NK cell subset responses to HIV

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August 2019

This thesis is submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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ABSTRACT

In 2017 around 37 million people worldwide were living with HIV and 1.8 million new infections occurred. Therefore, HIV infection continues to be an important global human pathogen. Although there have been remarkable achievements in treatment and strategies designed to develop effective vaccines against this virus, these have been insufficient to eradicate this virus for several reasons such as this virus' high rate of mutation/recombination. Several epidemiological and functional studies have highlighted the role of NK cells in inhibition of HIV viral replication. Some Killer Immunoglobulin-like Receptors//human leukocyte antigen (*KIR/HLA*) genotypes contribute to protection from HIV infection in individuals who remain uninfected despite HIV exposure and to HIV control as seen by slower time to AIDS in HIV infected persons who carry these genotypes. Of note, NK cells acquire their functional potential through a process called education, which requires the interaction of inhibitory NK receptors (NKR) with their cognate ligands.

In this thesis, I studied the role of NKRs in anti-HIV responses. The role of activating NKRs (aNKRs) in NK cell education is not fully understood. Nevertheless, based on NK education principles, if the ligand is constitutively expressed, the presence of aNKRs should tune down NK cell functional potential. However, in this thesis we demonstrated that NK cells bearing KIR3DS1, an activating KIR, responded more potently than KIR3DS1$^-$ NK cells, to HLA null cells and to autologous HIV infected CD4 cells by producing higher levels of CCL4, IFN-γ and CD107a. I also showed that the functionality of KIR3DS1$^+$ NK cells was not due to co-expression of other activating/inhibitory NKRs. Rather, the activation of KIR3DS1$^+$ NK cells

was due to the interaction between KIR3DS1 on NK cells and HLA-F expressed on HLA null and autologous HIV infected CD4 T cells.

I also showed that NK cell education influenced NK cells responses to HIV infected cells. In this thesis, I reported that NK cell education through KIR2DL receptor interactions with HLA-C calibrated anti-HIV NK responses. A higher frequency of educated, than uneducated, $KIR2DL1^+$ NK cells responded to autologous HIV-infected cells by secreting CCL4 and IFN-γ. I also showed that a higher frequency of educated, than uneducated, $KIR2DL2^+$ and $KIR2DL3^+$ NK cells produced CCL4 in response to autologous HIV infected cells. Using an antibody panel, flow cytometry and a gating strategy that I designed and optimized to gate on single positive $KIR2DL1^+$, $KIR2DL2^+$ and $KIR2DL3^+$ NK cells, I eliminated the effect of signals received through other inhibitory/activating NKRs. Furthermore, I confirmed that the HIV strain that we used in this study was able to reduce the expression of HLA-A, B and C on the surface of HIV infected CD4 T cells.

I demonstrated that the density of expression of both NKRs and their ligands and the avidity of NKR/ligand interactions affected NK cell education potency and the magnitude of the responses to HIV infected cells. For example, a *KIR/HLA* genotype combination called *3DL1high/Bw480I,* encodes a receptor and ligand with high cell surface expression levels that have a high affinity for one another, A higher frequency of NK cells from carriers of this genotype combination secreted CCL4 than NK cells from carriers of the other tested *KIR/HLA* genotypes.

Altogether, results presented in my thesis provide insights into functional mechanisms explaining the results of epidemiological studies that found associations between the carriage of certain *KIR/HLA* genotypes and HIV disease outcomes. These results highlight the important role of NK cells against HIV. More broadly, the data I have presented in this thesis on the effect of education on NK cell responses inform the role played by NK cells not only in infections such as HIV, but also in cancer, transplantation and pregnancy in which NK cells are determinants of clinical outcomes.

RESUMÉ

Selon le dernier rapport de l'ONUSIDA en 2017, environ 37 million de personne vive avec le virus de l'immunodéficience humain (VIH) dans le monde, avec 1,8 million de nouvelles infections durant la même année. Ces données montrent que l'infection par VIH continue à présenter une importante pathologie humain mondial. Bien que les avancés remarquables réaliser dans la disponibilité des traitements efficaces contre le virus ainsi que les stratégies employer pour le développement d'un vaccin. Cependant, ces avancées restent insuffisantes pour éradiquer le virus et cela pour plusieurs raisons dont le taux élever des mutations/recombinaisons dans le génome viral. Plusieurs études épidémiologiques et fonctionnelles ont reporté le rôle des cellules *Natural Killer* (NK) dans l'inhibition de la réplication du VIH. L'expression de quelques génotypes des récepteurs KIR/HLA, « *Killer Immunoglobulin-like Receptors/human leukocyte antigen* », contribue soit à la protection contre l'infection par VIH chez des individus non infectés et qui était en contact avec le virus, ou à retarder le développement du syndrome SIDA chez les individus infectés. A noté que les cellules NK acquièrent leur potentiel fonctionnel grâce à un processus appelé, éducation des cellules NK, qui nécessite l'interaction entre des récepteurs inhibiteurs des cellules NK (NKR) et leurs ligands.

Durant cette thèse, J'ai étudiée le rôle des récepteurs NKRs dans la réponse antivirale anti-VIH. Le rôle des récepteurs activateurs des cellules NK (aNKRs) dans l'éducation de ces dernières n'est pas encore complétement élucidé. Néanmoins, selon le principe de l'éducation des cellules NK, l'expression des aNKRs diminuera le potentiel fonctionnel des cellules NK. Cependant, dans cette thèse nous avons démontré que les cellules NK portant KIR3DS1 à leur surface (NK KIR3DS1^{+}), un récepteur activateur, réagissent plus fortement que les cellules dépourvues de ce récepteur (NK

KIR3DS1^{-}) aux cellules HLA-nulles, utilisées comme modèle de cellules infectées par VIH, et aux lymphocytes T CD4 autologues infectées par VIH. Cette réponse est marquée par la production à des niveaux plus élevés de CCL4, IFN-γ et CD107a. Nous avons aussi montré que le potentiel fonctionnel des cellules NK KIR3DS1^{+} n'est pas due à l'expression d'autres récepteurs activateurs/inhibiteurs NKRs, mais plutôt à l'interaction entre KIR3DS1 des cellules NK et HLA-F exprimés à la surface des cellules HLA-nulle et des lymphocytes T CD4 autologues infectées par VIH.

