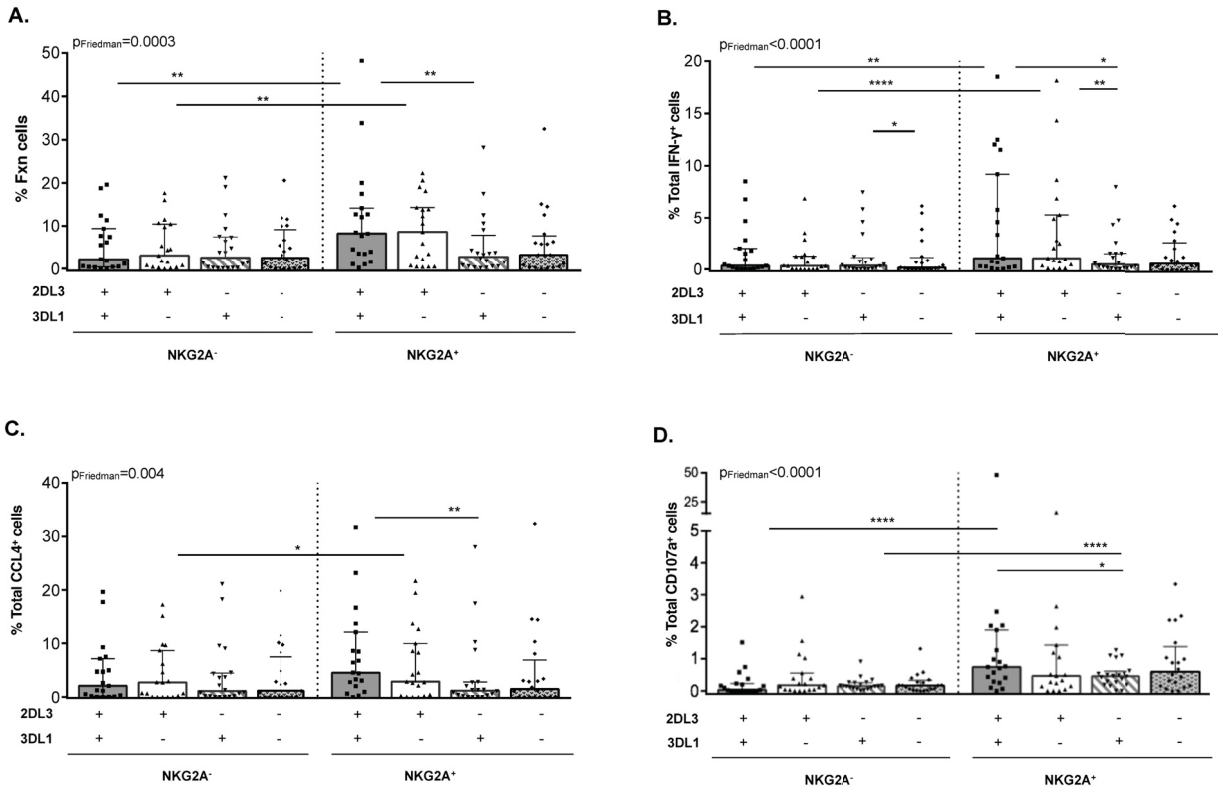


Figure 1: HIV iCD4 stimulation of functional NK cell subsets contributing to the differential responsiveness of $CD56^{Dim}NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ NK cell populations. The frequency of iCD4 stimulated NK cell populations characterized by total-responsiveness (C), total IFN-γ secretion (D), total CCL4 secretion (E) and total CD107a expression (F) response profiles are shown in the y-axis for each $CD56^{Dim}NKG2A^{+/-}2DL3^{+/-}3DL1^{+/-}$ population. Bar heights and error bars represent the mean and standard deviation (SD) for each group. Data from 20 individuals analyzed in duplicate were used to generate these results. Friedman ($p_{Friedman}$) and Wilcoxon (*) tests were used to determine the significance of differences between data sets. The data obtained were corrected for background using results obtained following stimulation with uninfected CD4 cells. % Fnx cells = Percent of cells exhibiting total responsiveness.

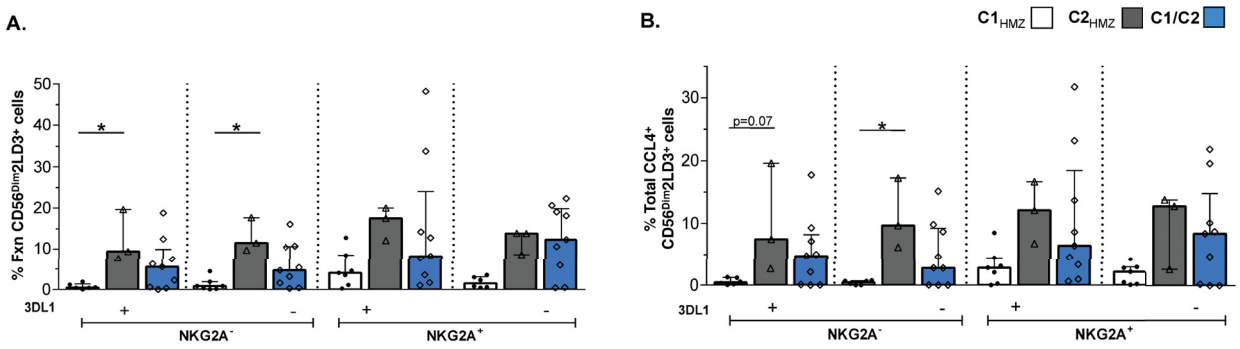


Figure 2: CD56^{Dim}2DL3^{+} NK cells from *C1* homozygotes have lower responses to autologous iCD4 than those from *C2* carriers. The frequency of iCD4 stimulated CD56^{Dim}NKG2A$^{+/-}$2DL3^{+}3DL1$^{+/-}$ NK cells from *C1* homozygotes ($C1_{HMZ}$; n=7), *C2* homozygotes ($C2_{HMZ}$; n=3) and heterozygous (C1/C2; n=9) characterized by total responsiveness (A) and total CCL4 secretion (B) response profiles are shown on the y-axis. Bar heights and error bars represent the median and IQR for each group. Data from 19 individuals positive for a *2DL3* allele, analyzed in duplicate, are plotted. Kruskal-Wallis with Dunn's post tests were used to determine the significance of between group differences. "*" = $p<0.05$. The data obtained were corrected for background using results obtained following stimulation with uninfected CD4 cells.

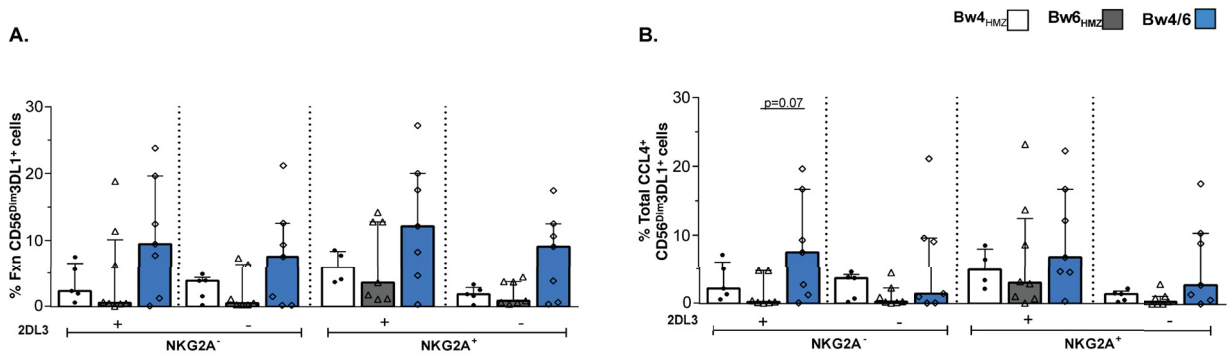
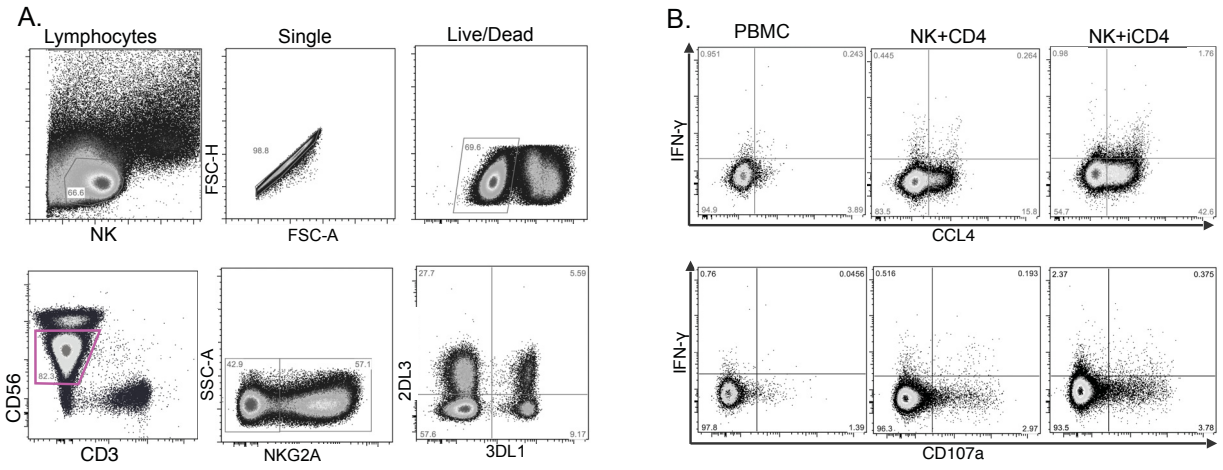
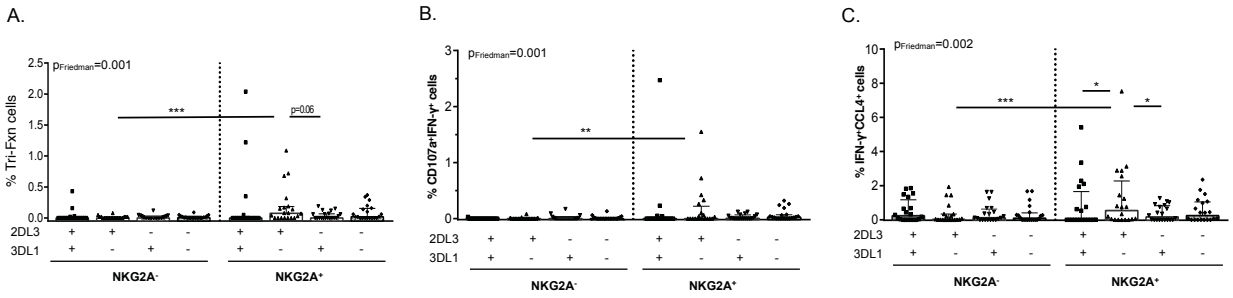


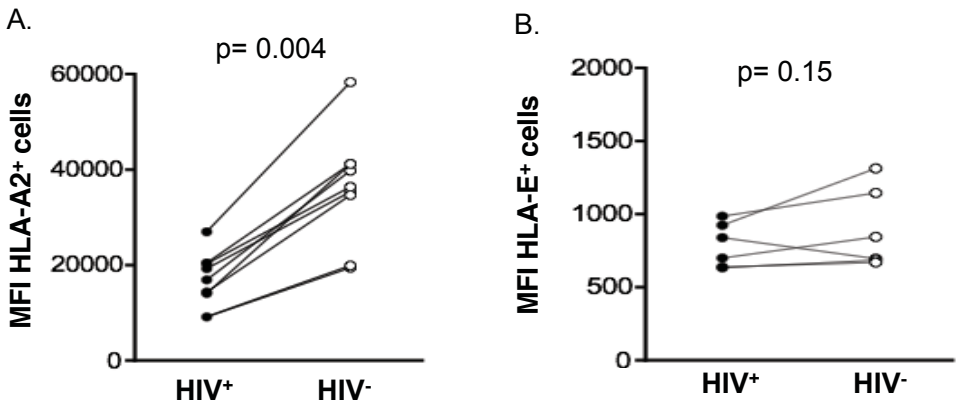
Figure 3: CD56^{Dim}3DL1^{+} NK cells from *Bw4/Bw6* heterozygotes have higher responses to iCD4 than those from *Bw6* homozygotes. The frequency of iCD4 stimulated CD56^{Dim}NKG2A$^{+/-}$2DL3$^{+/-}$3DL1^{+} NK cells from *Bw4* homozygotes (Bw4$_{HMZ}$; n=5), *Bw6* homozygotes (Bw6$_{HMZ}$; n=8) and heterozygotes (Bw4/6; n=7) characterized by total responsiveness (A) and total CCL4 (B) response profiles are shown on the y-axis. Bar heights and error bars represents the median and IQR for each group. Data from 20 individuals, analyzed in duplicate, are plotted. Kruskal-Wallis with Dunn's post tests were used to determine the significance between group differences. "*" = $p<0.05$. The data obtained were corrected for background using results obtained following stimulation with uninfected CD4 cells.



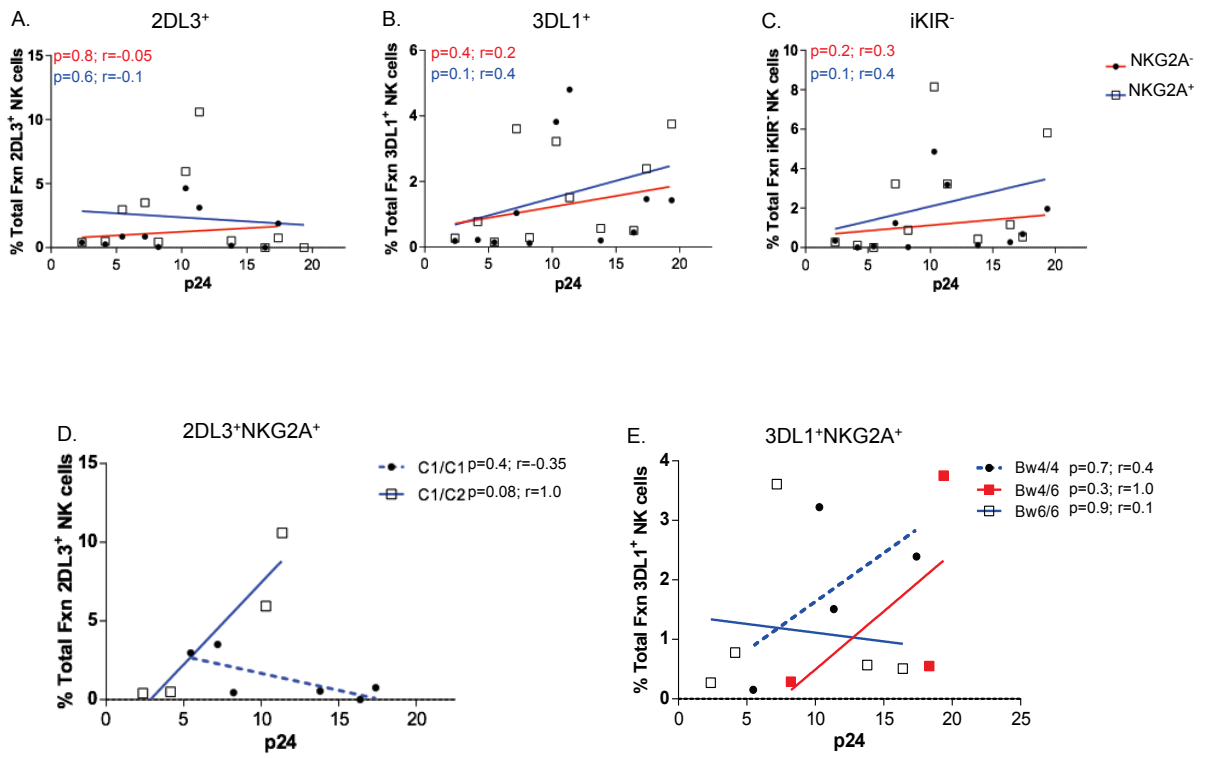
Supplementary Figure 1. The gating strategy used to identify iNKR population and functional subsets. (A). Cells were gated on the live singlet lymphocyte population. NK cells were defined as CD3-CD56+ and those that were CD56Dim were distinguished from CD56bright. NK cells positive for the iNKR NKG2A, 2DL3 and/or 3DL1 were analyzed from the CD56Dim gate. (B) Functional gates for IFN-γ, CCL4 and CD107a were set on gated CD56DimNK cells from HIV uninfected peripheral blood mononuclear cells (PBMC) (left hand panels). The background for NK cells stimulated with iCD4 (NK+iCD4) (right hand panels) was NK cells co-cultured with autologous uninfected CD4 cells (NK+CD4) (middle panels). The background for the NK+CD4 is higher than the PBMC gating control due to the media required to culture purified NK cells, R10 supplemented with IL-2, as described in the Materials and methods section.



Supplementary Figure 2. iCD4 stimulation of functional subsets contributing to the differential responsiveness of CD56DimNKG2A+2DL3+ NK cell populations. The frequency of iCD4 stimulated NK cell populations characterized by trifunctional (A), CD107a+IFN-γ+ (B) and IFN-γ+CCL4+ (C) response profiles are shown in the y-axis for each CD56DimNKG2A+/-2DL3+/-3DL1+/- population. Bar height represents the mean+/-SD for each group. Data from 16 individuals analyzed in duplicate were used to generate these results. Friedman (pFriedman) and Wilcoxon (*) tests were used to determine significance between data sets. P-values for between group comparisons are shown over lines linking the two groups being compared.