Nous avons montré aussi que l'éducation des cellules NK influence leur réponse contre les cellules infectées par VIH. Dans cette thèse, j'ai rapporté que l'éducation des cellules NK via l'interaction entre le récepteur KIR2DL et HLA-C ajuste la réponse anti-VIH des cellules NK. Une fréquence plus élevée de cellules NK KIR2DL1^{+} éduquées par rapport aux cellules non éduquées induit une réponse contre les lymphocytes T CD4 autologue infectées via la sécrétion de CCL4 et IFN γ. J'ai aussi montrée qu'un pourcentage plus élevé de cellules NK exprimant KIR2DL2 et KIR2DL3 qu'aux cellules NK non éduquées induit une production du CCL4 suite à la réponse contre les cellules CD4 autologues infectées. En utilisant une liste d'anticorps, un cytométrie de flux et une stratégie de sélection que j'ai optimisé, j'ai réussi à analyser les cellules NK exprimant individuellement les récepteurs KIR2DL1, KIR2DL2 et KIR2DL3 et ainsi éliminer l'effet des signaux reçus via d'autres récepteurs inhibiteurs/activateurs NKRs. En outre, J'ai confirmé que la souche du VIH utilisé dans cette étude est capable de diminuer l'expression du HLA-A, B et C à la surface des lymphocytes T CD4 infectées par VIH.

J’ai ensuite démontrée que la densité de l’expression à la fois des récepteurs NKRs et de leurs ligands ainsi que l’avidité de l’interaction NKR/ligand affecte le potentiel éducatif des cellules NK et l’ampleur de leur réponse aux cellules infectées par VIH. Par exemple, la combinaison de génotype *KIR/HL*A nommé 3DL1high/Bw480I, code pour un récepteur et un ligand qui ont un taux d’expression élevé à la surface des cellules et qui ont une forte affinité l’un pour l’autre. Une fréquence plus élevée de cellules NK exprimant la combinaison de génotype 3DL1high/Bw480I secrétant plus de CCL4 comparer au cellules NK portant autres génotypes *KIR/HLA* testés.

Globalement, les résultats présentés dans ma thèse démontrent des nouvelles données sur les mécanismes fonctionnels expliquant les observations reportées par des études épidémiologiques qui ont montrés des associations entre l’expression de certains génotypes *KIR/HLA* et la susceptibilité à l’infection par VIH ainsi que la progression du SIDA. Ces résultats mettent en évidence le rôle important des cellules NK contre l’infection par VIH. Plus généralement, les données que j'ai présentées dans cette thèse sur l'effet de l'éducation sur les réponses des cellules NK, nous informent non seulement sur le rôle joué par les cellules NK dans des infections telles que le VIH, mais aussi dans les cancers, les greffes et la grossesse dans lesquels les cellules NK sont déterminantes pour des meilleurs résultats cliniques.

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor, Dr. Nicole F. Bernard. . Her guidance and insightful comments were a great help throughout my Ph.D. research and thesis preparation I am thankful for her trust, which contributed to my development as an independent researcher.

I would like to thank all the past and present members of the Bernard laboratory. In particular, I am appreciative of the support and technical assistance provided by Ms. Tsoarello Mabanga and Ms. Xioayan Ni. I also wish to thank Dr. Dupuy, Dr. Song and Dr. Lisovsky for their training and guidance. I also wish to acknowledge Dr. Meziane and Dr. Dupuy for their help in translating my English thesis abstract into French.

My greatest thanks go to my supportive parents, Mehri and Taghi who encouraged all of my pursuits and inspired me to follow my dreams. Thank you for instilling in me a strong passion for higher learning. I am genuinely blessed to have them as my parents. Achieving this Ph.D. degree would not have been possible without them and words are insufficient to express my gratitude to them!

My deepest appreciation goes to my amazing husband, Hamed, who has been a constant source of encouragement and motivation during my challenging journey as a Ph.D. student. His patience, emotional support and scientific depth have been a source of insight and sustenance throughout this journey. I am truly fortunate to have him in my life!

CONTRIBUTION TO ORIGINAL KNOWLEDGE

- The interaction of KIR3DS1 expressed on NK cell clones with HLA-F expressed on cell lines was shown by others to lead to KIR3DS1$^+$ NK clone activation. In chapter 2, working with *ex vivo* primary NK cells, I showed for the first time that the interaction of KIR3DS1 on KIR3DS1$^+$ primary NK cells with HLA-F expressed on the 721.221 cell line was functional and resulted in the activation of these primary KIR3DS1$^+$ NK cells.

- In chapter 3, working with *ex vivo* primary NK cells, I showed for the first time that the interaction of KIR3DS1 on primary KIR3DS1$^+$ NK cells with HLA-F expressed on autologous HIV infected CD4 T cells was functional and activated primary KIR3DS1$^+$ NK cell to elicit anti-viral functions. A particularly novel aspect of this work was the use of sorted CD4- HIV infected T cells as the autologous HIV infected cell NK cell stimulus.

- In chapter 4, I investigated the responsiveness of single positive (sp)KIR2DL1$^+$, spKIR2DL2$^+$, spKIR2DL3$^+$ and spKIR3DL1$^+$ *ex vivo* NK cells to autologous HIV infected cells. Contributions to original knowledge in this chapter include: 1) The use of a fluorochrome-conjugated antibody panel and flow cytometry gating strategy that permitted gating on NK cells that were sp or four distinct inhibitory KIRs. 2) The ability to gate on and examine the function of spKIR2DL2$^+$ NK cells was particularly novel as no commercially available antibodies exist that are specific for this receptor. 3) A demonstration that educated spKIR2DL2$^+$ NK cells were more responsive than their uneducated counterparts to autologous HIV infected cell stimulation. 4) The inclusion of

fluorochrome conjugated antibodies in the panel that allowed the measurement of the secretion of the chemokine CCL4 as a functional marker of NK cell activity. Information of CCL4 secretion showed that this anti-viral activity dominated the direct response of NK cells to autologous HIV-infected cell stimulation.

CONTRIBUTION OF AUTHORS

Chapter 1:

Zahra Kiani prepared the chapter; Nicole F. Bernard, edited the chapter.

Chapter 2:

Zahra Kiani designed the study, performed the experiments, analyzed the results, prepared all figures and wrote the first of the manuscript; Franck P. Dupuy participated in the experiments for figure 3 and edited the manuscript; Julie Bruneau and Bertrand Lebouche provided clinical samples; Elise Jackson, Cindy X. Zhang and Irene Lisovsky performed some of the experiments for figure 4 and edited the manuscript; Sandrina da Fonseca trained Zahra Kiani in the performance of the experiments used to generate the results reported in this chapter and edited the manuscript; Daniel E. Geraghty provided the anti-HLA-F mAbs and edited the manuscript; Nicole F. Bernard designed the study and prepared and revised the manuscript. The authors declare no conflict of interest.

Chapter 3:

Zahra Kiani designed the study, performed the experiments and data analysis, prepared the all figures and wrote the first draft of the manuscript; Julie Bruneau provided clinical samples and edited the manuscript; Daniel E. Geraghty provided the anti-HLA-F mAbs and edited the

manuscript; Nicole F. Bernard designed the study, participated in data analysis and prepared the manuscript.

Chapter 4:

Zahra Kiani designed the study, performed the experiments and data analysis, prepared all the figures and wrote the first draft of the chapter; Franck P. Dupuy participated in some antibody panel optimization experiments; Julie Bruneau and Bertrand Lebouche, provided clinical samples; Christelle Retière, provided mAb 1F12; Daniel E. Geraghty performed KIR region genotyping and allotyping; Nicole F. Bernard designed the study, participated in data analysis and edited the chapter.

Chapter 5:

Zahra Kiani prepared the chapter; Nicole F. Bernard edited the chapter.

LIST OF ABBREVIATIONS

aa- amino acid

Ab – antibody

mAbs- Monoclonal antibodies

ADCC - antibody dependent cellular cytotoxicity

aKIR – activating killer immunoglobulin-like receptor

aNKR - activating natural killer cell receptor

AIDS - acquired immunodeficiency syndrome

APC – antigens presenting cell

APOBEC3G- Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 G

ART - antiretroviral therapy

cART – combination antiretroviral therapy

BAT3 - HLA -B-Associated Transcript 3

BM – bone marrow

BST-2- Bone marrow stromal cell antigen 2

CA - capsid (or p24)

CD4 - $CD4^+$ T cells

CLP - common lymphoid progenitor

CMV – cytomegalovirus

CRF - circulating recombinant form

CSW - commercial sex workers

CTLs - cytotoxic T lymphocytes

DAP10 – DNAX activating protein of 10kb

DAP12 - DNAX activating protein of 12kb

DC – dendritic cell

DNA - deoxyribonucleic acid

dsDNA – double stranded DNA

EC - elite controller

EIA - enzyme immunoassay

Env – Envelope

ESCRT - endosomal sorting complex required for transport I

FI - fusion inhibitor

GALT - gut associated lymphoid tissue

Gag- Group-specifc antigen

GWAS – genome-wide association studies

H - heavy

HA - hemagglutinin

HAART - highly active antiretroviral therapy

HCMV – human cytomegalovirus

HCV - hepatitis C virus

HESN - HIV exposed seronegative

HIV-1 - human immunodeficiency virus type -1

HLA - human leukocyte antigen

Hmz – homozygote

HSC - hematopoietic stem cell

HSPG - Heparin sulfate proteoglycan

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

iDC – immature dendritic cell

IDU - injection drug user

Ig - immunoglobulin

iKIR – inhibitory killer immunoglobulin-like receptor

ILC - innate lymphoid cell

iNK – immature NK

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

LMPP - lymphoid primed multipotent progenitor

LN – lymph node

LCR - leukocyte receptor complex

LTNP - long-term nonprogressor

LTR - long terminal repeat

MA - matrix (or p17)

mDC – mature dendritic cell

mAb – monoclonal antibody

MALT - mucosal associated lymphoid tissue

MHC - major histocompatibility complex

MICA - MHC class I-chain-related protein A

MICB - MHC class I-chain-related protein B

MIP-1α – macrophage inflammatory protein 1-alpha

MIP-1β – macrophage inflammatory protein 1- beta

mNK – mature NK cell

MSM - men who have sex with men

MTCT – mother to child transmission

NC - nucleocapsid (or p7)

NCAM - neural cell adhesion molecule

NCR - natural cytotoxicity receptor

NK - natural killer

NKC – natural killer complex

NKP – NK progenitor

NKR - natural killer cell receptor

Nef - negative regulatory factor

NHP – non-human primates

NNRTI - non nucleoside reverse transcriptase inhibitor

NRTI - nucleotide and nucleoside reverse transcriptase inhibitor

OC – open conformers

PBMC - peripheral blood mononuclear cell

PCNA - Proliferating cell nuclear antigen

PCR - polymerase chain reaction

PI - protease inhibitor

PIC – pre-integration complex

PLGF - placental growth factor

Pol- polymerase (DNA polymerase)