Supplementary Figure 3. Surface HLA levels following in vitro HIV infection of purified and stimulated CD4 cells. The frequency and MFI of HLA-A*02 (A & B) and HLA-E (C & D) are shown on the y-axis following in vitro HIV infection, as described in materials and methods. Individuals tested for HLA-E frequency were negative by allotyping for HLA-B*27, B*40, C*17 and C*04:03 alleles that have been shown to cross-react with 3D12 antibody. Wilcoxon tests were used to determine significance between data sets. P-values for between group comparisons are shown over lines linking the two groups being compared. HIV+: HIV-infected CD4 cells. HIV-: HIV-uninfected CD4 cells.



Supplementary Figure 4. Correlation between percentage of p24+iCD4 cells used for stimulation and total responsiveness of NK cell populations. Total functionality of 2DL3+ (A), 3DL1+ (B) and iKIR- (C) NK cells (y-axis) was correlated to level of HIV infectivity, as measured by p24 (x-axis). Both iNKR+ populations were positive for only the indicated iNKR (i.e. 2DL3+3DL1-). Both NKG2A+ (blue) and NKG2A- (red) sub-populations of NK cells were analyzed. The NKG2A+ populations of 2DL3+ (D) and 3DL1+ (E) Nk cells were further examined in a stratification analysis by the presence of the iNKR cognate ligand. Correlation was analyzed using Spearman and two-tailed p-value analysis.

4.8 REFERENCES

1. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187-376.

2. Munz C, Steinman RM, Fujii S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. The Journal of experimental medicine. 2005;202(2):203-7.

3. Forthal DN, Landucci G, Daar ES. Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells. Journal of virology. 2001;75(15):6953-61.

4. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. The Journal of experimental medicine. 2003;198(7):1069-76.

5. Alter G, Teigen N, Davis BT, Addo MM, Suscovich TJ, Waring MT, et al. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. Blood. 2005;106(10):3366-9.

6. 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. The Journal of experimental medicine. 2007;204(12):3027-36.

7. Alter G, Heckerman D, Schneidewind A, Fadda L, Kadie CM, Carlson JM, et al. HIV-1 adaptation to NK-cell-mediated immune pressure. Nature. 2011;476(7358): 96-100.

8. Song R, Lisovsky I, Lebouche B, Routy JP, Bruneau J, Bernard NF. HIV protective KIR3DL1/S1-HLA-B genotypes influence NK cell-mediated inhibition of HIV replication in autologous CD4 targets. PLoS Pathog. 2014;10(1):e1003867.

9. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature. 2005;436(7051):709-13.

10. Brodin P, Karre K, Hoglund P. NK cell education: not an on-off switch but a tunable rheostat. Trends in immunology. 2009;30(4):143-9.

11. 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;113:2434-41.

12. 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):331-42.

13. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. European journal of immunology. 1997;27(5):1164-9.

14. Yawata M, Yawata N, Draghi M, Partheniou F, Little AM, Parham P. MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. Blood. 2008;112(6):2369-80.

15. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. The Journal of experimental medicine. 1994;180(4):1235-42.

16. 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. Journal of immunology. 1986;137(11):3671-4.

17. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(24):12000-4.

18. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, et al. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. The Journal of experimental medicine. 1993;178(2):597-604.

19. Moesta AK, Parham P. Diverse functionality among human NK cell receptors for the C1 epitope of HLA-C: KIR2DS2, KIR2DL2, and KIR2DL3. Frontiers in immunology. 2012;3:336.

20. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. J Immunol. 2008;180(6):3969-79.

21. Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, et al. A whole-genome association study of major determinants for host control of HIV-1. Science. 2007;317(5840):944-7.

22. Bashirova AA, Thomas R, Carrington M. HLA/KIR restraint of HIV: surviving the fittest. Annu Rev Immunol. 2011;29:295-317.

23. Apps R, Qi Y, Carlson JM, Chen H, Gao X, Thomas R, et al. Influence of HLA-C expression level on HIV control. Science. 2013;340(6128):87-91.

24. International HIVCS, Pereyra F, Jia X, McLaren PJ, Telenti A, de Bakker PI, et al. The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. Science. 2010;330(6010):1551-7.

25. 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. Nature genetics. 2007;39(6):733-40.

26. 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):1487-91.

27. 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;184:2057-64.

28. Kamya P, Boulet S, Tsoukas CM, Routy JP, Thomas R, Cote P, 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. Journal of virology. 2011;85(12):5949-60.

29. Thomas R, Apps R, Qi Y, Gao X, Male V, O'HUigin C, et al. HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. Nature genetics. 2009;41(12):1290-4.

30. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity. 1999;10(6):661-71.

31. Apps R, Meng Z, Del Prete GQ, Lifson JD, Zhou M, Carrington M. Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. Journal of immunology. 2015;194(8):3594-600.

32. Lisovsky I, Isitman G, Song R, DaFonseca S, Tremblay-McLean A, Lebouche B, et al. A higher frequency of NKG2A+ than NKG2A- NK cells respond to autologous HIV-infected CD4 cells irrespective of whether they co-express KIR3DL1. J Virol. 2015.

33. Boulet S, Sharafi S, Simic N, Bruneau J, Routy JP, Tsoukas CM, et al. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. AIDS. 2008;22(5):595-9.

34. Kulkarni S, Martin MP, Carrington M. KIR genotyping by multiplex PCR-SSP. Methods Mol Biol. 2010;612:365-75.

35. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R, et al. CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. Journal of immunology. 2007;178(8): 4947-55.

36. 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.

37. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood. 2010;116(19):3853-64.

38. Beziat V, Dalgard O, Asselah T, Halfon P, Bedossa P, Boudifa A, et al. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. European journal of immunology. 2012;42(2):447-57.

39. Ravindranath MH, Pham T, El-Awar N, Kaneku H, Terasaki PI. Anti-HLA-E mAb 3D12 mimics MEM-E/02 in binding to HLA-B and HLA-C alleles: Web-tools validate the immunogenic epitopes of HLA-E recognized by the antibodies. Molecular immunology. 2011;48(4):423-30.

40. Bonaparte MI, Barker E. 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. 2004;104(7):2087-94.

41. Brodin P, Lakshmikanth T, Mehr R, Johansson MH, Duru AD, Achour A, et al. Natural killer cell tolerance persists despite significant reduction of self MHC class I on normal target cells in mice. PLoS One. 2010;5(10).

42. 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):1166-74.

43. Oliva A, Kinter AL, Vaccarezza M, Rubbert A, Catanzaro A, Moir S, et al. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. The Journal of clinical investigation. 1998;102(1):223-31.

44. Snary D, Barnstable CJ, Bodmer WF, Crumpton MJ. Molecular structure of human histocompatibility antigens: the HLA-C series. European journal of immunology. 1977;7(8):580-5.

45. Czaja K, Borer AS, Schmied L, Terszowski G, Stern M, Gonzalez A. A comprehensive analysis of the binding of anti-KIR antibodies to activating KIRs. Genes and immunity. 2014;15(1):33-7.

CHAPTER 5: HIGHLIGHTS AND SUMMARY OF ORIGINAL WORK

The main objective of this thesis was to characterize NK cell populations responding to HIV infected cells and analyze their functional profiles. Numerous studies have described the importance that NK cell education has on acquisition of NK cell functional potential. Therefore, we stratified our analyses of NK cell populations by taking into consideration genotypes encoding iNKR-HLA receptor ligand combinations. We also sought to better understand the mechanisms underlying protection from infection of 3DL1$^+$ NK cells that were previously unknown. The summary of our key findings from each thesis chapter are presented below.

Chapter 2:

1. NK cells from subjects carrying **h/*y+B*57* suppress viral replication as well as those from carriers of the *3DS1+*80I* pair and better than those from of carriers of **l/*x+B*57* and *Bw6hmz* subjects.
2. Bulk NK and 3DL1$^+$ NK cells from *h/*y+B*57* subjects secrete more CC-chemokines in response to iCD4 cells than those from Bw6hmz and **l/*x+B*57* carriers.
3. This suggests that NK cell education influences the potency of NK cell mediated inhibition of HIV replication that is partially due to secretion of CC-chemokines and in part mediated by 3DL1$^+$ NK cells.

Chapter 3:

1. $3DL1^{+}NKG2A^{+}$ NK cells were significantly more responsive to HLA-null cells than the other 3 $NKG2A^{+/-}3DL1^{+/-}$ populations.
2. A higher frequency of $NKG2A^{+}$ than $NKG2A^{-}$ NK cells with tri-functional, $CD107a^{+}IFN\gamma^{+}$, total $CD107a^{+}$ and total $IFN\gamma^{+}$ functional profiles respond to iCD4 cells. Thus the less mature $CD56^{bright}$ NK cell population responds most potently to iCD4 cells.
3. Co-expression of 3DL1 on $NKG2A^{+/-}$ populations did not enhance responses to iCD4 cells.

Chapter 4:

1. The expression or co-expression of 3DL1 has a minimal impact on NK cells responses to iCD4.
2. In contrast, 2DL3 has an impact on NK cells functionality to iCD4, which is enhanced by NKG2A co-expression.
3. A higher frequency of $3DL1^{+}$ NK cells from $Bw4^{+}$ individuals than Bw6hmz controls responded to iCD4 by secreting CCL4.
4. Lower frequency of $2DL3^{+}$ NK cells from individuals positive for C1, versus C2 homozygotes responded to iCD4.
5. Responses of NK cell populations to iCD4 are influenced by NK cell education through specific KIR/HLA pairs and differential HIV mediated changes in HLA expression levels.

Together these observations provide additional support for the importance of NK cells and populations of these cells as mediators of responses to HIV infected cells. These findings also demonstrate the dynamic role of NK cell education on aspects of NK cell functions, both in the context of broad stimulation by HLA null cells and responses to iCD4 cells.

CHAPTER 6: DISCUSSION

The functional heterogeneity of educated NK cell populations; implications for evaluation methods

NK cells acquire functional competence via the process of NK cell education. Education depends on a number of factors, such as: the iNKRs expressed by an NK cell, the HLA type of the NK cell donor, the number of iNKRs to self ligands an NK cell expresses and the potency of particular iNKR-HLA ligand combination. There are many publications that highlight the functional benefits of educated compared to un-educated NK cell populations. NK cell functional competence depends on how potently they were educated and can be measured by activating NK cells with a stimulus such as HLA null cells and measuring various effector functions (e.g. secretion of cytokines and chemokine, apoptosis/killing assays and Ab activation and cytotoxicity). In other words, the stronger the interactions are between iKIR and HLA, the more functional the NK cell population will be compared to NK cells educated through weaker iKIR/HLA pairs or those expressing either iKIR without their ligands or vice versa.

The term “functional potential” refers to NK cell responsiveness induced by HLA-null cell stimulation. The HLA-null 721 and K562 cell lines are commonly used to activate NK cells educated through the interaction of iNKRs with HLA antigens. Their lack of cell surface HLA abrogates negative signaling through iNKRs, which inhibit NK cell function. HLA null cell stimulation results in a level of NK cell activation that is correlated with the extent to which NK cells were educated. NK cell activation also requires the

engagement of aNKRs with their ligands present on HLA null cells. Several studies highlight differences between 721 and K562 in terms of which aNKRs ligands they express. These differences are likely to be important for the differential activation profiles measured in bulk NK cells and NK cell populations following stimulation with these two HLA-null cell lines. Our results using 721 and K562 to stimulate NK cells illustrate a dichotomy of responses in which K562 cells preferentially stimulate CD107a driven responses, while 721 cells stimulate a response profile driven by IFN-γ and CCL4 secretion [379]. Therefore, these HLA-null cell lines have the potential to induce different functional profiles in NK cells and their subsets. Indeed, we have previously shown that 721 and K562 induce a different level and pattern of activation of bulk and $3DL1^+$ NK cells. We also measured higher levels of total responsiveness following stimulation with 721 than K562 cells in $3DL1^-$ cells that were $2DL1^+$, $2DL2/S2^+$ and $NKG2A^+$ (unpublished data). Together, these results suggest that the nature of the stimulus has a significant impact on the level of NK cell activation and the NK cell population engaged.

In chapter 3 we assessed whether co-expression of the iNKRs NKG2A and 3DL1 affects functional responses of NK cell populations responding to 721 and K562. 721 induced a higher frequency of functional NK cells than K562 within the four populations expressing all possible combinations of NKG2A and 3DL1. One notable exception to this tendency was that a higher frequency of single positive NKG2A than double $iNKR^+$ NK cells responded to HLA null stimulation by expressing CD107a; this response for K562 was greater than 721 stimulation. We previously studied the differential response

profiles of bulk and 3DL1$^{+/-}$ NK cells in response to 721 and K562 [379]. Briefly, when overall functionality was dissected in order examine individual functions we found that 721 and K562 induced IFN-γ, CCL4 and CD107a expression differentially. For example, K562 stimulation induced a higher frequency of CD107a expression from bulk and 3DL1$^{+/-}$ NK cells, while 721 was superior to K562 for inducing the secretion of IFN-γ and CCL4 [379]. The reason behind this dichotomy may be due to two possible factors: NK cell activation and trafficking pathways. NK cell activation requires not only the interruption of negative signaling through iNKR, but also the interaction of aNKRs with ligands on target cells. The lack of surface HLA expression on 721 and K562 is common factor for both cell lines and fulfills the first criterion for activation. However, expression of ligands for aNKR, the other factor involved in activation, differ between these cells lines [374, 377, 456-458]. These differences are likely to be important for the differential activation profiles measured in bulk NK cells and NK cell populations following stimulation with these two HLA-null cell lines. There is accumulating evidence that cytokines are trafficked and secreted by different pathways than cytotoxic granules, suggesting that cytotoxicity and cytokine/chemokine production in NK cells can be uncoupled [457, 459, 460]. For example, NKG2D engagement is known to initiate a signaling cascade leading to cytotoxicity [457]. K562 expresses several ligands for NKG2D that can activate NK cells through this aNKR, while 721 does not [461]. In contrast, 721 expresses ligands for aNKRs, such as NCRs and CD48, which is not found on K562 cells. The co-expression of CD48 with other NCR specific activating ligands is consistent with 721's superior ability to induce the secretion of IFN-γ and/or CCL4 compared to K562 [377, 461].

In the second part of chapter 3 we investigated the responses of NK cell populations defined by NKG2A and 3DL1 expression to autologous HIV iCD4. It should come as no surprise that this stimulus induced a different pattern of NK cell activation than HLA null cells. Following stimulation with iCD4 cells the frequency of functional double iNKR^{+} NK cells was not superior to that of the other 3 NK cell populations studied. Rather, the frequency of functional single NKG2A^{+} cells was comparable, and sometimes superior, to that of NKG2A^{+}3DL1^{+} NK cells. This further supports the concept we identified in our study of bulk NK and 3DL1$^{+/-}$ NK cells using 721 and K562 [379] that NK cell populations are triggered differentially by different stimuli. This is likely due to difference in the configuration of ligands for aNKR and iNKR present on the different target cells, i.e. 721 vs K562 or iCD4 that are stimulating NK cells. Therefore, each stimulus is unique and is likely to engage with NK cells in a specific capacity and stimulate a unique signature of responses. In conclusion, results of functional NK cell and NK cell population responses may not be transferable from one stimulus to another and must be interpreted carefully. Each stimulant needs to be assessed and considered independently with their respective controls.

Pivotal studies on discovering and understanding the mechanisms of NK cell education by Kim *et al.*, Anfossi *et al.* and Moesta *et al.* examined educated versus non-educated NK cells by defining these populations using expression of one to several iKIRs and iNKRs. It is important to make the distinction that uneducated NK cell populations in these studies were not ‘non-functional’ (i.e. zero functional potential), rather, they

exhibited lower functionality compared to their educated counterparts. This is a 'fail/safe' mechanism that prevents responsiveness to self [462]. The measured functionality in the uneducated NK cell populations could be due to the co-expression of aKIRs, or in some cases other iNKRs that may not be a strong iKIR/HLA pair and thus have a limited contribution to the functional potential. Therefore, it would be desirable and informative to examine NK cell populations that exclusively express a single iNKR and to analyze its individual and additive (in combination with other iNKRs) effect on NK cell functional potential. At the time the experiments described in this thesis were done an examination of the functional repertoire of all NK cell populations defined by the various iNKRs responding to a given stimuli was not feasible. This was primarily due to practical limitations relating to the availability of Abs detecting specific iNKR, and particularly KIR. Either Abs specific for a particular receptor do not exist or they cross-react with other iNKRs and/or the aKIR counterpart of iKIRs that share high sequence homology. This situation represents a limitation of the results presented in this thesis. However, given that little information was published and known on NK cell populations responding to HIV infected cells, efforts in this thesis describe analyses of NK cell populations defined by three iNKRs, which results in a comprehensive analysis of 8 distinct NK cell populations. With advances made in Ab availability and strategies for analyzing complex NK cell panels [463], I was able to develop, test and optimize an Ab panel that can study simultaneously NK cell populations defined by 5 different iNKR and aNKRs: KIR2DL1, KIR2DL2/S2, KIR2DL3, KIR3DL1 and NKG2A. This panel generated results for stimulations with HLA-null cells (both K562 and 721) and Ab-induced NK cell activation (unpublished results and Appendix 1). The Bernard lab has since improved on

the Ab panel and the new Ab panel can distinguish KIR2DL1,and KIR2DL2 from KIR2DL3 and KIR2DS2. This has been made possible by the acquisition of Abs than are not available commercially. This new Ab panel will contribute to a more complete assessment of the response of NK cell populations to stimuli and deepen our understanding of the interaction of NK cells with autologous iCD4.