PR – protease

RAG - recombinant-activating gene

RANTES – regulated upon activation, normal T cell expressed and secreted

Rev - regulator of expression of virion proteins

RM - rhesus macaque

RNA - ribonucleic acid

RRE - Rev response element

RT - reverse transcriptase

SDF-1 - stromal cell derived factor-1

SIV - simian immunodeficiency virus

SH2 - Src homology 2

SHIP-1 – SH2-containing inositol phosphatase 1

SHP-2 - SH2-containing phosphatases 2

SLT - secondary lymphoid tissue

SLMC - spontaneous lymphocyte-mediated cytotoxicity

SP - slow progressor

ss – single standed

ssDNA – single stranded DNA

ssRNA – single stranded RNA

SYK – spleen typosine kinase

Tat- Trans-Activator of Transcription

TB -mycobacterium tuberculosis

TCR – T cell receptor

Tat - trans-activating protein

TRIM5α- tripartitie motif-containing protein 5 α

VEGF - vascular endothelial growth factor

Vif - viral infectivity factor

VL –viral load

Vpr - viral protein R

Vpu - viral protein U

ULBP - UL16 binding protein

URF - unique recombinant form

ZAP70 - Zeta-chain associated tyrosine kinase-70

CHAPTER 1

Introduction and Literature Review

HIV

The HIV/AIDS GLOBAL PANDEMIC

The acquired immunodeficiency syndrome (AIDS) was identified in 1981 when the Centers for Disease Control and Prevention (CDC) reported Kaposi's sarcoma and *Pneumocystis carinii* (now *jirovecii*) pneumonia in five homosexual men in the United States. One year later, two articles were published and proposed that this syndrome was due to infection with a new virus. Early after it was first described, this disease was believed to be a disease only found in homosexuals. As a result, it was named gay-related immune deficiency (GRID) (1, 2). In 1982, this cellular immune deficiency disease was found in females who were sexual partners of infected individuals and among people who received blood transfusions as well. Therefore, the term "GRID" was changed to AIDS. The following year, Drs. Luc Montagnier and Françoise Barré-Sinoussi isolated a T-lymphotropic retrovirus from a patient at risk of AIDS. This was the discovery that HIV was the cause of AIDS (3). In this thesis, the term HIV refers to HIV-1 unless otherwise specified.

According to the 2017 UNAIDS report, around 36.9 million people worldwide were living with HIV/AIDS. Of these, 1.8 million were children (<15 years old) and 1.8 million individuals became newly HIV infected, which is about 5,000 new infections per day (Fig 1). In 2004, 1.9 million (range 1.4-2.7 million) people died from AIDS-related illnesses which was the peak of annual AIDS- related deaths. This number has decreased to 940,000 (range 670,000-1.3 million)

in 2017, which is a 51% reduction from its peak in 2004. In 2017, African regions were the most affected areas, making up almost 70% of global HIV infection and over two thirds of new infections globally.

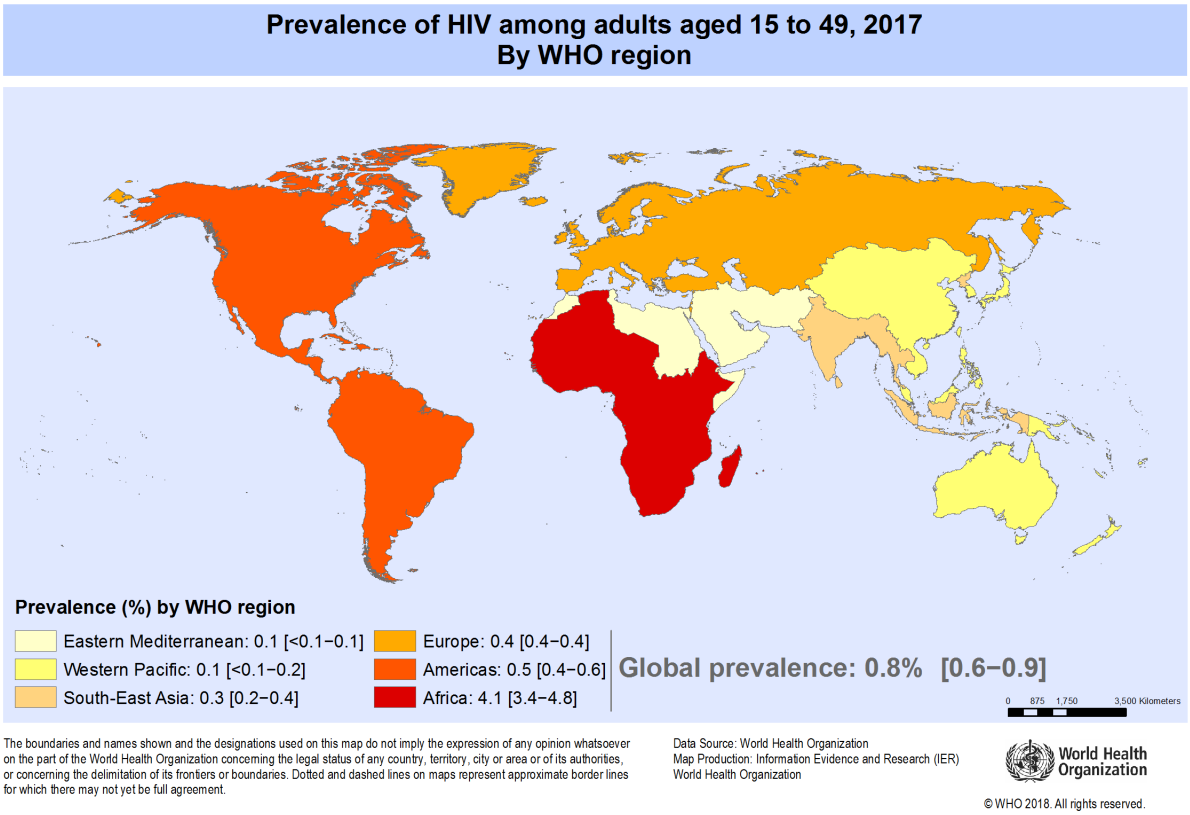

Figure 1. Worldwide prevalence of HIV among adults, 2017. Figure adapted from WHO. https://www.who.int/gho/hiv/hiv_013.pngua=1

HIV ORIGIN AND GENETIC DIVERSITY

There are two evolutionarily different HIV lineages, HIV-1 and HIV-2. These viruses are similar morphologically but distinct antigenically (4). The first cases of AIDS caused by HIV-2 were reported in 1986 in West Africa. Although, HIV-2 is related to HIV-1, it is more closely related to a simian immunodeficiency virus (SIV), which is non-pathogenic in sooty mangabeys but causes immunodeficiency in rhesus macaques (RMs) (5, 6). In subsequent years, other SIVs were found in other primate species from sub Saharan Africa. These viruses generally do not cause disease in their natural hosts. However, several of these viruses have crossed species to infect new hosts. Such a transmission is thought to be the origin of HIV infection in humans (7).