Superior functionality of NKG2A$^+$ NK cell populations in response to stimulation with HIV infected cells

NK cell populations are involved in responses to HIV and may mediate both protection from infection and protection from disease progression. The types of responses associated with both outcomes are not well characterized. The focus of this thesis was protective immune responses and thus the system chosen to evaluate this phenomenon relied on purified NK and autologous *in vitro* HIV iCD4 cells from HIV-negative donors. At the time my thesis research project was initiated, little was known regarding whether iCD4 stimulated NK cell populations differentially and if so, which populations and functional subsets they activated. The NK cell population that was first shown to inhibit HIV replication was the 3DS1$^+$ population from 3DS1/3DL1 heterozygous donors [447]. In Chapter, 2 we showed that 3DL1$^+$ NK cells from donors who express both high expression 3DL1 variants and a potent ligand for 3DL1, i.e. HLA-B*57 not only limit HIV replication *in vivo*, but also get preferentially stimulated by autologous HIV iCD4 targets to secrete CCL3, 4 and 5 chemokines. The level of this stimulation is higher than that of

3DL1 NK cells from carriers of low expression 3DL1 variants (even when co-expressed with HLA-B*57) and from carriers of 3DL1 not co-expressing an HLA-B ligand. The results described in Chapter 2 illustrate the impact on NK cell functionality of strong, versus weak, versus no educating signals between iKIR/HLA pairs on functional responses to HIV infected cells.

Given that NK cell functional potential also depends on the number of educating iNKR signals received during education we assessed the impact of the expression of multiple iNKR on NK cell populations (i.e. other than 3DL1) in response to autologous iCD4 cells in Chapters 3 and 4. We reasoned that NK cells with greater functional potential, in other words with more iNKRs to self HLA, should be more responsive to stimulation in accordance with theory of NK cell education and as confirmed in Chapter 3 using two different HLA-null cell lines. Thus we expected that the double iNKR^{+} population would be more responsive to autologous iCD4 than the other 3 populations defined by NKG2A or 3DL1 alone or neither. We were surprised to find that the double iNKR^{+} population did not show the highest functional responses to iCD4. HIV iCD4 targets stimulated a higher frequency of responses in NKG2A^{+} NK cell populations compared to NKG2A^{-}, irrespective of 3DL1 co-expression. Even more surprising was the observation that the highest frequency of NK cells responding to iCD4 cells were the CD56^{Bright}NKG2A^{+} population. The reason this is surprising is that, 1) NKG2A is an iNKR who's HLA-ligand is not downmodulated during HIV infection, raising questions regarding how this NK cell population is activated by iCD4. 2) CD56^{Bright}NKG2A^{+} cells are less mature compared to CD56^{Dim}iKIR^{+} populations. According to studies that analyzed the correlation between

NK cell development and functionality, less mature cells should be less responsive to stimulation. Together these results reaffirm the notion that higher NK cell functional potential, as predicted by the theory of NK cell education and confirmed by assessment of NK cell functional potential, does not always predict which NK cell population will be quantitatively superior in its responses to stimulation.

These findings also lead to another important question regarding the role of NKG2A$^+$ NK cells in HIV infection: i.e. do NKG2A$^+$ NK cells protect from infection or does this phenomenon represent a viral diversion mechanism that steers the anti-HIV responses away from controlling the virus? One way to assess the anti-iCD4 functionality of NKG2A$^+$ NK cells would be to isolate NKG2A$^+$ and NKG2A$^-$ NK cells and test them side by side in an inhibition of viral replication assay. This has yet to be done. We did block the interaction of NKG2A, with HLA-E, in order to ascertain its functional role as an inhibitory receptor. Blocking the NKG2A/HLA-E axis resulted in enhanced NKG2A$^+$ NK cell responses to autologous iCD4. This led us to the possibility that HIV may affect the pool of leader peptides available to bind HLA-E, which could have an impact on interactions between HLA-E and NKG2A. Indeed a recent study by Davis *et al.* [464] confirmed our observations and identified a possible mechanism underlying the enhanced NKG2A$^+$ NK responses to iCD4 cells. They used HLA-E tetramers loaded with a conserved Gag peptide to probe the interaction of HLA-E/HIV peptide with NKG2A. Their results show that the presentation of this conserved HIV Gag 9-mer peptide abrogated the binding of HLA-E to NKG2A, which is also evident at the level of NKG2A$^+$ NK cell responses to autologous iCD4. Thus, when HIV infection occurs and

this 9-mer peptide is presented by HLA-E, the binding interactions between NKG2A and HLA-E are disrupted, reducing the inhibitory input through this receptor and resulting in a greater activation of $NKG2A^{+}$ NK cell populations. Davis *et al.* also noted greater responses to iCD4 by $CD56^{Bright}$ compared to $CD56^{Dim}$ $NKG2A^{+}$ population. The latter finding is also in agreement with our data and can be easily reconciled given the predominant expression of NKG2A on $CD56^{Bright}$ NK cells.

In future projects it would be interesting to test these findings in NK cells from individuals in the chronic phase of HIV infection and compare the responses of these NK cell populations in HIV controllers and typical-progressors. In other words, it would be interesting to know whether NK cell populations from individuals in chronic phase HIV infection respond similarly to iCD4 at the population level and inhibit HIV replication similarly to NK cells from uninfected subjects. Future experiments should assess whether HIV infection has a negative impact on NK cell functionality at the NK cell population levels, particularly $NKG2A^{+}$ NK cells, as has been reported for $3DL1^{+}$ NK cells from carriers of a *Bw4* allele [465]. If so, it would be worth knowing whether any negative impacts are correlated with HIV VL.

NK cell responses to HIV infected cells are CC-chemokine driven

The timing of NK cell responses suggests that they may have a role in initial control of HIV infection [447]. This early role is supported by epidemiological and functional

studies that show that VL decline occurs before adaptive immune responses (primarily meditated by T cells) are fully engaged and the higher frequency of functional NK cells in HESN, particularly those secreting CC-chemokines [187]. CC-chemokine secretion by NK cells is a common theme in this thesis. In Chapter 2, we measured greater CCL3, 4 and 5 secretion by NK cells from B57 donors in response to autologous iCD4. We showed that when these NK cells were co-cultured with iCD4 targets their inhibition of HIV replication is driven in part by CC-chemokines that can outcompete the virus for binding the entry co-receptor CCR5. The contribution of additional iNKRs to the responses of educated 3DL1^{+} NK cells was investigated in Chapters 3 and 4. The co-expresison of either 2DL3 or NKG2A enhanced the CCL4 secretion by 3DL1^{+} NK cells from Bw4 donors compared to Bw6 controls. We chose to focus on CCL4 secretions instead of CCL3 as both chemokines are co-produced and the signal of the latter is harder to detect by flow cytometry (unpublished data). Altogether, these data suggest that NK, and particularly educated 3DL1^{+} NK cells, limit HIV replication at least at the entry level.

These findings raise some interesting questions about additional potential mechanisms that NK cells can use to inhibit HIV spread or HIV replication. Similarly to what has been observed with 3DL1^{+} NK cells, can other NK cell population respond to HIV infected cells? Is it possible that NK cells interfere with HIV replication at other points of the replication cycle than entry? Would this be mediated by a particular NK cell population or bulk NK cells? While the timing of NK cell expansions in acute HIV infection precedes that of HIV-specific CTL expansions, it is likely that the major contribution of NK cell

mediated inhibition of HIV infection dissemination occurs at an early stage of infection. Given the diverse functional profiles of NK cells and the complexity of the human immune system it is probable that NK cells can also exert restrictive pressure on HIV replication and/or assist with replication control at different stages of infection. Previously, NK cells were found to be poor “killers” of autologous iCD4 cells [466]. However, this study examined purified NK cell activity against isolated and *in vitro* HIV infected autologous T cells in the absence of IL-2. This cytokine would have been produced naturally in the cytokine milieu following establishment of infection. In a subsequent article this technical limitation was overcome and the field has been using IL-2 in co-culture systems of purified target-effector cells in the context of HIV [467]. Nevertheless, to fully address these questions new studies need to be designed. Results presented in Chapters 3 and 4 provide insights into the multi-functionality of immune responses triggered by NK cell populations responding to HIV-specific stimulation.

Immune responses to HIV are multifactorial and heterogeneous, as is the pathophysiology of this virus. Therefore, it is logical to hypothesize that NK cell populations may respond heterogeneously as well. As previously described, $3DL1^+$ NK cells from carriers of the high-affinity B*57 ligand can inhibit HIV at the level of entry via the secretion of CC-chemokines. Similarly to $3DL1^+$ NK cells, a higher frequency of $2DL3^+$ cells from donors who carry 1 copy, or no C1 alleles (i.e. C2 homozygotes), responded to iCD4 by secreting CCL4 following iCD4 stimulation. Thus $2DL3^+$ NK cells from ‘less educated’ HLA backgrounds are likely to be involved at limiting HIV replication

at the entry level, since their responses are mainly characterized by CCL4 secretion. The role of 2DL3^{+} NK cell population in controlling HIV replication and infection dissemination is further highlighted by HIV evasion mechanisms from NK cells. A recent study found that an HLA-C restricted Gag variant activated 2DL3^{+} NK cells poorly; this was associated with viral escape from 2DL3^{+} NK cells [468]. A better understanding of the mechanisms by which HIV-1 evades NK-cell-mediated immune pressure will facilitate the development of novel targeted immune interventions aimed at harnessing the antiviral activities of NK cells.

We have identified a previously uncharacterized NK cell population that has high responses to HIV infected cells. Indeed, as presented in Chapters 3 and 4, NKG2A^{+} NK cells respond to HIV infected cells in a multi-functional fashion by not only secreting CCL4, but also secreting IFN-γ and degranulating via CD107a upregulation. These responses were measured in both CD56^{Bright} and CD56^{Dim} NKG2A^{+} NK cells, but were dominant in the former compartment, likely due to the high prevalence of NKG2A on CD56^{Bright} NK cells. These multi-functional responses may also aid in shaping immune responses that interfere with other steps in HIV replication via the secretion of IFN-γ or additional cytokines.

Given the high level of responsiveness measured in NKG2A^{+} NK cells following stimulation with autologous iCD4 cells we wanted to ascertain whether these cells can also act in other ways and limit HIV infection. NK cell activation via antibody-dependent stimulation has been on the forefront of recent research due to results from a secondary

analysis of the RV 144 phase III HIV vaccine trial. In a post hoc analysis ADCC, which is mediated by NK cells, was shown to be an important correlate of protection. However recent computational analyses highlight the importance of Ab-mediated NK cell activation that can drive other immune responses than cellular cytotoxicity [285]. In addition, host Ab-dependent functions directed against HIV infected cells have been implicated in protection from infection induced by protective HIV vaccines [271]. These Ab-dependent functions are almost certainly more potent than direct NK cell responses (e.g. direct killing) to iCD4 and dependent on the presence of anti-HIV Env specific Abs. These Abs would not be present in HIV susceptible subjects before infection, unless they were induced by immunization with an appropriate vaccine or adoptively transferred to uninfected hosts. Currently, the effector function of $NKG2A^{+}$ NK cells in anti-HIV Ab-dependent activation is not known. The process of Ab-dependent NK cell activation is influenced by CD16, which is expressed predominately on the $CD56^{Dim}$ compartment [307]. This is in contrast to the cellular distribution of NKG2A, which is expressed on essentially all immature $CD56^{Bright}$ cells, but only on a subset of $CD56^{Dim}$ NK cells [243]. Accordingly, we hypothesized that, unlike responses to HIV iCD4, a lower frequency of $CD56^{Bright}NKG2A^{+}$ than $NKG2A^{-}$ NK cells would respond to anti-HIV Ab-dependent activation. To assess this we examined Ab dependent NK cell activation using CEM.NKr.CCR5 target cells coated with gp120 as stimuli, NK cells as effectors and pooled anti-HIV Env Abs from a single source. This experimental set-up permitted the simultaneous assessment of responses of 6 distinct NK cell populations to Ab-dependent stimulation of cells from 16 different donors. We focused on responses from NK cell populations expressing NKG2A and $CD56^{Dim}KIR^{+}$ NK cells: $CD56^{Bright}NKG2A^{+}$,

$CD56^{Dim}NKG2A^{+}$, $2DL1^{+}$, $2DL2/S2^{+}$, $2DL3^{+}$ and $3DL1^{+}$ (Appendix Figure 1). Results showed a dichotomy of responses between $CD56^{Bright}$ and $CD56^{Dim}NKG2A^{+}$ NK cells, where a lower frequency of the former than the later population responded to the stimulus (Appendix Figure 2). In addition, $CD56^{Bright}$ NK cells were the least responsive NK population among the 6 populations analyzed (Appendix Figure 2). In contrast, $CD56^{Dim}NKG2A^{+}$ cells had moderate responses, which were comparable to $2DL1^{+}$ and $2DL2/S2^{+}$ populations, while lower than $2DL3^{+}$ and $3DL1^{+}$ populations. In addition, the frequency of total $CD107a^{+}$ $CD56^{Dim}NKG2A^{+}$ NK cells was comparable to that of KIR^{+} populations (Appendix Figure 2). CCL4 responses were measured for all NK cell populations analyzed. However, the frequency of $CD56^{Dim}NKG2A^{+}$ and KIR^{+} populations cells secreting CCL4 were not significantly different (data not shown). Altogether these results and conclusions from Chapters 3 and 4 suggest that $NKG2A^{+}$ NK cells have important multi-functional responses to HIV-specific target cells. $CD56^{Bright}NKG2A^{+}$ and $CD56^{Dim}NKG2A^{+}$ NK cells are both stimulated by HIV-infected cells, however the frequency of $CD56^{Bright}$ cells responding to iCD4 is higher than that of $CD56^{Dim}$ NK cells. In contrast, the frequency of $CD56^{Dim}NKG2A^{+}$ NK cells responding to anti-HIV Ab-dependent stimulations is higher than that of $CD56^{Bright}NKG2A^{+}$ NK cells. Altogether, these findings support the notion that in addition to $3DL1^{+}$ NK cell, other NK cell populations can interfere with HIV replication and possibly at other points of the replication cycle than entry (i.e. via mechanisms that are not based on CC-chemokine secretion).

The dynamic plasticity of NK cell education in the context of HIV infection

NK cells are classically classified as belonging to the innate arm of the immune system. However, over the years, numerous studies have presented evidence that NK cells can display more “adaptive-like” characteristics. A number of similarities exist between T cells and NK cells including differentiation, memory and functional plasticity. The phenotype of functional plasticity is a relatively new concept in immunology and has been reported in immune cells, such as Th17. For example, the Th17 lineage, which develops from naive T cells, promotes pro-inflammatory processes by secreting primarily IL-17 and facilitates the elimination of extracellular bacteria and fungi [469]. However, recent studies have identified $Th17^{+}$ cells that, not only produce IL-17, but also co-express markers for regulatory T cells that retain a suppressive functional phenotype [470, 471]. In addition, Th17 cells found in the tumor micro-environment secrete effector cytokines such as IL-2, IFN-γ and TNF-α that are classically characterized as part of a Th1 cytokine-profile [472]. These findings highlight the dynamic nature of T cell subsets and support a paradigm for subset functionality that extends beyond the conventional classification.

Similar features that highlight functional plasticity can also be seen in NK cell populations. For example, NK cells are classically dichotomized by CD56 expression into Bright and Dim populations. Classically, $CD56^{Bright}$ NK cells responds to targets cells via secretion of chemokines and cytokines, while $CD56^{Dim}$ via degranulation. Results from our group and others, showed that the two CD56 populations are capable

of both cytokine/chemokine secretion and degranulation profiles. In Chapter 3 we show that $CD56^{Bright}$ NK cells in response to autologous HIV infected CD4 cells are able to produce IFN-γ and up-regulate CD107a equally well. In fact, the up regulation of CD107a appears to be a functional output most frequently associated with $NKG2A^{+}$ NK cells, whether $CD56^{Bright}$ or $CD56^{Dim}$, and in response to either iCD4 or HLA-null cell lines.

NK cell education is an NK specific process that shows a degree of plasticity, which was well documented in innovative approaches using mouse models. NK cell development and education take place primarily in the bone marrow [473, 474]. However, evidence is emerging that NK cells can continue the maturation process outside the bone marrow in SLT or the periphery. Two important studies by Elliot *et al.* and Joncker *et al.* show that mature functional NK cells can be “reprogrammed” by exposing them to an environment with different MHC-I ligands than the one in which they originally developed [370, 371]. In these studies murine splenic NK cells that matured in a setting where they were exposed to specific MHC-I ligands (i.e. sufficient environment) were transferred into new host devoid of this MHC ligand (i.e. deficient environment). These functional mature NK cells were anergic in the new host. In reciprocal experiments, anergic NK cells that developed in an MHC-I deficient host gained effector functions when transferred into a new host that was MHC-I sufficient. Another way that the host MHC environment can change is due to infection. Murine studies of MCMV show that anergic uneducated NK cells that do not express the iNKR for the MHC-I expressed by the host, but express an aNKR, can still be activated in this host in the context of viral infection. Adoptive transfer

of uneducated, rather than educated, NK cells in neonate mice resulted in protection against challenge with MCMV [475]. Altogether, these findings highlight the dynamic nature of NK cell education and provide additional supporting evidence for NK cell functional plasticity.

The latter example of how viral infection can alter NK cell effector function also highlights our finding from Chapter 4 and addresses whether education offers benefits in protection from HIV infection. We showed in Chapter 3 that the more educated combinations of iNKR-HLA are present on an NK cell, the greater its functional potential as measured by responses to K562 and 721 stimulation. However, this pattern was not seen when NK cells were stimulated with autologous iCD4 cells. In Chapter 4, we analyzed the responsiveness to iCD4 of NK populations educated, or not, through two different educating pairs, 3DL1-Bw4 and 2DL3-C1. We observed a differential impact of education through these two iKIRs on the response of NK cell populations to HIV iCD4. A higher frequency of educated, compared to uneducated, $3DL1^+$ NK cells secreted CCL4 in response to iCD4 cells, while a lower frequency of educated versus uneducated $2DL3^+$ NK cells, responded to iCD4 by secreting CCL4. The selective downregulation of HLA surface expression by HIV explains how iCD4 cells from donors with KIR/HLA pairs, where the ligand remains on the surface, can inhibit NK cells expressing this KIR; as well as, on the contrary, cases where the ligand is downregulated and inhibition mediated through the remaining HLA ligands pairs on iCD4 falls below the threshold required to inhibit NK cell responses via signals mediated by the partner KIR. Furthermore, HIV can present viral peptides that stabilize

interactions between iNKRs and respective HLA ligands, thus resulting in transmission of inhibitory input into the NK cell. Together, these results show that the functionality of NK cell populations to HIV is not only dependent on the educating iKIR/HLA combinations carried by the donor, but also on environmental changes in HLA expression levels driven by HIV infection.

In conclusion, work presented in this thesis uncovers additional parts of a puzzle that describes the role of NK cells and NK cell populations in response to HIV. Knowledge of what protective mechanisms NK cells exert at different stages of infection, other than entry via CC chemokines, could offer invaluable insight into designing effective HIV prophylactic and possibly therapeutic vaccines. A recent study from Dr. Galit Alter's laboratory used an integrative System Serology approach to analyze humoral immunity network profiles induced by different HIV vaccines [271]. The only protective vaccine thus far, the one used in the RV144 vaccine trial, offered a modest protection of ~31%, which presented with heightened NK cell responses contributing to Ab-induced activation [271, 476]. The NK cells remain an important factor in protective HIV immune responses. What needs to be addressed in future research is which NK populations contribute to anti-HIV activity, in addition to what has been described in this thesis, and a description of the mechanisms they use to exert anti-HIV activity.

7. APPENDIX

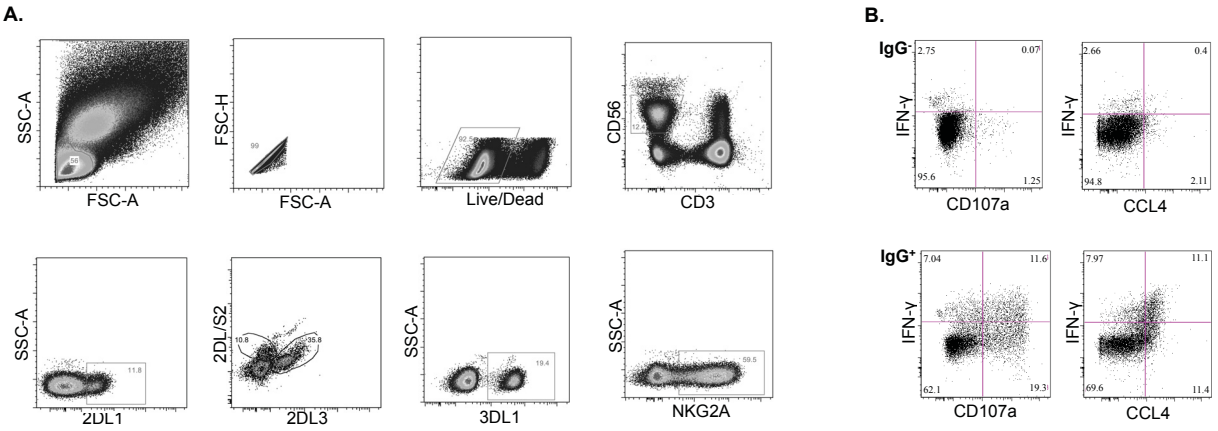


Figure 1. Gating strategy to identify 5 populations of NK cells and their functional responses. (A) Live lymphocyte singlets were gated on. NK cells were defined as $CD3^-$ $CD56^{Dim}$. The 2DL1, 2DL3, 2DL2/S2, 3DL1 and NKG2A populations were derived from the $CD56^{Dim}$ NK cell population. We also examined the contribution of $CD56^{Bright}NKG2A^+$ NK cells to Ab-dependant NK cell activation. This strategy permitted us to analyze 6-distinct NK cell populations (B) Functional responses for CD107, IFN-γ and CCL4 were analyzed using Boolean gating. Background for anti-HIVIG rgp120-coated CEM cell stimulation was stimulation of PBMCs with rgp120-coated CEM cells (no HIVIG). Results reported were background subtracted.

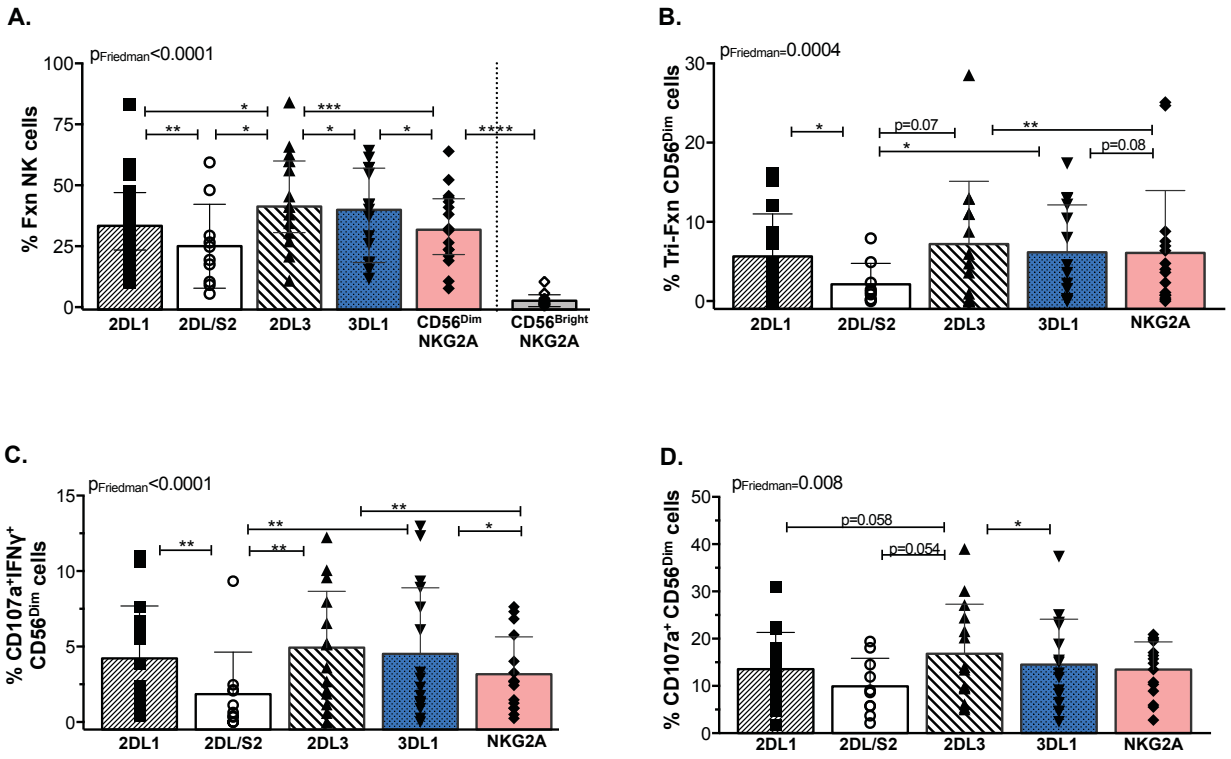


Figure 2: Low functionality of CD56^{Dim}2DL/S2 and CD56^{Bright}NKG2A^{+} NK cells in response to anti-HIV Ab-dependent NK cell activation. The frequency of NK cell populations stimulated bin an Ab-dependent fashion characterized by total responsiveness (A), tri-functionality (B), CD107a^{+}IFN-γ^{+} (C) and CD107a^{+} (D) response profiles are shown in the y-axis for each CD56^{Dim}2DL1, 2DL2/S2, 2DL3, 3DL1, NKG2A and CD56^{Bright}NKG2A populations. Total responsiveness, defined as the sum of the frequency of all possible combination of CD107a expression and IFN-γ & CCL4 secretion, was plotted on the y-axis. Bar height represents the mean+/-SD for each group. Data from 16 individuals analyzed in duplicate were used to generate these results. Friedman ($p_{Friedman}$) and Wilcoxon (*) tests were used to determine significance between data sets. P-values for between-group comparisons are shown over lines linking the two groups being compared.

8. REFERENCES

1. Centers for Disease C. Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. *MMWR Morb Mortal Wkly Rep* 1981,**30**:305-308.

2. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA*, 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**:1425-1431.

3. Masur H, Michelis MA, Greene JB, Onorato I, Stouwe RA, Holzman RS*, et al.* An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med* 1981,**305**:1431-1438.

4. Centers for Disease C. Possible transfusion-associated acquired immune deficiency syndrome (AIDS) - California. *MMWR Morb Mortal Wkly Rep* 1982,**31**:652-654.

5. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J*, et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983,**220**:868-871.

6. 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**:497-500.

7. Wain-Hobson S, Vartanian JP, Henry M, Chenciner N, Cheynier R, Delassus S*, et al.* LAV revisited: origins of the early HIV-1 isolates from Institut Pasteur. *Science* 1991,**252**:961-965.

8. Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N*, et al.* Human immunodeficiency viruses. *Science* 1986,**232**:697.

9. Taylor BS, Sobieszczyk ME, McCutchan FE, Hammer SM. The challenge of HIV-1 subtype diversity. *N Engl J Med* 2008,**358**:1590-1602.

10. UNAIDS. Fact sheet 2015 - Statistics. 2016.

11. Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK*, et al.* HIV-1 nomenclature proposal. *Science* 2000,**288**:55-56.

12. Hemelaar J. The origin and diversity of the HIV-1 pandemic. *Trends Mol Med* 2012,**18**:182-192.

13. Korber B, Gaschen B, Yusim K, Thakallapally R, Kesmir C, Detours V. Evolutionary and immunological implications of contemporary HIV-1 variation. *Br Med Bull* 2001,**58**:19-42.

14. Clavel F, Hance AJ. HIV drug resistance. *N Engl J Med* 2004,**350**:1023-1035.

15. Kaleebu P, French N, Mahe C, Yirrell D, Watera C, Lyagoba F*, et al.* Effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression in a large cohort of HIV-1-positive persons in Uganda. *J Infect Dis* 2002,**185**:1244-1250.

16. Snoeck J, Kantor R, Shafer RW, Van Laethem K, Deforche K, Carvalho AP*, et al.* Discordances between interpretation algorithms for genotypic resistance to protease and reverse transcriptase inhibitors of human immunodeficiency virus are subtype dependent. *Antimicrob Agents Chemother* 2006,**50**:694-701.

17. Wainberg MA, Zaharatos GJ, Brenner BG. Development of antiretroviral drug resistance. *N Engl J Med* 2011,**365**:637-646.

18. Koehler RN, Alter G, Tovanabutra S, Saathoff E, Arroyo MA, Walsh AM*, et al.* Natural killer cell-mediated innate sieve effect on HIV-1: the impact of KIR/HLA polymorphism on HIV-1 subtype-specific acquisition in east Africa. *J Infect Dis* 2013,**208**:1250-1254.

19. Kuiken C, Leitner T, Foley B, Hahn B, Marx P, McCutchan F, et al. HIV sequence compendium 2009. Los Alamos, New Mexico: Los Alamos National Laboratory, Theoretical Biology and Biophysics. 2009.

20. UNAIDS. Fact sheet 2015 - Statistics. In; 2016.

21. Aldrich C, Hemelaar J. Global HIV-1 diversity surveillance. *Trends Mol Med* 2012,**18**:691-694.

22. Siemieniuk RA, Beckthold B, Gill MJ. Increasing HIV subtype diversity and its clinical implications in a sentinel North American population. *Can J Infect Dis Med Microbiol* 2013,**24**:69-73.

23. Briggs JA, Wilk T, Welker R, Krausslich HG, Fuller SD. Structural organization of authentic, mature HIV-1 virions and cores. *EMBO J* 2003,**22**:1707-1715.

24. Freed EO, Myers DJ, Risser R. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proc Natl Acad Sci U S A* 1990,**87**:4650-4654.

25. Weiss CD. HIV-1 gp41: mediator of fusion and target for inhibition. *AIDS Rev* 2003,**5**:214-221.

26. Yuan X, Yu X, Lee TH, Essex M. Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. *J Virol* 1993,**67**:6387-6394.

27. Hill CP, Worthylake D, Bancroft DP, Christensen AM, Sundquist WI. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proc Natl Acad Sci U S A* 1996,**93**:3099-3104.

28. Goel A, Mazur SJ, Fattah RJ, Hartman TL, Turpin JA, Huang M*, et al.* Benzamide-based thiolcarbamates: a new class of HIV-1 NCp7 inhibitors. *Bioorg Med Chem Lett* 2002,**12**:767-770.

29. Sierra S, Kupfer B, Kaiser R. Basics of the virology of HIV-1 and its replication. *J Clin Virol* 2005,**34**:233-244.

30. Frankel AD, Young JA. HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem* 1998,**67**:1-25.

31. Kondo E, Mammano F, Cohen EA, Gottlinger HG. The p6gag domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. *J Virol* 1995,**69**:2759-2764.

32. Damgaard CK, Andersen ES, Knudsen B, Gorodkin J, Kjems J. RNA interactions in the 5' region of the HIV-1 genome. *J Mol Biol* 2004,**336**:369-379.

33. Wilkinson KA, Gorelick RJ, Vasa SM, Guex N, Rein A, Mathews DH*, et al.* High-throughput SHAPE analysis reveals structures in HIV-1 genomic RNA strongly conserved across distinct biological states. *PLoS Biol* 2008,**6**:e96.

34. Watts JM, Dang KK, Gorelick RJ, Leonard CW, Bess JW, Jr., Swanstrom R*, et al.* Architecture and secondary structure of an entire HIV-1 RNA genome. *Nature* 2009,**460**:711-716.

35. Starcich B, Ratner L, Josephs SF, Okamoto T, Gallo RC, Wong-Staal F. Characterization of long terminal repeat sequences of HTLV-III. *Science* 1985,**227**:538-540.

36. Louis JM, Nashed NT, Parris KD, Kimmel AR, Jerina DM. Kinetics and mechanism of autoprocessing of human immunodeficiency virus type 1 protease from an analog of the Gag-Pol polyprotein. *Proc Natl Acad Sci U S A* 1994,**91**:7970-7974.

37. Louis JM, Clore GM, Gronenborn AM. Autoprocessing of HIV-1 protease is tightly coupled to protein folding. *Nat Struct Biol* 1999,**6**:868-875.

38. Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, Hendrickson WA*, et al.* A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* 1998,**280**:1949-1953.

39. Wrin T, Crawford L, Sawyer L, Weber P, Sheppard HW, Hanson CV. Neutralizing antibody responses to autologous and heterologous isolates of human immunodeficiency virus. *J Acquir Immune Defic Syndr* 1994,**7**:211-219.

40. Burton DR, Stanfield RL, Wilson IA. Antibody vs. HIV in a clash of evolutionary titans. *Proc Natl Acad Sci U S A* 2005,**102**:14943-14948.

41. Belair M, Dovat M, Foley B, Mayerat C, Pantaleo G, Graziosi C. The polymorphic nature of HIV type 1 env V4 affects the patterns of potential N-glycosylation sites in proviral DNA at the intrahost level. *AIDS Res Hum Retroviruses* 2009,**25**:199-206.

42. Harrich D, Hooker B. Mechanistic aspects of HIV-1 reverse transcription initiation. *Rev Med Virol* 2002,**12**:31-45.

43. Harrich D, Ulich C, Garcia-Martinez LF, Gaynor RB. Tat is required for efficient HIV-1 reverse transcription. *EMBO J* 1997,**16**:1224-1235.

44. Fischer U, Pollard VW, Luhrmann R, Teufel M, Michael MW, Dreyfuss G*, et al.* Rev-mediated nuclear export of RNA is dominant over nuclear retention and is coupled to the Ran-GTPase cycle. *Nucleic Acids Res* 1999,**27**:4128-4134.

45. Suhasini M, Reddy TR. Cellular proteins and HIV-1 Rev function. *Curr HIV Res* 2009,**7**:91-100.

46. Balliet JW, Kolson DL, Eiger G, Kim FM, McGann KA, Srinivasan A*, et al.* Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate. *Virology* 1994,**200**:623-631.

47. Connor RI, Chen BK, Choe S, Landau NR. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* 1995,**206**:935-944.

48. Nitahara-Kasahara Y, Kamata M, Yamamoto T, Zhang X, Miyamoto Y, Muneta K*, et al.* Novel nuclear import of Vpr promoted by importin alpha is crucial for human immunodeficiency virus type 1 replication in macrophages. *J Virol* 2007,**81**:5284-5293.

49. Kogan M, Rappaport J. HIV-1 accessory protein Vpr: relevance in the pathogenesis of HIV and potential for therapeutic intervention. *Retrovirology* 2011,**8**:25.

50. Lu YL, Spearman P, Ratner L. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J Virol* 1993,**67**:6542-6550.

51. Poeschla EM. Integrase, LEDGF/p75 and HIV replication. *Cell Mol Life Sci* 2008,**65**:1403-1424.

52. Di Marzio P, Choe S, Ebright M, Knoblauch R, Landau NR. Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. *J Virol* 1995,**69**:7909-7916.