SIV from the chimpanzee is the most likely origin of HIV-1. Two lineages have been identified, SIVcpzPts (Pan troglodytes schweinfurthii) and SIVcpzPtt (Pan troglodytes) (7, 8). Of note, viruses from these two lineages have Gag, Pol and Env protein sequences that are quite different from each other (9).

HIV-1 falls into different groups due to the above-mentioned cross-species transmissions. These include groups M (Main), N (New or nonmain), O (Outlier) and P (Putative) (7, 10, 11). Group M is the cause of the global pandemic. It has caused the HIV-1 related deaths of millions of people around the world. This group is divided into different clades (A, B, C, D, F, G, H, J and K) (7). These clades are also divided into diverse sub-clades such as A1, A2, A3, A4, F1, F2 (12, 13). Moreover, due to recombination between subtypes, there exist HIV-1 “circulating” and unique “recombinant forms” (CRFs) (12, 14). HIV-1 exhibits great divergence genetically and

phenotypically (15, 16), due to its viral reverse transcriptase (RT) lacking a proofreading mechanism, its high recombination rate and its rapid rate of viral replication. There is up to 20% variability in the Env protein within clades and up to 40% between clades (15-18). This phenomenon can influence the virus' transmission fitness, infection rate, course of disease and response to treatments (14, 19-22).

Globally, approximately 60% of HIV cases are due to HIV clade C, which has a high prevalence in India, Sub-Saharan Africa and China whereas in Western Europe, Australia, and the Americas the predominant clade responsible for HIV infection is clade B (18). Sub-Saharan Africa is the most pandemic region with the highest rate of rare subtypes and CRFs. It is the region with the highest viral diversity (23).

HOST ADAPTATION

It has been shown that all primates have a large number of "host restriction factors" as part of their innate immune response against all pathogens (24-26). However, HIV has generated mechanisms to overcome host restriction factors in order to productively replicate in infected host cells (27) (28).

There are three groups of restriction factors that can interfere with HIV replication in the infected cells:

1) Tripartite motif-containing protein 5 α (TRIM5α), interferes with viral uncoating (29), 2) Tetherin (also called bone marrow stromal cell antigen 2 (BST-2) or CD317), inhibits the budding of the virus from infected cells (30) and 3) Apoliprotein B mRNA editing enzyme, catalytic polypeptide-like 3 G (APOBEC3G), inhibits the RT viral enzyme (31). Viral escape mechanisms from these restriction factors are different for different species.

TRANSMISSION

HIV is transmitted through exposure at mucosal surfaces (sexual exposure) or via percutaneous injections (drug use or blood transfusion). It also can be transmitted vertically from mother to child before or during birth or by breast feeding after birth (32). There is not enough evidence to confirm the diverse transmission efficiencies among different HIV subtypes, except for CRF01-AE with a defined transmission efficacy among Thai injection drug users (IDUs) (32). However, it is unclear whether epidemiological, host, or viral factors were responsible for this (33). Based on UNAIDS reports, in 2009 there was a decrease in the rate of newly infected people compared to other years. Interestingly, the rate of this reduction was not uniform in all infected areas and countries, highlighting the role of distinct transmission routes and other possible factors in influencing HIV transmission (Table 1).

HIV transmission site	Transmission fluid	Probability of transmission/ exposure	Contribution to global HIV infection
Female genital tract	Semen, blood	1 in 200 - 1 in 2000	12.6 million
Male genital tract	Cervicovaginal and rectal secretions; blood	1 in 700 - 1 in 3000	10.2 million[a]
Male rectum	Semen	1 in 20 - 1 in 300	3.9 million[b]
Placenta	Maternal blood	1 in 10 - 1 in 20	480,000[c]
Blood stream	Blood products; sharps	95 in 100 - 1 in 150	2.6 million[d]

Table 1. HIV transmission routes. Information from UNAIDS/WHO AIDS epidemic update and and Hladik and McEleath, Nat Rev Immunol (2008). Made by Zahra Kiani.

a, MSM, bisexual and heterosexual men.

b, MSM, bisexual men and women infected through anal receptive intercourse.

c. mother to child transmission.

d, mainly IDUs; however, contains infections due to transfusions and health-care related accidents.

Socioeconomic factors may also be indirect risk factors in HIV transmission. Viral load (VL) is another risk factor in this regard as HIV transmission in discordant couples increased 2.5-fold for every 10-fold increase in VL (34, 35). Anti-HIV treatments reduce HIV VL and decrease HIV transmission by 96% (36).

Stage of infection (acute, chronic and AIDS) is another transmission risk factor with acute infection having a higher risk of transmission from infected partners than chronic phase infection (37-40). This is due to higher VL and absence of neutralizing antibodies (Abs) in acute phase infection, compared to that in other disease stages. Neutralizing Abs are thought to be important in inactivating circulating virus in chronically infected people. HIV is most infectious during its acute phase (37, 41-43).

HIV strains isolated from acute and chronic phase infection are quite different. CCR5 is the HIV co-receptor that is predominantly used by HIV strains in acute infection. CXCR4 becomes the predominant HIV co-receptor around the time that AIDS is diagnosed. Its use coincides with rapid progression to AIDS, although both using CCR5 (R5) and CXCR4 (X4) isolates are equally cytopathic (44). It is proposed that the faster HIV disease progression rate seen when X4 viruses predominate is due to the higher frequency of target cells expressing CXCR4 than CCR5. Also, sexually transmitted diseases causing genital inflammation in HIV transmission result in faster HIV disease progression. Some of these are associated with HIV shedding into the genital tract resulting in an increased rate of transmission (45). On the other hand, circumcision reduces the risk of transmission by 60% (46-49).