53. Andersen JL, Le Rouzic E, Planelles V. HIV-1 Vpr: mechanisms of G2 arrest and apoptosis. *Exp Mol Pathol* 2008,**85**:2-10.

54. Sato A, Yoshimoto J, Isaka Y, Miki S, Suyama A, Adachi A*, et al.* Evidence for direct association of Vpr and matrix protein p17 within the HIV-1 virion. *Virology* 1996,**220**:208-212.

55. Sheehy AM, Gaddis NC, Choi JD, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 2002,**418**:646-650.

56. Marin M, Rose KM, Kozak SL, Kabat D. HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nat Med* 2003,**9**:1398-1403.

57. Stopak K, de Noronha C, Yonemoto W, Greene WC. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. *Mol Cell* 2003,**12**:591-601.

58. Harris RS, Petersen-Mahrt SK, Neuberger MS. RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. *Mol Cell* 2002,**10**:1247-1253.

59. Petersen-Mahrt SK, Harris RS, Neuberger MS. AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification. *Nature* 2002,**418**:99-103.

60. Garcia JV, Miller AD. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature* 1991,**350**:508-511.

61. Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. *Immunity* 2002,**16**:493-497.

62. Dube M, Bego MG, Paquay C, Cohen EA. Modulation of HIV-1-host interaction: role of the Vpu accessory protein. *Retrovirology* 2010,**7**:114.

63. Kestler HW, 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD*, et al.* Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* 1991,**65**:651-662.

64. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N Engl J Med* 1995,**332**:228-232.

65. Ewart GD, Sutherland T, Gage PW, Cox GB. The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. *J Virol* 1996,**70**:7108-7115.

66. Neil SJ, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 2008,**451**:425-430.

67. Moore JP, Nara PL. The role of the V3 loop of gp120 in HIV infection. *AIDS* 1991,**5 Suppl 2**:S21-33.

68. Doms RW, Moore JP. HIV-1 membrane fusion: targets of opportunity. *J Cell Biol* 2000,**151**:F9-14.

69. Hu WS, Temin HM. Retroviral recombination and reverse transcription. *Science* 1990,**250**:1227-1233.

70. Peliska JA, Benkovic SJ. Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. *Science* 1992,**258**:1112-1118.

71. Jonckheere H, Anne J, De Clercq E. The HIV-1 reverse transcription (RT) process as target for RT inhibitors. *Med Res Rev* 2000,**20**:129-154.

72. Popov S, Rexach M, Zybarth G, Reiling N, Lee MA, Ratner L*, et al.* Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. *EMBO J* 1998,**17**:909-917.

73. Kamata M, Nitahara-Kasahara Y, Miyamoto Y, Yoneda Y, Aida Y. Importin-alpha promotes passage through the nuclear pore complex of human immunodeficiency virus type 1 Vpr. *J Virol* 2005,**79**:3557-3564.

74. Le Rouzic E, Benichou S. The Vpr protein from HIV-1: distinct roles along the viral life cycle. *Retrovirology* 2005,**2**:11.

75. Brown PO, Bowerman B, Varmus HE, Bishop JM. Correct integration of retroviral DNA in vitro. *Cell* 1987,**49**:347-356.

76. Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 2002,**110**:521-529.

77. Jordan A, Defechereux P, Verdin E. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *EMBO J* 2001,**20**:1726-1738.

78. Pollard VW, Malim MH. The HIV-1 Rev protein. *Annu Rev Microbiol* 1998,**52**:491-532.

79. Jacks T, Power MD, Masiarz FR, Luciw PA, Barr PJ, Varmus HE. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature* 1988,**331**:280-283.

80. Sundquist WI, Krausslich HG. HIV-1 assembly, budding, and maturation. *Cold Spring Harb Perspect Med* 2012,**2**:a006924.

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

82. Zimmerman C, Klein KC, Kiser PK, Singh AR, Firestein BL, Riba SC*, et al.* Identification of a host protein essential for assembly of immature HIV-1 capsids. *Nature* 2002,**415**:88-92.

83. Kleiman L, Cen S. The tRNALys packaging complex in HIV-1. *Int J Biochem Cell Biol* 2004,**36**:1776-1786.

84. Haase AT. Targeting early infection to prevent HIV-1 mucosal transmission. *Nature* 2010,**464**:217-223.

85. Wolinsky SM, Wike CM, Korber BT, Hutto C, Parks WP, Rosenblum LL*, et al.* Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* 1992,**255**:1134-1137.

86. Zhu T, Wang N, Carr A, Nam DS, Moor-Jankowski R, Cooper DA*, 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**:3098-3107.

87. Derdeyn CA, Decker JM, Bibollet-Ruche F, Mokili JL, Muldoon M, Denham SA*, et al.* Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. *Science* 2004,**303**:2019-2022.

88. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG*, et al.* Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 2008,**105**:7552-7557.

89. Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H*, et al.* Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med* 2009,**206**:1273-1289.

90. Morgan C, Marthas M, Miller C, Duerr A, Cheng-Mayer C, Desrosiers R*, et al.* The use of nonhuman primate models in HIV vaccine development. *PLoS Med* 2008,**5**:e173.

91. Haaland RE, Hawkins PA, Salazar-Gonzalez J, Johnson A, Tichacek A, Karita E*, et al.* Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. *PLoS Pathog* 2009,**5**:e1000274.

92. Weiss RA. How does HIV cause AIDS? *Science* 1993,**260**:1273-1279.

93. Schacker T, Collier AC, Hughes J, Shea T, Corey L. Clinical and epidemiologic features of primary HIV infection. *Ann Intern Med* 1996,**125**:257-264.

94. Gurunathan S, Habib RE, Baglyos L, Meric C, Plotkin S, Dodet B*, et al.* Use of predictive markers of HIV disease progression in vaccine trials. *Vaccine* 2009,**27**:1997-2015.

95. Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, Peddada L*, et al.* Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS* 2003,**17**:1871-1879.

96. Mellors JW, Munoz A, Giorgi JV, Margolick JB, Tassoni CJ, Gupta P*, et al.* Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Ann Intern Med* 1997,**126**:946-954.

97. McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol* 2010,**10**:11-23.

98. Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, Knight HL*, et al.* Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 1998,**280**:427-431.

99. Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A*, et al.* Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* 2003,**77**:11708-11717.

100. Stebbing J, Gazzard B, Douek DC. Where does HIV live? *N Engl J Med* 2004,**350**:1872-1880.

101. Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D*, et al.* HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nat Immunol* 2008,**9**:301-309.

102. Grossman Z, Meier-Schellersheim M, Paul WE, Picker LJ. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat Med* 2006,**12**:289-295.

103. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X*, et al.* Antibody neutralization and escape by HIV-1. *Nature* 2003,**422**:307-312.

104. Ackerman ME, Dugast AS, Alter G. Emerging concepts on the role of innate immunity in the prevention and control of HIV infection. *Annu Rev Med* 2012,**63**:113-130.

105. Aasa-Chapman MM, Holuigue S, Aubin K, Wong M, Jones NA, Cornforth D*, et al.* Detection of antibody-dependent complement-mediated inactivation of both autologous and heterologous virus in primary human immunodeficiency virus type 1 infection. *J Virol* 2005,**79**:2823-2830.

106. Huber M, Fischer M, Misselwitz B, Manrique A, Kuster H, Niederost B*, et al.* Complement lysis activity in autologous plasma is associated with lower viral loads during the acute phase of HIV-1 infection. *PLoS Med* 2006,**3**:e441.

107. Weiss RA. Special anniversary review: twenty-five years of human immunodeficiency virus research: successes and challenges. *Clin Exp Immunol* 2008,**152**:201-210.

108. Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, Huang FP*, et al.* Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 1995,**375**:408-411.

109. Wu H, Zhu H, Miao H, Perelson AS. Parameter identifiability and estimation of HIV/AIDS dynamic models. *Bull Math Biol* 2008,**70**:785-799.

110. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR Recomm Rep* 1992,**41**:1-19.

111. Ananworanich J, Fletcher JL, Pinyakorn S, van Griensven F, Vandergeeten C, Schuetz A*, et al.* A novel acute HIV infection staging system based on 4th generation immunoassay. *Retrovirology* 2013,**10**:56.

112. Kuritzkes DR. HIV-1 entry inhibitors: an overview. *Curr Opin HIV AIDS* 2009,**4**:82-87.

113. Jochmans D. Novel HIV-1 reverse transcriptase inhibitors. *Virus Res* 2008,**134**:171-185.

114. de Bethune MP. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989-2009). *Antiviral Res* 2010,**85**:75-90.

115. Quashie PK, Sloan RD, Wainberg MA. Novel therapeutic strategies targeting HIV integrase. *BMC Med* 2012,**10**:34.

116. Deeks SG, Smith M, Holodniy M, Kahn JO. HIV-1 protease inhibitors. A review for clinicians. *JAMA* 1997,**277**:145-153.

117. Martinez-Cajas JL, Wainberg MA. Protease inhibitor resistance in HIV-infected patients: molecular and clinical perspectives. *Antiviral Res* 2007,**76**:203-221.

118. Survival after introduction of HAART in people with known duration of HIV-1 infection. The CASCADE Collaboration. Concerted Action on SeroConversion to AIDS and Death in Europe. *Lancet* 2000,**355**:1158-1159.

119. Hammer SM, Katzenstein DA, Hughes MD, Gundacker H, Schooley RT, Haubrich RH*, et al.* A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. AIDS Clinical Trials Group Study 175 Study Team. *N Engl J Med* 1996,**335**:1081-1090.

120. Krentz HB, Kliewer G, Gill MJ. Changing mortality rates and causes of death for HIV-infected individuals living in Southern Alberta, Canada from 1984 to 2003. *HIV Med* 2005,**6**:99-106.

121. Hammer SM, Saag MS, Schechter M, Montaner JS, Schooley RT, Jacobsen DM*, et al.* Treatment for adult HIV infection: 2006 recommendations of the International AIDS Society-USA panel. *JAMA* 2006,**296**:827-843.

122. Palella FJ, Jr., Baker RK, Moorman AC, Chmiel JS, Wood KC, Brooks JT*, et al.* Mortality in the highly active antiretroviral therapy era: changing causes of death and disease in the HIV outpatient study. *J Acquir Immune Defic Syndr* 2006,**43**:27-34.

123. Martinez-Cajas JL, Pant-Pai N, Klein MB, Wainberg MA. Role of genetic diversity amongst HIV-1 non-B subtypes in drug resistance: a systematic review of virologic and biochemical evidence. *AIDS Rev* 2008,**10**:212-223.

124. Anta L, Blanco JL, Llibre JM, Garcia F, Perez-Elias MJ, Aguilera A*, et al.* Resistance to the most recent protease and non-nucleoside reverse transcriptase inhibitors across HIV-1 non-B subtypes. *J Antimicrob Chemother* 2013,**68**:1994-2002.

125. Bruner KM, Hosmane NN, Siliciano RF. Towards an HIV-1 cure: measuring the latent reservoir. *Trends Microbiol* 2015,**23**:192-203.

126. Ho YC, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DI*, et al.* Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* 2013,**155**:540-551.

127. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A* 1998,**95**:8869-8873.

128. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 1995,**373**:123-126.

129. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P*, et al.* Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 1995,**373**:117-122.

130. Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H*, et al.* Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* 1997,**387**:183-188.

131. Chun TW, Davey RT, Jr., Ostrowski M, Shawn Justement J, Engel D, Mullins JI*, et al.* Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. *Nat Med* 2000,**6**:757-761.

132. Deeks SG, Wrin T, Liegler T, Hoh R, Hayden M, Barbour JD*, et al.* Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *N Engl J Med* 2001,**344**:472-480.

133. Ruff CT, Ray SC, Kwon P, Zinn R, Pendleton A, Hutton N*, et al.* Persistence of wild-type virus and lack of temporal structure in the latent reservoir for human immunodeficiency virus type 1 in pediatric patients with extensive antiretroviral exposure. *J Virol* 2002,**76**:9481-9492.

134. Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB*, et al.* Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat Med* 2003,**9**:727-728.

135. Ramratnam B, Mittler JE, Zhang L, Boden D, Hurley A, Fang F*, et al.* The decay of the latent reservoir of replication-competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. *Nat Med* 2000,**6**:82-85.

136. Sedaghat AR, Siliciano JD, Brennan TP, Wilke CO, Siliciano RF. Limits on replenishment of the resting CD4+ T cell reservoir for HIV in patients on HAART. *PLoS Pathog* 2007,**3**:e122.

137. Archin NM, Margolis DM. Emerging strategies to deplete the HIV reservoir. *Curr Opin Infect Dis* 2014,**27**:29-35.

138. Dean M, Carrington M, Goedert J, O'Brien S J. Participants in HIV Study: Correction. *Science* 1996,**274**:1069a.

139. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R*, et al.* Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 1996,**86**:367-377.

140. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM*, et al.* Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 1996,**382**:722-725.

141. Margolis L, Shattock R. Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved? *Nat Rev Microbiol* 2006,**4**:312-317.

142. Rappaport J, Cho YY, Hendel H, Schwartz EJ, Schachter F, Zagury JF. 32 bp CCR-5 gene deletion and resistance to fast progression in HIV-1 infected heterozygotes. *Lancet* 1997,**349**:922-923.

143. de Roda Husman AM, Koot M, Cornelissen M, Keet IP, Brouwer M, Broersen SM*, et al.* Association between CCR5 genotype and the clinical course of HIV-1 infection. *Ann Intern Med* 1997,**127**:882-890.

144. Hutter G, Nowak D, Mossner M, Ganepola S, Mussig A, Allers K*, et al.* Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* 2009,**360**:692-698.

145. Balotta C, Bagnarelli P, Violin M, Ridolfo AL, Zhou D, Berlusconi A*, et al.* Homozygous delta 32 deletion of the CCR-5 chemokine receptor gene in an HIV-1-infected patient. *AIDS* 1997,**11**:F67-71.

146. Kaslow RA, McNicholl JM. Genetic determinants of HIV-1 infection and its manifestations. *Proc Assoc Am Physicians* 1999,**111**:299-307.

147. Glass WG, McDermott DH, Lim JK, Lekhong S, Yu SF, Frank WA*, et al.* CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J Exp Med* 2006,**203**:35-40.

148. Rossol M, Pierer M, Arnold S, Keysser G, Burkhardt H, Baerwald C*, et al.* Negative association of the chemokine receptor CCR5 d32 polymorphism with systemic inflammatory response, extra-articular symptoms and joint erosion in rheumatoid arthritis. *Arthritis Res Ther* 2009,**11**:R91.

149. Bai J, Gorantla S, Banda N, Cagnon L, Rossi J, Akkina R. Characterization of anti-CCR5 ribozyme-transduced CD34+ hematopoietic progenitor cells in vitro and in a SCID-hu mouse model in vivo. *Mol Ther* 2000,**1**:244-254.

150. Anderson J, Li MJ, Palmer B, Remling L, Li S, Yam P*, et al.* Safety and efficacy of a lentiviral vector containing three anti-HIV genes--CCR5 ribozyme, tat-rev siRNA, and TAR decoy--in SCID-hu mouse-derived T cells. *Mol Ther* 2007,**15**:1182-1188.

151. Kumar P, Ban HS, Kim SS, Wu H, Pearson T, Greiner DL*, et al.* T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* 2008,**134**:577-586.

152. Holt N, Wang J, Kim K, Friedman G, Wang X, Taupin V*, et al.* Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. *Nat Biotechnol* 2010,**28**:839-847.

153. Meyers AF, Fowke KR. International symposium on natural immunity to HIV: a gathering of the HIV-exposed seronegative clan. *J Infect Dis* 2010,**202 Suppl 3**:S327-328.

154. Salkowitz JR, Purvis SF, Meyerson H, Zimmerman P, O'Brien TR, Aledort L*, et al.* Characterization of high-risk HIV-1 seronegative hemophiliacs. *Clin Immunol* 2001,**98**:200-211.

155. Coovadia HM, Rollins NC, Bland RM, Little K, Coutsoudis A, Bennish ML*, et al.* Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study. *Lancet* 2007,**369**:1107-1116.

156. Kesho Bora Study G, de Vincenzi I. Triple antiretroviral compared with zidovudine and single-dose nevirapine prophylaxis during pregnancy and breastfeeding for prevention of mother-to-child transmission of HIV-1 (Kesho Bora study): a randomised controlled trial. *Lancet Infect Dis* 2011,**11**:171-180.

157. Schouten EJ, Jahn A, Midiani D, Makombe SD, Mnthambala A, Chirwa Z*, et al.* Prevention of mother-to-child transmission of HIV and the health-related Millennium Development Goals: time for a public health approach. *Lancet* 2011,**378**:282-284.

158. Patel P, Borkowf CB, Brooks JT, Lasry A, Lansky A, Mermin J. Estimating per-act HIV transmission risk: a systematic review. *AIDS* 2014,**28**:1509-1519.

159. Fowke KR, Nagelkerke NJ, Kimani J, Simonsen JN, Anzala AO, Bwayo JJ*, et al.* Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *Lancet* 1996,**348**:1347-1351.

160. Lau JT, Wang M, Wong HN, Tsui HY, Jia M, Cheng F*, et al.* Prevalence of bisexual behaviors among men who have sex with men (MSM) in China and associations between condom use in MSM and heterosexual behaviors. *Sex Transm Dis* 2008,**35**:406-413.

161. Luchters S, Chersich MF, Rinyiru A, Barasa MS, King'ola N, Mandaliya K*, et al.* Impact of five years of peer-mediated interventions on sexual behavior and sexually transmitted infections among female sex workers in Mombasa, Kenya. *BMC Public Health* 2008,**8**:143.

162. Paxton WA, Martin SR, Tse D, O'Brien TR, Skurnick J, VanDevanter NL*, et al.* Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. *Nat Med* 1996,**2**:412-417.

163. Hladik F, Desbien A, Lang J, Wang L, Ding Y, Holte S*, et al.* Most highly exposed seronegative men lack HIV-1-specific, IFN-gamma-secreting T cells. *J Immunol* 2003,**171**:2671-2683.

164. Templeton DJ, Jin F, Mao L, Prestage GP, Donovan B, Imrie J*, et al.* Circumcision and risk of HIV infection in Australian homosexual men. *AIDS* 2009,**23**:2347-2351.

165. Ranki A, Mattinen S, Yarchoan R, Broder S, Ghrayeb J, Lahdevirta J*, et al.* T-cell response towards HIV in infected individuals with and without zidovudine therapy, and in HIV-exposed sexual partners. *AIDS* 1989,**3**:63-69.

166. Clerici M, Giorgi JV, Chou CC, Gudeman VK, Zack JA, Gupta P*, et al.* Cell-mediated immune response to human immunodeficiency virus (HIV) type 1 in seronegative homosexual men with recent sexual exposure to HIV-1. *J Infect Dis* 1992,**165**:1012-1019.

167. Rowland-Jones SL, Nixon DF, Aldhous MC, Gotch F, Ariyoshi K, Hallam N*, et al.* HIV-specific cytotoxic T-cell activity in an HIV-exposed but uninfected infant. *Lancet* 1993,**341**:860-861.

168. Clerici M, Levin JM, Kessler HA, Harris A, Berzofsky JA, Landay AL*, et al.* HIV-specific T-helper activity in seronegative health care workers exposed to contaminated blood. *JAMA* 1994,**271**:42-46.

169. Pinto LA, Sullivan J, Berzofsky JA, Clerici M, Kessler HA, Landay AL*, et al.* ENV-specific cytotoxic T lymphocyte responses in HIV seronegative health care workers occupationally exposed to HIV-contaminated body fluids. *J Clin Invest* 1995,**96**:867-876.

170. Rowland-Jones SL, Dong T, Fowke KR, Kimani J, Krausa P, Newell H*, et al.* Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *J Clin Invest* 1998,**102**:1758-1765.

171. Eyeson J, King D, Boaz MJ, Sefia E, Tomkins S, Waters A*, et al.* Evidence for Gag p24-specific CD4 T cells with reduced susceptibility to R5 HIV-1 infection in a UK cohort of HIV-exposed-seronegative subjects. *AIDS* 2003,**17**:2299-2311.

172. Lo Caputo S, Trabattoni D, Vichi F, Piconi S, Lopalco L, Villa ML*, et al.* Mucosal and systemic HIV-1-specific immunity in HIV-1-exposed but uninfected heterosexual men. *AIDS* 2003,**17**:531-539.

173. John R, Arango-Jaramillo S, Finny GJ, Schwartz DH. Risk associated HIV-1 cross-clade resistance of whole peripheral blood mononuclear cells from exposed uninfected individuals with wild-type CCR5. *J Acquir Immune Defic Syndr* 2004,**35**:1-8.

174. Pallikkuth S, Wanchu A, Bhatnagar A, Sachdeva RK, Sharma M. Human immunodeficiency virus (HIV) gag antigen-specific T-helper and granule-dependent CD8 T-cell activities in exposed but uninfected heterosexual partners of HIV type 1-infected individuals in North India. *Clin Vaccine Immunol* 2007,**14**:1196-1202.

175. Clerici M, Shearer GM. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol Today* 1994,**15**:575-581.

176. Promadej N, Costello C, Wernett MM, Kulkarni PS, Robison VA, Nelson KE*, et al.* Broad human immunodeficiency virus (HIV)-specific T cell responses to conserved HIV proteins in HIV-seronegative women highly exposed to a single HIV-infected partner. *J Infect Dis* 2003,**187**:1053-1063.

177. Jennes W, Vuylsteke B, Borget MY, Traore-Ettiegne V, Maurice C, Nolan M*, et al.* HIV-specific T helper responses and frequency of exposure among HIV-exposed seronegative female sex workers in Abidjan, Cote d'Ivoire. *J Infect Dis* 2004,**189**:602-610.

178. Kebba A, Kaleebu P, Rowland S, Ingram R, Whitworth J, Imami N*, et al.* Distinct patterns of peripheral HIV-1-specific interferon- gamma responses in exposed HIV-1-seronegative individuals. *J Infect Dis* 2004,**189**:1705-1713.

179. McLaren PJ, Ball TB, Wachihi C, Jaoko W, Kelvin DJ, Danesh A*, et al.* HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. *J Infect Dis* 2010,**202 Suppl 3**:S339-344.

180. Beyrer C, Artenstein AW, Rugpao S, Stephens H, VanCott TC, Robb ML*, et al.* Epidemiologic and biologic characterization of a cohort of human immunodeficiency virus type 1 highly exposed, persistently seronegative female sex workers in northern Thailand. Chiang Mai HEPS Working Group. *J Infect Dis* 1999,**179**:59-67.

181. Kaul R, Plummer F, Clerici M, Bomsel M, Lopalco L, Broliden K. Mucosal IgA in exposed, uninfected subjects: evidence for a role in protection against HIV infection. *AIDS* 2001,**15**:431-432.

182. Nguyen M, Pean P, Lopalco L, Nouhin J, Phoung V, Ly N*, et al.* HIV-specific antibodies but not t-cell responses are associated with protection in seronegative partners of HIV-1-infected individuals in Cambodia. *J Acquir Immune Defic Syndr* 2006,**42**:412-419.

183. Mazzoli S, Lopalco L, Salvi A, Trabattoni D, Lo Caputo S, Semplici F*, et al.* Human immunodeficiency virus (HIV)-specific IgA and HIV neutralizing activity in the serum of exposed seronegative partners of HIV-seropositive persons. *J Infect Dis* 1999,**180**:871-875.

184. Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P*, et al.* Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells. *J Immunol* 2000,**165**:5170-5176.

185. Clerici M, Barassi C, Devito C, Pastori C, Piconi S, Trabattoni D*, et al.* Serum IgA of HIV-exposed uninfected individuals inhibit HIV through recognition of a region within the alpha-helix of gp41. *AIDS* 2002,**16**:1731-1741.

186. Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, Trabattoni D*, et al.* Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G: a possible role in the resistance to HIV of HIV-exposed seronegative individuals. *J Infect Dis* 2007,**195**:960-964.

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

188. Cao Y, Qin L, Zhang L, Safrit J, Ho DD. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med* 1995,**332**:201-208.

189. Pantaleo G, Koup RA. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat Med* 2004,**10**:806-810.

190. Hubert JB, Burgard M, Dussaix E, Tamalet C, Deveau C, Le Chenadec J*, et al.* Natural history of serum HIV-1 RNA levels in 330 patients with a known date of infection. The SEROCO Study Group. *AIDS* 2000,**14**:123-131.

191. Moir S, Chun TW, Fauci AS. Pathogenic mechanisms of HIV disease. *Annu Rev Pathol* 2011,**6**:223-248.

192. Strathdee SA, Veugelers PJ, Page-Shafer KA, McNulty A, Moss AR, Schechter MT*, et al.* Lack of consistency between five definitions of nonprogression in cohorts of HIV-infected seroconverters. *AIDS* 1996,**10**:959-965.

193. Pereyra F, Addo MM, Kaufmann DE, Liu Y, Miura T, Rathod A*, et al.* Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J Infect Dis* 2008,**197**:563-571.

194. Saez-Cirion A, Hamimi C, Bergamaschi A, David A, Versmisse P, Melard A*, et al.* Restriction of HIV-1 replication in macrophages and CD4+ T cells from HIV controllers. *Blood* 2011,**118**:955-964.

195. Olson AD, Meyer L, Prins M, Thiebaut R, Gurdasani D, Guiguet M*, et al.* An evaluation of HIV elite controller definitions within a large seroconverter cohort collaboration. *PLoS One* 2014,**9**:e86719.

196. Prins M, Veugelers PJ. Comparison of progression and non-progression in injecting drug users and homosexual men with documented dates of HIV-1 seroconversion. European Seroconverter Study and the Tricontinental Seroconverter Study. *AIDS* 1997,**11**:621-631.

197. Madec Y, Boufassa F, Rouzioux C, Delfraissy JF, Meyer L, Group SS. Undetectable viremia without antiretroviral therapy in patients with HIV seroconversion: an uncommon phenomenon? *Clin Infect Dis* 2005,**40**:1350-1354.

198. Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ*, et al.* Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* 1995,**270**:988-991.

199. Wang B, Ge YC, Palasanthiran P, Xiang SH, Ziegler J, Dwyer DE*, et al.* Gene defects clustered at the C-terminus of the vpr gene of HIV-1 in long-term nonprogressing mother and child pair: in vivo evolution of vpr quasispecies in blood and plasma. *Virology* 1996,**223**:224-232.

200. Yamada T, Iwamoto A. Comparison of proviral accessory genes between long-term nonprogressors and progressors of human immunodeficiency virus type 1 infection. *Arch Virol* 2000,**145**:1021-1027.

201. Churchill MJ, Rhodes DI, Learmont JC, Sullivan JS, Wesselingh SL, Cooke IR*, et al.* Longitudinal analysis of human immunodeficiency virus type 1 nef/long terminal repeat sequences in a cohort of long-term survivors infected from a single source. *J Virol* 2006,**80**:1047-1052.

202. Okulicz JF, Marconi VC, Landrum ML, Wegner S, Weintrob A, Ganesan A*, et al.* Clinical outcomes of elite controllers, viremic controllers, and long-term nonprogressors in the US Department of Defense HIV natural history study. *J Infect Dis* 2009,**200**:1714-1723.

203. Bailey JR, Lassen KG, Yang HC, Quinn TC, Ray SC, Blankson JN*, et al.* Neutralizing antibodies do not mediate suppression of human immunodeficiency virus type 1 in elite

suppressors or selection of plasma virus variants in patients on highly active antiretroviral therapy. *J Virol* 2006,**80**:4758-4770.

204. Blankson JN, Bailey JR, Thayil S, Yang HC, Lassen K, Lai J*, et al.* Isolation and characterization of replication-competent human immunodeficiency virus type 1 from a subset of elite suppressors. *J Virol* 2007,**81**:2508-2518.

205. Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R*, et al.* Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* 1996,**273**:1856-1862.

206. Katzenstein TL, Eugen-Olsen J, Hofmann B, Benfield T, Pedersen C, Iversen AK*, et al.* HIV-infected individuals with the CCR delta32/CCR5 genotype have lower HIV RNA levels and higher CD4 cell counts in the early years of the infection than do patients with the wild type. Copenhagen AIDS Cohort Study Group. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997,**16**:10-14.

207. Meyer L, Magierowska M, Hubert JB, Rouzioux C, Deveau C, Sanson F*, et al.* Early protective effect of CCR-5 delta 32 heterozygosity on HIV-1 disease progression: relationship with viral load. The SEROCO Study Group. *AIDS* 1997,**11**:F73-78.

208. Michael NL, Chang G, Louie LG, Mascola JR, Dondero D, Birx DL*, et al.* The role of viral phenotype and CCR-5 gene defects in HIV-1 transmission and disease progression. *Nat Med* 1997,**3**:338-340.

209. Kaslow RA, Carrington M, Apple R, Park L, Munoz A, Saah AJ*, et al.* Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med* 1996,**2**:405-411.

210. O'Brien SJ, Gao X, Carrington M. HLA and AIDS: a cautionary tale. *Trends Mol Med* 2001,**7**:379-381.

211. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. *Annu Rev Med* 2003,**54**:535-551.

212. Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M*, et al.* A whole-genome association study of major determinants for host control of HIV-1. *Science* 2007,**317**:944-947.

213. International HIVCS, Pereyra F, Jia X, McLaren PJ, Telenti A, de Bakker PI*, et al.* The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science* 2010,**330**:1551-1557.

214. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW*, et al.* HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 2002,**3**:1061-1068.

215. Bailey JR, Williams TM, Siliciano RF, Blankson JN. Maintenance of viral suppression in HIV-1-infected HLA-B*57+ elite suppressors despite CTL escape mutations. *J Exp Med* 2006,**203**:1357-1369.

216. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J*, et al.* HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 2006,**107**:4781-4789.

217. Migueles SA, Osborne CM, Royce C, Compton AA, Joshi RP, Weeks KA*, et al.* Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* 2008,**29**:1009-1021.

218. Emu B, Sinclair E, Favre D, Moretto WJ, Hsue P, Hoh R*, et al.* Phenotypic, functional, and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment. *J Virol* 2005,**79**:14169-14178.

219. Pitcher CJ, Quittner C, Peterson DM, Connors M, Koup RA, Maino VC*, et al.* HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med* 1999,**5**:518-525.

220. Emu B, Sinclair E, Hatano H, Ferre A, Shacklett B, Martin JN*, et al.* HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control. *J Virol* 2008,**82**:5398-5407.

221. Deeks SG, Schweighardt B, Wrin T, Galovich J, Hoh R, Sinclair E*, et al.* Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. *J Virol* 2006,**80**:6155-6164.

222. Bailey JR, Sedaghat AR, Kieffer T, Brennan T, Lee PK, Wind-Rotolo M*, et al.* Residual human immunodeficiency virus type 1 viremia in some patients on antiretroviral therapy is

dominated by a small number of invariant clones rarely found in circulating CD4+ T cells. *J Virol* 2006,**80**:6441-6457.

223. Ackerman ME, Mikhailova A, Brown EP, Dowell KG, Walker BD, Bailey-Kellogg C*, et al.* Polyfunctional HIV-Specific Antibody Responses Are Associated with Spontaneous HIV Control. *PLoS Pathog* 2016,**12**:e1005315.

224. De Maria A, Fogli M, Costa P, Murdaca G, Puppo F, Mavilio D*, 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**:2410-2418.

225. Barker E, Martinson J, Brooks C, Landay A, Deeks S. Dysfunctional natural killer cells, in vivo, are governed by HIV viremia regardless of whether the infected individual is on antiretroviral therapy. *AIDS* 2007,**21**:2363-2365.

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

227. Vieillard V, Fausther-Bovendo H, Samri A, Debre P, French Asymptomatiques a Long Terme A-COSG. 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**:564-573.

228. Rosmaraki EE, Douagi I, Roth C, Colucci F, Cumano A, Di Santo JP. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* 2001,**31**:1900-1909.

229. Huntington ND, Vosshenrich CA, Di Santo JP. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol* 2007,**7**:703-714.

230. Yu J, Freud AG, Caligiuri MA. Location and cellular stages of natural killer cell development. *Trends Immunol* 2013,**34**:573-582.

231. Chan A, Hong DL, Atzberger A, Kollnberger S, Filer AD, Buckley CD*, et al.* CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. *J Immunol* 2007,**179**:89-94.

232. Romagnani C, Juelke K, Falco M, Morandi B, D'Agostino A, Costa R*, et al.* CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J Immunol* 2007,**178**:4947-4955.

233. Lanier LL, Testi R, Bindl J, Phillips JH. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med* 1989,**169**:2233-2238.

234. Caligiuri MA. Human natural killer cells. *Blood* 2008,**112**:461-469.

235. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* 1986,**136**:4480-4486.

236. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001,**22**:633-640.

237. Freud AG, Becknell B, Roychowdhury S, Mao HC, Ferketich AK, Nuovo GJ*, et al.* A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* 2005,**22**:295-304.

238. Orange JS. Unraveling human natural killer cell deficiency. *J Clin Invest* 2012,**122**:798-801.

239. Orange JS. Natural killer cell deficiency. *J Allergy Clin Immunol* 2013,**132**:515-525; quiz 526.

240. Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989,**47**:187-376.

241. Jost S, Altfeld M. Control of human viral infections by natural killer cells. *Annu Rev Immunol* 2013,**31**:163-194.

242. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *Journal of Immunological Methods* 2004,**294**:15-22.

243. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA*, et al.* Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* 2010,**116**:3853-3864.

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

245. Juelke K, Killig M, Luetke-Eversloh M, Parente E, Gruen J, Morandi B*, et al.* CD62L expression identifies a unique subset of polyfunctional CD56dim NK cells. *Blood* 2010,**116**:1299-1307.

246. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M*, et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med* 1999,**5**:405-411.

247. Ferlazzo G, Pack M, Thomas D, Paludan C, Schmid D, Strowig T*, et al.* Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc Natl Acad Sci U S A* 2004,**101**:16606-16611.

248. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 2002,**195**:343-351.

249. Della Chiesa M, Vitale M, Carlomagno S, Ferlazzo G, Moretta L, Moretta A. The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. *Eur J Immunol* 2003,**33**:1657-1666.

250. Reitano KN, Kottilil S, Gille CM, Zhang X, Yan M, O'Shea MA*, et al.* Defective plasmacytoid dendritic cell-NK cell cross-talk in HIV infection. *AIDS Res Hum Retroviruses* 2009,**25**:1029-1037.

251. Lanier LL. NK cell recognition. *Annu Rev Immunol* 2005,**23**:225-274.

252. Monticelli S, Natoli G. Short-term memory of danger signals and environmental stimuli in immune cells. *Nat Immunol* 2013,**14**:777-784.

253. Netea MG. Training innate immunity: the changing concept of immunological memory in innate host defence. *Eur J Clin Invest* 2013,**43**:881-884.

254. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature* 2009,**457**:557-561.

255. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* 2006,**7**:507-516.

256. Cooper MA, Elliott JM, Keyel PA, Yang L, Carrero JA, Yokoyama WM. Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci U S A* 2009,**106**:1915-1919.