ANTI-HIV THERAPEUTIC APPROACHES

So far, more than 20 approved antiretroviral (ARV) drugs are available to use, classified into 6 groups including the nucleoside/nucleotide RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors (FIs) and integrase strand transfer inhibitors (INSTIs), which all target critical steps in viral entry/replication, which decrease plasma VL. Based on The Department of Health and Human Services (HHS) panel on ARV Guidelines for Adults and Adolescents recommendation, starting ART is recommended on the day of HIV diagnosis.

Most of the time, an initial combination ART (cART) regimen (known as highly active antiretroviral therapy (HAART) consists of two NRTIs in combination with an NNRTI, a PI, an INSTI, or a fusion inhibitor (http://aidsinfo.nih.gov/guidelines). This multiple drug therapy has been more successful compared to mono-therapy in which only one viral protein is targeted. The discovery of HAART was a remarkable achievement in increasing life expectancy for HIV infected persons. However, not all infected people have access to these treatments. In addition, anti-HIV drugs are extremely expensive and have numerous side effects including diarrhea, fatigue, insomnia, constipation, rash, abdominal pain as mild side effects and diabetes, osteoporosis, lipodystrophy, liver failure, cardiovascular and neurological diseases as severe side effects (50) (http://aidsinfo.nih.gov/guidelines). Moreover, infected people under HAART need to take long-term medication, which could result in the development of drug resistance mutations (51). Altogether, all abovementioned drawbacks complicate the use of HAART. Therefore, there is a need for less toxic and more effective strategies for HIV treatment.

Broadly neutralizing Abs (bNAbs) have been introduced as a novel candidate for HIV prevention and treatment over last few years (52). Although the first monoclonal Abs (mAbs) against HIV failed to show promising results, a new generation of bNAbs have demonstrated encouraging outcomes by neutralizing most viral strains (53). As shown in Figure 2, there is a list of new generation of bNAbs, which are being tested in clinical trials. Abs against the HIV envelope's (Env) CD4 binding site (3BNC117, VRC01) and V3 loop (10-1074) are currently being tested in human clinical trials. However, as repeatedly passively administering bNAbs is not practical, sustaining above threshold concentration of bNAbs is one of the obstacles to the use these Abs against HIV, which could be solved by introducing transgenic bNAbs expression. Adeno-

associated viruses (AAVs) are potential candidates for this purpose due to their ability to infect both dividing and non-dividing cells and due to their allowing persistent expression without toxicity effects (55). There are two phase I clinical trials (NCT01937455 and NCT03374202) studying the safety and immunogenicity of AVV-mediated bNAbs against viral Env's CD4 binding site (PG9 and VRC07, respectively). Moreover, it has been proposed that, similar to cART, combinations of bNAbs may achieve higher protection and avoid resistance to administered bNAbs.

In conclusion, bNAb-based therapies have been introduced as potential alternatives to ART or in combination with ART for prevention for those who are at high risk of HIV acquisition and also for treatment for HIV infected individuals to prolong the course their disease.