257. Romee R, Schneider SE, Leong JW, Chase JM, Keppel CR, Sullivan RP*, et al.* Cytokine activation induces human memory-like NK cells. *Blood* 2012,**120**:4751-4760.

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

259. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK*, et al.* Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J Immunol* 2012,**189**:5082-5088.

260. O'Sullivan TE, Johnson LR, Kang HH, Sun JC. BNIP3- and BNIP3L-Mediated Mitophagy Promotes the Generation of Natural Killer Cell Memory. *Immunity* 2015,**43**:331-342.

261. Xu X, Araki K, Li S, Han JH, Ye L, Tan WG*, et al.* Autophagy is essential for effector CD8(+) T cell survival and memory formation. *Nat Immunol* 2014,**15**:1152-1161.

262. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 2008,**9**:495-502.

263. Ting AT, Karnitz LM, Schoon RA, Abraham RT, Leibson PJ. Fc gamma receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-gamma 1 and PLC-gamma 2 in natural killer cells. *J Exp Med* 1992,**176**:1751-1755.

264. Cella M, Fujikawa K, Tassi I, Kim S, Latinis K, Nishi S*, et al.* Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. *J Exp Med* 2004,**200**:817-823.

265. Tassi I, Colonna M. The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells. *J Immunol* 2005,**175**:7996-8002.

266. Faure M, Barber DF, Takahashi SM, Jin T, Long EO. Spontaneous clustering and tyrosine phosphorylation of NK cell inhibitory receptor induced by ligand binding. *J Immunol* 2003,**170**:6107-6114.

267. Stewart CA, Laugier-Anfossi F, Vely F, Saulquin X, Riedmuller J, Tisserant A*, et al.* Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A* 2005,**102**:13224-13229.

268. Graef T, Moesta AK, Norman PJ, Abi-Rached L, Vago L, Older Aguilar AM*, et al.* KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. *J Exp Med* 2009,**206**:2557-2572.

269. Beziat V, Duffy D, Quoc SN, Le Garff-Tavernier M, Decocq J, Combadiere B*, et al.* CD56brightCD16+ NK cells: a functional intermediate stage of NK cell differentiation. *J Immunol* 2011,**186**:6753-6761.

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

271. Chung AW, Kumar MP, Arnold KB, Yu WH, Schoen MK, Dunphy LJ*, et al.* Dissecting Polyclonal Vaccine-Induced Humoral Immunity against HIV Using Systems Serology. *Cell* 2015,**163**:988-998.

272. Richards JO, Karki S, Lazar GA, Chen H, Dang W, Desjarlais JR. Optimization of antibody binding to FcgammaRIIa enhances macrophage phagocytosis of tumor cells. *Mol Cancer Ther* 2008,**7**:2517-2527.

273. Smith P, DiLillo DJ, Bournazos S, Li F, Ravetch JV. Mouse model recapitulating human Fcgamma receptor structural and functional diversity. *Proc Natl Acad Sci U S A* 2012,**109**:6181-6186.

274. Bournazos S, Ravetch JV. Fcgamma receptor pathways during active and passive immunization. *Immunol Rev* 2015,**268**:88-103.

275. Forthal DN, Landucci G, Daar ES. Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells. *J Virol* 2001,**75**:6953-6961.

276. Smith PD, Meng G, Salazar-Gonzalez JF, Shaw GM. Macrophage HIV-1 infection and the gastrointestinal tract reservoir. *J Leukoc Biol* 2003,**74**:642-649.

277. Holl V, Peressin M, Decoville T, Schmidt S, Zolla-Pazner S, Aubertin AM*, et al.* Nonneutralizing antibodies are able to inhibit human immunodeficiency virus type 1 replication in macrophages and immature dendritic cells. *J Virol* 2006,**80**:6177-6181.

278. Ackerman ME, Dugast AS, McAndrew EG, Tsoukas S, Licht AF, Irvine DJ*, et al.* Enhanced phagocytic activity of HIV-specific antibodies correlates with natural production of immunoglobulins with skewed affinity for FcgammaR2a and FcgammaR2b. *J Virol* 2013,**87**:5468-5476.

279. Smalls-Mantey A, Connors M, Sattentau QJ. Comparative efficiency of HIV-1-infected T cell killing by NK cells, monocytes and neutrophils. *PLoS One* 2013,**8**:e74858.

280. Boesch AW, Alter G, Ackerman ME. Prospects for engineering HIV-specific antibodies for enhanced effector function and half-life. *Curr Opin HIV AIDS* 2015,**10**:160-169.

281. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. 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**:1109-1114.

282. Hatjiharissi E, Xu L, Santos DD, Hunter ZR, Ciccarelli BT, Verselis S*, 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**:2561-2564.

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

284. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM*, et al.* Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med* 2012,**366**:1275-1286.

285. Choi I, Chung AW, Suscovich TJ, Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S*, et al.* Machine learning methods enable predictive modeling of antibody feature:function relationships in RV144 vaccinees. *PLoS Comput Biol* 2015,**11**:e1004185.

286. Isitman G, Lisovsky I, Tremblay-McLean A, Parsons MS, Shoukry NH, Wainberg MA*, et al.* Natural killer cell education does not affect the magnitude of granzyme B delivery to target cells by antibody-dependent cellular cytotoxicity. *AIDS* 2015,**29**:1433-1443.

287. Lee WS, Richard J, Lichtfuss M, Smith AB, 3rd, Park J, Courter JR*, et al.* Antibody-Dependent Cellular Cytotoxicity against Reactivated HIV-1-Infected Cells. *J Virol* 2015,**90**:2021-2030.

288. Gooneratne SL, Center RJ, Kent SJ, Parsons MS. Functional advantage of educated KIR2DL1(+) natural killer cells for anti-HIV-1 antibody-dependent activation. *Clin Exp Immunol* 2016,**184**:101-109.

289. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC*, et al.* Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* 2001,**19**:197-223.

290. Koch J, Steinle A, Watzl C, Mandelboim O. Activating natural cytotoxicity receptors of natural killer cells in cancer and infection. *Trends Immunol* 2013,**34**:182-191.

291. Hecht ML, Rosental B, Horlacher T, Hershkovitz O, De Paz JL, Noti C*, et al.* Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences. *J Proteome Res* 2009,**8**:712-720.

292. Arnon TI, Achdout H, Levi O, Markel G, Saleh N, Katz G*, et al.* Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol* 2005,**6**:515-523.

293. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B*, et al.* The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med* 2009,**206**:1495-1503.

294. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* 2001,**31**:2680-2689.

295. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y*, et al.* Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 2001,**409**:1055-1060.

296. 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**:10981-10986.

297. Brown D, Trowsdale J, Allen R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. *Tissue Antigens* 2004,**64**:215-225.

298. Cosman D, Fanger N, Borges L, Kubin M, Chin W, Peterson L*, et al.* A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* 1997,**7**:273-282.

299. Lepin EJ, Bastin JM, Allan DS, Roncador G, Braud VM, Mason DY*, et al.* Functional characterization of HLA-F and binding of HLA-F tetramers to ILT2 and ILT4 receptors. *Eur J Immunol* 2000,**30**:3552-3561.

300. Beck S, Barrell BG. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. *Nature* 1988,**331**:269-272.

301. Chapman TL, Heikeman AP, Bjorkman PJ. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity* 1999,**11**:603-613.

302. Houchins JP, Yabe T, McSherry C, Bach FH. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J Exp Med* 1991,**173**:1017-1020.

303. Iwaszko M, Bogunia-Kubik K. Clinical significance of the HLA-E and CD94/NKG2 interaction. *Arch Immunol Ther Exp (Warsz)* 2011,**59**:353-367.

304. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS*, et al.* HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998,**391**:795-799.

305. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur J Immunol* 1997,**27**:1164-1169.

306. Llano M, Lee N, Navarro F, Garcia P, Albar JP, Geraghty DE*, et al.* HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol* 1998,**28**:2854-2863.

307. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H*, et al.* CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* 2010,**116**:3865-3874.

308. Wu J, Song Y, Bakker AB, Bauer S, Spies T, Lanier LL*, et al.* An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 1999,**285**:730-732.

309. Nolting A, Dugast AS, Rihn S, Luteijn R, Carrington MF, Kane K*, et al.* MHC class I chain-related protein A shedding in chronic HIV-1 infection is associated with profound NK cell dysfunction. *Virology* 2010,**406**:12-20.

310. Richard J, Sindhu S, Pham TN, Belzile JP, Cohen EA. HIV-1 Vpr up-regulates expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing. *Blood* 2010,**115**:1354-1363.

311. Norman JM, Mashiba M, McNamara LA, Onafuwa-Nuga A, Chiari-Fort E, Shen W*, et al.* The antiviral factor APOBEC3G enhances the recognition of HIV-infected primary T cells by natural killer cells. *Nat Immunol* 2011,**12**:975-983.

312. Cerboni C, Neri F, Casartelli N, Zingoni A, Cosman D, Rossi P*, 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**:242-250.

313. Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D. Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *J Biol Chem* 2004,**279**:7792-7798.

314. Bashirova AA, Thomas R, Carrington M. HLA/KIR restraint of HIV: surviving the fittest. *Annu Rev Immunol* 2011,**29**:295-317.

315. Trowsdale J, Jones DC, Barrow AD, Traherne JA. Surveillance of cell and tissue perturbation by receptors in the LRC. *Immunol Rev* 2015,**267**:117-136.

316. Middleton D, Gonzelez F. The extensive polymorphism of KIR genes. *Immunology* 2010,**129**:8-19.

317. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B*, et al.* Human diversity in killer cell inhibitory receptor genes. *Immunity* 1997,**7**:753-763.

318. Martin AM, Freitas EM, Witt CS, Christiansen FT. The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. *Immunogenetics* 2000,**51**:268-280.

319. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D*, et al.* Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A* 2000,**97**:4778-4783.

320. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* 2002,**20**:217-251.

321. Maxwell LD, Wallace A, Middleton D, Curran MD. A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey. *Tissue Antigens* 2002,**60**:254-258.

322. Middleton D, Gonzalez A, Gilmore PM. Studies on the expression of the deleted KIR2DS4*003 gene product and distribution of KIR2DS4 deleted and nondeleted versions in different populations. *Hum Immunol* 2007,**68**:128-134.

323. Marsh SG, Parham P, Dupont B, Geraghty DE, Trowsdale J, Middleton D*, et al.* Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics* 2003,**55**:220-226.

324. Martin AM, Kulski JK, Gaudieri S, Witt CS, Freitas EM, Trowsdale J*, et al.* Comparative genomic analysis, diversity and evolution of two KIR haplotypes A and B. *Gene* 2004,**335**:121-131.

325. Pyo CW, Guethlein LA, Vu Q, Wang R, Abi-Rached L, Norman PJ*, et al.* Different patterns of evolution in the centromeric and telomeric regions of group A and B haplotypes of the human killer cell Ig-like receptor locus. *PLoS One* 2010,**5**:e15115.

326. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Marsh SG*, et al.* Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J Immunol* 2014,**192**:4592-4600.

327. Rajalingam R, Parham P, Abi-Rached L. Domain shuffling has been the main mechanism forming new hominoid killer cell Ig-like receptors. *J Immunol* 2004,**172**:356-369.

328. Rajagopalan S, Long EO. KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front Immunol* 2012,**3**:258.

329. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A* 1993,**90**:12000-12004.

330. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R*, et al.* P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med* 1993,**178**:597-604.

331. Moesta AK, Parham P. Diverse functionality among human NK cell receptors for the C1 epitope of HLA-C: KIR2DS2, KIR2DL2, and KIR2DL3. *Front Immunol* 2012,**3**:336.

332. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol* 2008,**180**:3969-3979.

333. 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**:633-645.

334. 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**:733-740.

335. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J Exp Med* 1994,**180**:1235-1242.

336. 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**:1133-1144.

337. 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**:3671-3674.

338. Luque I, Solana R, Galiani MD, Gonzalez R, Garcia F, Lopez de Castro JA*, et al.* Threonine 80 on HLA-B27 confers protection against lysis by a group of natural killer clones. *Eur J Immunol* 1996,**26**:1974-1977.

339. O'Connor GM, Vivian JP, Widjaja JM, Bridgeman JS, Gostick E, Lafont BA*, et al.* Mutational and structural analysis of KIR3DL1 reveals a lineage-defining allotypic dimorphism that impacts both HLA and peptide sensitivity. *J Immunol* 2014,**192**:2875-2884.

340. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. *J Immunol* 2005,**175**:5222-5229.

341. 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**:1487-1491.

342. 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**:3053-3058.

343. 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**:2057-2064.

344. Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A1,2. *J Immunol* 1996,**156**:3098-3101.

345. Pende D, Biassoni R, Cantoni C, Verdiani S, Falco M, di Donato C*, et al.* The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. *J Exp Med* 1996,**184**:505-518.

346. Hansasuta P, Dong T, Thananchai H, Weekes M, Willberg C, Aldemir H*, et al.* Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol* 2004,**34**:1673-1679.

347. Wong-Baeza I, Ridley A, Shaw J, Hatano H, Rysnik O, McHugh K*, et al.* KIR3DL2 binds to HLA-B27 dimers and free H chains more strongly than other HLA class I and promotes the expansion of T cells in ankylosing spondylitis. *J Immunol* 2013,**190**:3216-3224.

348. Gillespie GM, Bashirova A, Dong T, McVicar DW, Rowland-Jones SL, Carrington M. Lack of KIR3DS1 binding to MHC class I Bw4 tetramers in complex with CD8+ T cell epitopes. *AIDS Res Hum Retroviruses* 2007,**23**:451-455.

349. O'Connor GM, Vivian JP, Gostick E, Pymm P, Lafont BA, Price DA*, et al.* Peptide-Dependent Recognition of HLA-B*57:01 by KIR3DS1. *J Virol* 2015,**89**:5213-5221.

350. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M*, et al.* The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med* 1996,**183**:645-650.

351. Moesta AK, Graef T, Abi-Rached L, Older Aguilar AM, Guethlein LA, Parham P. Humans differ from other hominids in lacking an activating NK cell receptor that recognizes the C1 epitope of MHC class I. *J Immunol* 2010,**185**:4233-4237.

352. Hilton HG, Vago L, Older Aguilar AM, Moesta AK, Graef T, Abi-Rached L*, et al.* Mutation at positively selected positions in the binding site for HLA-C shows that KIR2DL1 is a more refined but less adaptable NK cell receptor than KIR2DL3. *J Immunol* 2012,**189**:1418-1430.

353. Saulquin X, Gastinel LN, Vivier E. Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158j). *J Exp Med* 2003,**197**:933-938.

354. Katz G, Markel G, Mizrahi S, Arnon TI, Mandelboim O. Recognition of HLA-Cw4 but not HLA-Cw6 by the NK cell receptor killer cell Ig-like receptor two-domain short tail number 4. *J Immunol* 2001,**166**:7260-7267.

355. Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol* 2002,**168**:6208-6214.

356. Kikuchi-Maki A, Catina TL, Campbell KS. Cutting edge: KIR2DL4 transduces signals into human NK cells through association with the Fc receptor gamma protein. *J Immunol* 2005,**174**:3859-3863.

357. Goodridge JP, Witt CS, Christiansen FT, Warren HS. KIR2DL4 (CD158d) genotype influences expression and function in NK cells. *J Immunol* 2003,**171**:1768-1774.

358. Rajagopalan S, Bryceson YT, Kuppusamy SP, Geraghty DE, van der Meer A, Joosten I*, et al.* Activation of NK cells by an endocytosed receptor for soluble HLA-G. *PLoS Biol* 2006,**4**:e9.

359. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L*, et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 2005,**436**:709-713.

360. 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**:331-342.

361. Yokoyama WM, Kim S. How do natural killer cells find self to achieve tolerance? *Immunity* 2006,**24**:249-257.

362. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol* 2006,**6**:520-531.

363. 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**:2434-2441.

364. 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**:4572-4580.

365. 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**:1166-1174.

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

367. Kamya P, Boulet S, Tsoukas CM, Routy JP, Thomas R, Cote P*, 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**:5949-5960.

368. Sun JC, Lanier LL. Cutting edge: viral infection breaks NK cell tolerance to "missing self". *J Immunol* 2008,**181**:7453-7457.

369. Sun JC. Re-educating natural killer cells. *J Exp Med* 2010,**207**:2049-2052.

370. 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**:2065-2072.

371. 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**:2073-2079.

372. Klein E, Ben-Bassat H, Neumann H, Ralph P, Zeuthen J, Polliack A*, et al.* Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. *Int J Cancer* 1976,**18**:421-431.

373. Kavathas P, Bach FH, DeMars R. Gamma ray-induced loss of expression of HLA and glyoxalase I alleles in lymphoblastoid cells. *Proc Natl Acad Sci U S A* 1980,**77**:4251-4255.

374. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL*, et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999,**285**:727-729.

375. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B*, et al.* Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med* 2003,**198**:557-567.

376. Chalupny NJ, Sutherland CL, Lawrence WA, Rein-Weston A, Cosman D. ULBP4 is a novel ligand for human NKG2D. *Biochem Biophys Res Commun* 2003,**305**:129-135.

377. Sandusky MM, Messmer B, Watzl C. Regulation of 2B4 (CD244)-mediated NK cell activation by ligand-induced receptor modulation. *Eur J Immunol* 2006,**36**:3268-3276.

378. Sanchez-Correa B, Gayoso I, Bergua JM, Casado JG, Morgado S, Solana R*, et al.* Decreased expression of DNAM-1 on NK cells from acute myeloid leukemia patients. *Immunol Cell Biol* 2012,**90**:109-115.

379. Lisovsky I, Isitman G, Bruneau J, Bernard NF. Functional analysis of NK cell subsets activated by 721.221 and K562 HLA-null cells. *J Leukoc Biol* 2015,**97**:761-767.