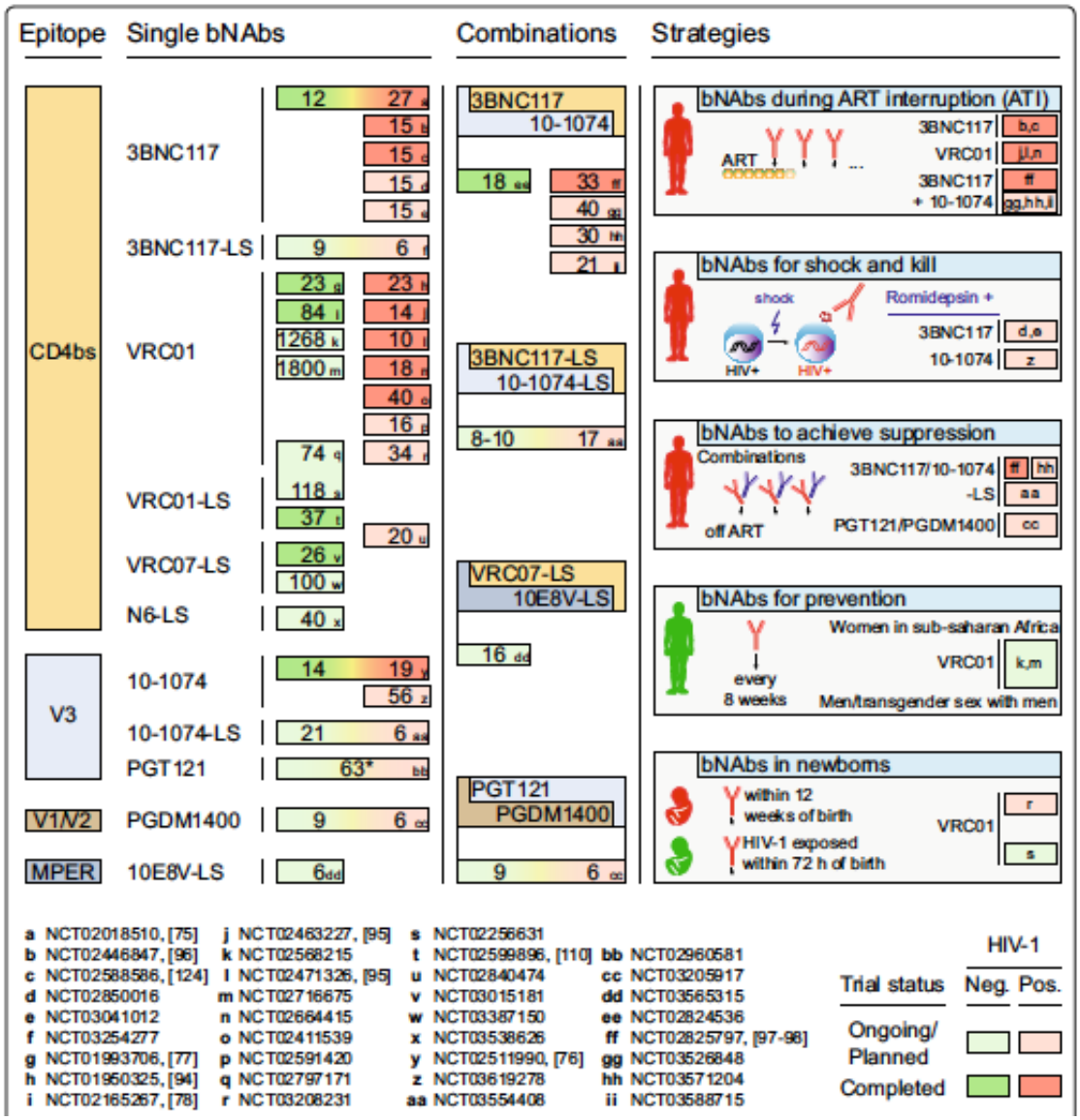

Figure 2. List of clinical trials of bNAbs. Numbers stand for participants receiving bNAb(s). Asterisk shows number of participants and placebo recipients as well. Adapted from Gruell H and Klein F, Retrovirology (2018).

HIV STRUCTURE

HIV is a spherical virus of 120nm in diameter, which is 60 times smaller than red blood cells (56). HIV is a member of the lentivirus genus, a subfamily of the retrovirus family, which uses RNA instead of DNA as its genetic material (Fig 3). HIV has two single stranded (ss) positive-sense RNA molecules as its genome that encode nine viral genes, which are translated into three structural and six regulatory and accessory proteins. The genes encoding structural proteins are *gag*, *pol*, and *env;* the genes encoding regulatory and accessory proteins are *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu.* Sometimes a tenth gene, *tev*, as a fusion of *tat*, *env* and *rev* is also present (57, 58). HIV capsid (CA) assembles into a conical core that contains the two copies of RNA attached to nucleocapsid (NC) proteins. Matrix (MA) encloses the capsid, which itself is surrounded by a phospholipid bilayer derived from the host. Env incorporates into the phospholipid bilayer. Env is a trimer assembled of heterodimers composed of gp41 and gp120 glycoproteins that shape homotrimeric "viral spikes" (56, 59). HIV virions contain enzymes essential for viral replication such as reverse transcriptase, integrase and protease.

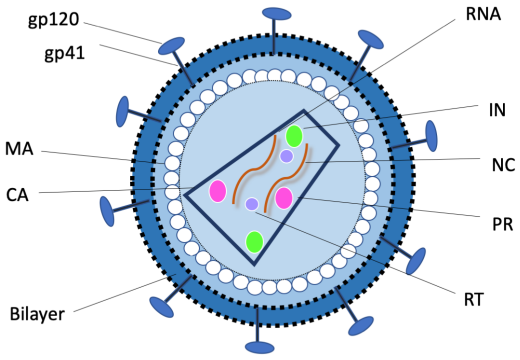

Figure 3. Schematic structure of HIV virion. Made by Zahra Kiani.

Viral genome

The size of the HIV genome is around 9.7 kb. The HIV genome is composed of two ssRNA molecules that are flanked at both ends by long terminal repeats (LTRs) (56) (Fig 4). As mentioned above, the HIV genome encodes three main and structural proteins, Gag, Pol and Env and six regulatory and accessory proteins, which play important roles in HIV infectivity, replication and pathogenesis. However, after further proteolysis, HIV will have 15 essential proteins for its replication. The functions of the viral gene products are summarized in Table 2.

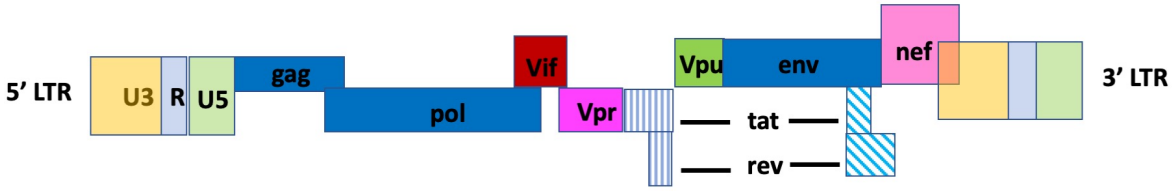

Figure 4. Organization of the HIV genome. The HIV genome is almost 10kb in length and includes 9 genes, which encode 15 viral proteins. The functions of the viral gene products are summarized in the Table 2. Made by Zahra Kiani.

Viral gene	Viral gene function
LTR	Involved in host transcription factor binding, initiation of transcription
gag	Encodes MA (p17), CA (p24), NC (p7), RNA binding protein (p6)
Pol	Encodes viral enzymes such as PR, RT and RNAseH, IN
Vif	Acts against APOBEC function, inhibits hypermutation and DNA viral degradation
Vpr	Stimulates G2 cell-cycle arrest. Involved in HIV infection of macrophages
Vpu	Elicits CD4 degradation and helps virion release

env	Encodes gp120 and gp41. Gp120 moderates CD4 and CCR5 binding. Gp41 moderates viral fusion into host cell
rev	Regulates viral gene expression. Mediates nuclear export of incompletely spliced viral RNA molecules
nef	Reduces CD4 and major histocompatibity complex class I HLA-B and -B expression. Inhibits apoptosis and increases viral infectivity. Involved in progression to AIDS
Tat	Acts as a transcriptional activator, increases RNA Pol II elongation on the viral DNA template

Table 2. HIV viral gene product functions. Made by Zahra Kiani.

The viral replication cycle

Virus entry

The process of virus entry starts by gp120 binding to CD4 receptors on the surface of host cells (60-65) (Fig 5). Gp120-co-receptor binding leads to more conformational changes, which allows gp41 to penetrate the cell membrane. Repeat sequences in gp41 leads to the formation of a six-helical hairpin structure, which brings the virus and the host cell membranes close together. Subsequently, viral envelope and cell membranes fuse together releasing capsid into the host cell (56, 66-68). As soon as a $CD4^+$ cell becomes infected, virus downmodulates the expression of CD4 to prevent superinfection and to avoid retaining budding virions through gp120-CD4 interactions (57).