380. Prod'homme V, Griffin C, Aicheler RJ, Wang EC, McSharry BP, Rickards CR*, et al.* The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells. *J Immunol* 2007,**178**:4473-4481.

381. Tomasec P, Braud VM, Rickards C, Powell MB, McSharry BP, Gadola S*, et al.* Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 2000,**287**:1031.

382. Guma M, Budt M, Saez A, Brckalo T, Hengel H, Angulo A*, et al.* Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* 2006,**107**:3624-3631.

383. Sun JC, Lanier LL. The Natural Selection of Herpesviruses and Virus-Specific NK Cell Receptors. *Viruses* 2009,**1**:362.

384. Loewendorf A, Benedict CA. Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything. *J Intern Med* 2010,**267**:483-501.

385. Guidotti LG, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006,**1**:23-61.

386. Alter G, Jost S, Rihn S, Reyor LL, Nolan BE, Ghebremichael M*, et al.* Reduced frequencies of NKp30+NKp46+, CD161+, and NKG2D+ NK cells in acute HCV infection may predict viral clearance. *J Hepatol* 2011,**55**:278-288.

387. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR*, et al.* Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* 2006,**131**:1887-1898.

388. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J*, et al.* HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 2004,**305**:872-874.

389. Romero V, Azocar J, Zuniga J, Clavijo OP, Terreros D, Gu X*, et al.* Interaction of NK inhibitory receptor genes with HLA-C and MHC class II alleles in Hepatitis C virus infection outcome. *Mol Immunol* 2008,**45**:2429-2436.

390. Knapp S, Warshow U, Hegazy D, Brackenbury L, Guha IN, Fowell A*, et al.* Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. *Hepatology* 2010,**51**:1168-1175.

391. Marangon AV, Silva GF, de Moraes CF, Grotto RM, Pardini MI, de Pauli DS*, et al.* KIR genes and their human leukocyte antigen ligands in the progression to cirrhosis in patients with chronic hepatitis C. *Hum Immunol* 2011,**72**:1074-1078.

392. Organization WH. Influenza surveillance outputs. 2016.

393. Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. *Immunol Rev* 1997,**159**:105-117.

394. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. *Emerg Infect Dis* 2006,**12**:48-54.

395. Draghi M, Pashine A, Sanjanwala B, Gendzekhadze K, Cantoni C, Cosman D*, et al.* NKp46 and NKG2D recognition of infected dendritic cells is necessary for NK cell activation in the human response to influenza infection. *J Immunol* 2007,**178**:2688-2698.

396. Du N, Zhou J, Lin X, Zhang Y, Yang X, Wang Y*, et al.* Differential activation of NK cells by influenza A pseudotype H5N1 and 1918 and 2009 pandemic H1N1 viruses. *J Virol* 2010,**84**:7822-7831.

397. Ahlenstiel G, Martin MP, Gao X, Carrington M, Rehermann B. Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. *J Clin Invest* 2008,**118**:1017-1026.

398. La D, Czarnecki C, El-Gabalawy H, Kumar A, Meyers AF, Bastien N*, et al.* Enrichment of variations in KIR3DL1/S1 and KIR2DL2/L3 among H1N1/09 ICU patients: an exploratory study. *PLoS One* 2011,**6**:e29200.

399. Aranda-Romo S, Garcia-Sepulveda CA, Comas-Garcia A, Lovato-Salas F, Salgado-Bustamante M, Gomez-Gomez A*, et al.* Killer-cell immunoglobulin-like receptors (KIR) in severe A (H1N1) 2009 influenza infections. *Immunogenetics* 2012,**64**:653-662.

400. Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C*, et al.* Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology* 2004,**14**:1229-1246.

401. Owen RE, Yamada E, Thompson CI, Phillipson LJ, Thompson C, Taylor E*, et al.* Alterations in receptor binding properties of recent human influenza H3N2 viruses are associated with reduced natural killer cell lysis of infected cells. *J Virol* 2007,**81**:11170-11178.

402. Achdout H, Manaster I, Mandelboim O. Influenza virus infection augments NK cell inhibition through reorganization of major histocompatibility complex class I proteins. *J Virol* 2008,**82**:8030-8037.

403. Alter G, Teigen N, Ahern R, Streeck H, Meier A, Rosenberg ES*, et al.* Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J Infect Dis* 2007,**195**:1452-1460.

404. Alter G, Altfeld M. NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. *J Intern Med* 2009,**265**:29-42.

405. Alter G, Heckerman D, Schneidewind A, Fadda L, Kadie CM, Carlson JM*, et al.* HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature* 2011,**476**:96-100.

406. Brenner BG, Dascal A, Margolese RG, Wainberg MA. Natural killer cell function in patients with acquired immunodeficiency syndrome and related diseases. *J Leukoc Biol* 1989,**46**:75-83.

407. Lucia MB, Froio N, Tacconelli E, Tumbarello M, Rutella S, Rumi C*, et al.* CD16+CD56+CD8+ natural killer (NK) cells are decreased during HIV infection. *Eur J Histochem* 1997,**41 Suppl 2**:197-198.

408. Goodier MR, Imami N, Moyle G, Gazzard B, Gotch F. Loss of the CD56hiCD16- NK cell subset and NK cell interferon-gamma production during antiretroviral therapy for HIV-1: partial recovery by human growth hormone. *Clin Exp Immunol* 2003,**134**:470-476.

409. Alter G, Teigen N, Davis BT, Addo MM, Suscovich TJ, Waring MT*, et al.* Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. *Blood* 2005,**106**:3366-3369.

410. Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E*, et al.* Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci U S A* 2005,**102**:2886-2891.

411. Taylor JM, Fahey JL, Detels R, Giorgi JV. CD4 percentage, CD4 number, and CD4:CD8 ratio in HIV infection: which to choose and how to use. *J Acquir Immune Defic Syndr* 1989,**2**:114-124.

412. Amadori A, Zamarchi R, De Silvestro G, Forza G, Cavatton G, Danieli GA*, et al.* Genetic control of the CD4/CD8 T-cell ratio in humans. *Nat Med* 1995,**1**:1279-1283.

413. Margolick JB, Munoz A, Donnenberg AD, Park LP, Galai N, Giorgi JV*, et al.* Failure of T-cell homeostasis preceding AIDS in HIV-1 infection. The Multicenter AIDS Cohort Study. *Nat Med* 1995,**1**:674-680.

414. Amadori A, Zamarchi R, Chieco-Bianchi L. CD4: CD8 ratio and HIV infection: the "tap-and-drain' hypothesis. *Immunol Today* 1996,**17**:414-417.

415. Franco JM, Leon-Leal JA, Leal M, Cano-Rodriguez A, Pineda JA, Macias J*, et al.* CD4+ and CD8+ T lymphocyte regeneration after anti-retroviral therapy in HIV-1-infected children and adult patients. *Clin Exp Immunol* 2000,**119**:493-498.

416. Kaufmann GR, Furrer H, Ledergerber B, Perrin L, Opravil M, Vernazza P*, et al.* Characteristics, determinants, and clinical relevance of CD4 T cell recovery to <500 cells/microL in HIV type 1-infected individuals receiving potent antiretroviral therapy. *Clin Infect Dis* 2005,**41**:361-372.

417. Ndumbi P, Gillis J, Raboud J, Cooper C, Hogg RS, Montaner JS*, et al.* Characteristics and determinants of T-cell phenotype normalization in HIV-1-infected individuals receiving long-term antiretroviral therapy. *HIV Med* 2014,**15**:153-164.

418. McLaren PJ, Coulonges C, Ripke S, van den Berg L, Buchbinder S, Carrington M*, et al.* Association study of common genetic variants and HIV-1 acquisition in 6,300 infected cases and 7,200 controls. *PLoS Pathog* 2013,**9**:e1003515.

419. Fellay J, Ge D, Shianna KV, Colombo S, Ledergerber B, Cirulli ET*, et al.* Common genetic variation and the control of HIV-1 in humans. *PLoS Genet* 2009,**5**:e1000791.

420. Databaes IP. In: EMBL-EBI; 2016.

421. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L*, et al.* HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* 2000,**97**:2709-2714.

422. Gao X, Bashirova A, Iversen AK, Phair J, Goedert JJ, Buchbinder S*, et al.* AIDS restriction HLA allotypes target distinct intervals of HIV-1 pathogenesis. *Nat Med* 2005,**11**:1290-1292.

423. Altfeld M, Addo MM, Rosenberg ES, Hecht FM, Lee PK, Vogel M*, et al.* Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *AIDS* 2003,**17**:2581-2591.

424. Migueles SA, Laborico AC, Imamichi H, Shupert WL, Royce C, McLaughlin M*, et al.* The differential ability of HLA B*5701+ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences. *J Virol* 2003,**77**:6889-6898.

425. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M*, et al.* HIV evolution: CTL escape mutation and reversion after transmission. *Nat Med* 2004,**10**:282-289.

426. Brumme ZL, Brumme CJ, Carlson J, Streeck H, John M, Eichbaum Q*, et al.* Marked epitope- and allele-specific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection. *J Virol* 2008,**82**:9216-9227.

427. Crawford H, Lumm W, Leslie A, Schaefer M, Boeras D, Prado JG*, et al.* Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. *J Exp Med* 2009,**206**:909-921.

428. Schneidewind A, Brockman MA, Yang R, Adam RI, Li B, Le Gall S*, et al.* Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *J Virol* 2007,**81**:12382-12393.

429. Goulder PJ, Phillips RE, Colbert RA, McAdam S, Ogg G, Nowak MA*, et al.* Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 1997,**3**:212-217.

430. Kelleher AD, Long C, Holmes EC, Allen RL, Wilson J, Conlon C*, et al.* Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med* 2001,**193**:375-386.

431. Feeney ME, Tang Y, Roosevelt KA, Leslie AJ, McIntosh K, Karthas N*, et al.* Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening

of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child. *J Virol* 2004,**78**:8927-8930.

432. Migueles SA, Connors M. Success and failure of the cellular immune response against HIV-1. *Nat Immunol* 2015,**16**:563-570.

433. Scorza Smeraldi R, Fabio G, Lazzarin A, Eisera NB, Moroni M, Zanussi C. HLA-associated susceptibility to acquired immunodeficiency syndrome in Italian patients with human-immunodeficiency-virus infection. *Lancet* 1986,**2**:1187-1189.

434. Itescu S, Mathur-Wagh U, Skovron ML, Brancato LJ, Marmor M, Zeleniuch-Jacquotte A*, et al.* HLA-B35 is associated with accelerated progression to AIDS. *J Acquir Immune Defic Syndr* 1992,**5**:37-45.

435. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ*, et al.* HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science* 1999,**283**:1748-1752.

436. Streeck H, Lichterfeld M, Alter G, Meier A, Teigen N, Yassine-Diab B*, et al.* Recognition of a defined region within p24 gag by CD8+ T cells during primary human immunodeficiency virus type 1 infection in individuals expressing protective HLA class I alleles. *J Virol* 2007,**81**:7725-7731.

437. Gao X, Nelson GW, Karacki P, Martin MP, Phair J, Kaslow R*, et al.* Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS. *N Engl J Med* 2001,**344**:1668-1675.

438. Huang J, Goedert JJ, Sundberg EJ, Cung TD, Burke PS, Martin MP*, et al.* HLA-B*35-Px-mediated acceleration of HIV-1 infection by increased inhibitory immunoregulatory impulses. *J Exp Med* 2009,**206**:2959-2966.

439. Stranger BE, Forrest MS, Clark AG, Minichiello MJ, Deutsch S, Lyle R*, et al.* Genome-wide associations of gene expression variation in humans. *PLoS Genet* 2005,**1**:e78.

440. Thomas R, Apps R, Qi Y, Gao X, Male V, O'HUigin C*, et al.* HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. *Nat Genet* 2009,**41**:1290-1294.

441. Kulkarni S, Savan R, Qi Y, Gao X, Yuki Y, Bass SE*, et al.* Differential microRNA regulation of HLA-C expression and its association with HIV control. *Nature* 2011,**472**:495-498.

442. Apps R, Qi Y, Carlson JM, Chen H, Gao X, Thomas R*, et al.* Influence of HLA-C expression level on HIV control. *Science* 2013,**340**:87-91.

443. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ*, et al.* Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* 2002,**31**:429-434.

444. Qi Y, Martin MP, Gao X, Jacobson L, Goedert JJ, Buchbinder S*, et al.* KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. *PLoS Pathog* 2006,**2**:e79.

445. Boulet S, Sharafi S, Simic N, Bruneau J, Routy JP, Tsoukas CM*, et al.* Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. *AIDS* 2008,**22**:595-599.

446. 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**:647-651.

447. 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**:3027-3036.

448. Song R, Lisovsky I, Lebouche B, Routy JP, Bruneau J, Bernard NF. HIV protective KIR3DL1/S1-HLA-B genotypes influence NK cell-mediated inhibition of HIV replication in autologous CD4 targets. *PLoS Pathog* 2014,**10**:e1003867.

449. Johansson S, Johansson M, Rosmaraki E, Vahlne G, Mehr R, Salmon-Divon M*, et al.* Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J Exp Med* 2005,**201**:1145-1155.

450. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL*, et al.* The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* 1999,**10**:661-671.

451. Korner C, Granoff ME, Amero MA, Sirignano MN, Vaidya SA, Jost S*, et al.* Increased frequency and function of KIR2DL1-3(+) NK cells in primary HIV-1 infection are determined by HLA-C group haplotypes. *Eur J Immunol* 2014,**44**:2938-2948.

452. Apps R, Del Prete GQ, Chatterjee P, Lara A, Brumme ZL, Brockman MA*, et al.* HIV-1 Vpu Mediates HLA-C Downregulation. *Cell Host Microbe* 2016,**19**:686-695.

453. Terahara K, Yamamoto T, Mitsuki YY, Shibusawa K, Ishige M, Mizukoshi F*, et al.* Fluorescent Reporter Signals, EGFP, and DsRed, Encoded in HIV-1 Facilitate the Detection of Productively Infected Cells and Cell-Associated Viral Replication Levels. *Front Microbiol* 2011,**2**:280.

454. Allam A, Majji S, Peachman K, Jagodzinski L, Kim J, Ratto-Kim S*, et al.* TFH cells accumulate in mucosal tissues of humanized-DRAG mice and are highly permissive to HIV-1. *Sci Rep* 2015,**5**:10443.

455. Ward J, Bonaparte M, Sacks J, Guterman J, Fogli M, Mavilio D*, et al.* HIV modulates the expression of ligands important in triggering natural killer cell cytotoxic responses on infected primary T-cell blasts. *Blood* 2007,**110**:1207-1214.

456. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W*, et al.* ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 2001,**14**:123-133.

457. Chen X, Trivedi PP, Ge B, Krzewski K, Strominger JL. Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. *Proc Natl Acad Sci U S A* 2007,**104**:6329-6334.

458. Stanietsky N, Rovis TL, Glasner A, Seidel E, Tsukerman P, Yamin R*, et al.* Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur J Immunol* 2013,**43**:2138-2150.

459. Reefman E, Kay JG, Wood SM, Offenhauser C, Brown DL, Roy S*, et al.* Cytokine secretion is distinct from secretion of cytotoxic granules in NK cells. *J Immunol* 2010,**184**:4852-4862.

460. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol* 2013,**31**:227-258.

461. Pende D, Rivera P, Marcenaro S, Chang CC, Biassoni R, Conte R*, et al.* Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Res* 2002,**62**:6178-6186.

462. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 2005,**105**:4416-4423.

463. Beziat V, Liu LL, Malmberg JA, Ivarsson MA, Sohlberg E, Bjorklund AT*, et al.* NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood* 2013,**121**:2678-2688.

464. Davis ZB, Cogswell A, Scott H, Mertsching A, Boucau J, Wambua D*, et al.* A Conserved HIV-1-Derived Peptide Presented by HLA-E Renders Infected T-cells Highly Susceptible to Attack by NKG2A/CD94-Bearing Natural Killer Cells. *PLoS Pathog* 2016,**12**:e1005421.

465. 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**:4488-4495.

466. Bonaparte MI, Barker E. Inability of natural killer cells to destroy autologous HIV-infected T lymphocytes. *AIDS* 2003,**17**:487-494.

467. Bonaparte MI, Barker E. 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* 2004,**104**:2087-2094.

468. Holzemer A, Thobakgale CF, Jimenez Cruz CA, Garcia-Beltran WF, Carlson JM, van Teijlingen NH*, et al.* Selection of an HLA-C*03:04-Restricted HIV-1 p24 Gag Sequence Variant Is Associated with Viral Escape from KIR2DL3+ Natural Killer Cells: Data from an Observational Cohort in South Africa. *PLoS Med* 2015,**12**:e1001900; discussion e1001900.

469. Basu R, Hatton RD, Weaver CT. The Th17 family: flexibility follows function. *Immunol Rev* 2013,**252**:89-103.

470. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C*, et al.* IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* 2009,**113**:4240-4249.

471. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K*, et al.* Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* 2009,**106**:4793-4798.

472. Kryczek I, Wei S, Szeliga W, Vatan L, Zou W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 2009,**114**:357-359.

473. Yokoyama WM, Kim S, French AR. The dynamic life of natural killer cells. *Annu Rev Immunol* 2004,**22**:405-429.

474. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Richard-Le Goff O*, et al.* A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol* 2006,**7**:1217-1224.

475. Orr MT, Murphy WJ, Lanier LL. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol* 2010,**11**:321-327.

476. Chung AW, Ghebremichael M, Robinson H, Brown E, Choi I, Lane S*, et al.* Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. *Sci Transl Med* 2014,**6**:228ra238.