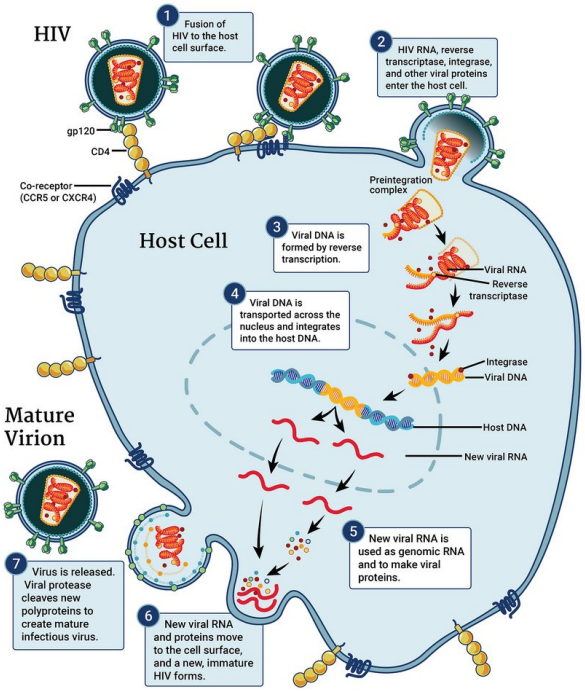

Figure 5. HIV replication cycle. The HIV replication cycle starts by HIV virions fusing to the cell surface of the target cell (step1) and ends once the virion is released from the infected cell. Different steps in the replication cycle are numbered in the figure. Adapted from National Institute of Allergy and Infectious Diseases (NIAID), https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle

Un-coating

Once the virus enters the cell, MA and CA proteins are digested, releasing viral enzymes and viral RNA molecules (Fig 5). The RT uses host nucleotides to convert viral RNA into cDNA. RT is prone to making errors because of its low proof-reading ability. The cDNA is then transcribed into double stranded DNA (dsDNA) (69). Subsequently, the viral dsDNA along with IN (known

as the pre-integration complex, PIC), are imported into the nucleus through nuclear ports using the cellular nuclear import machinery (70). Of note, there are also other viral components contributing to the transport of the PIC to the nucleolus, such as the central DNA flap, IN, MA and Vpr (71). Within the nucleus, IN insert the dsDNA into a host chromosome (72, 73). This integrated viral DNA either lies dormant in the host chromosome referred to as "latent stage" infection or transcribes viral components, referred to as the "productive stage of infection" (69).

Following host cell activation, host transcription factors bind to the viral LTRs and start viral transcription using cellular RNA polymerase II (74). Multiply spliced mRNAs are generated, which are translated into viral regulatory and accessory proteins such as Rev, Tat, Nef, Vpr, Vpu and Vif in the "Rev-independent" stage of viral transcription. Rev then binds to single-spliced and unspliced viral mRNA molecules and transports them into the cytoplasm where other structural viral proteins and genomic RNA are produced in the "Rev-dependent" stage of viral transcription (75-77).

In the cytoplasm, Gag-Pol transcripts are translated into Gag and Gag-Pol polyproteins through a ribosomal frameshift (78). Gag precursor polyproteins are the origin of the MA, CA, NC and p6 gene products whereas Gag-Pol precursors generate MA, CA, NC, protease (PR), RT and IN gene products (79).

Assembly, transportation

In the first step in the viral assembly, Gag binds to the viral genomic RNA molecules, multimerizes and migrates to areas of the plasma membrane called "lipid raft microdomains".

Env is also is incorporated into the assembling particles with Gag-Pol precursors. The endosomal sorting complex required for transport I (ESCRT-I) is recruited, which is followed by recruitment of ESCRT-III and eventually immature virions are released (79). The immature viral particles become mature through proteolytic cleavage of Gag and Gag-Pol polyproteins into structural viral proteins and enzymes, respectively by viral PR and the formation of a conical core made up of CA (80, 81).

HIV LATENCY & RESERVOIRS

Antiretroviral therapy (ART) for HIV infection can successfully block HIV replication and new infections. However, ART cannot eliminate HIV from resting cells that already harbor integrated proviral DNA. This is illustrated by the rapid VL rebound observed within days to weeks of treatment interruption (82, 83). HIV latency in reservoirs has been implicated as the main obstacle to HIV cure. The cells harboring latent virus are resting long-lived memory $CD4^+$ T cells carrying untranscribed proviral DNA in their genome (84). These cells express no viral proteins and are not recognized by cytolytic effector cells such as CD8 T cells and NK cells (85).

Much of the research directed towards eliminating HIV reservoirs has focused on “shock and kill” strategies (86). The goal of these approaches is to activate resting HIV infected cells to express viral proteins so that they can be killed via Vpr-mediated cycle arrest and apoptosis (87, 88) or through CD8 and NK cells recognition. Based on some modeling studies more than 4 logs of reservoir reduction would be needed to cure HIV (85), which is not easily achievable.

Of note, various attempts have been made to apply “shock and kill” strategies. Although in some of these attempts the virus was reactivated, a reduced in HIV DNA reservoirs did not occur. Treatments able to successfully activate the expression of HIV from latently infected cells are often toxic. Virus reactivation must be accompanied by effective control by HIV specific cytotoxic T cells (CTLs) or NK cells to kill HIV infected cells and prevent the reseeding of HIV reservoirs (89, 90). Another concern would be that the reactivation of reservoirs may not end in cell death (91).

COURSE OF INFECTION

HIV infection has three major stages characterized by biological markers and clinical manifestations (Fig 6). The first stage is the “acute phase” After 2-4 weeks of infection, there is a peak of viral replication as the virus reaches lymphoid organs and spreads throughout the body. In this stage, p24 antigen (Ag) is detectable in the blood. Around 70 to 80% of newly infected people experience an acute retroviral syndrome that can include symptoms such as fever, headache, rash, sore throat and malaise (92). Acute infection is followed by the “chronic or latent phase”. Although this phase is frequently asymptomatic, large amounts of virus is made and dies daily; large numbers of CD4 T cells are generated and die each day, as well. In this stage, the host immune system works hard to recognize and control HIV infected cells.

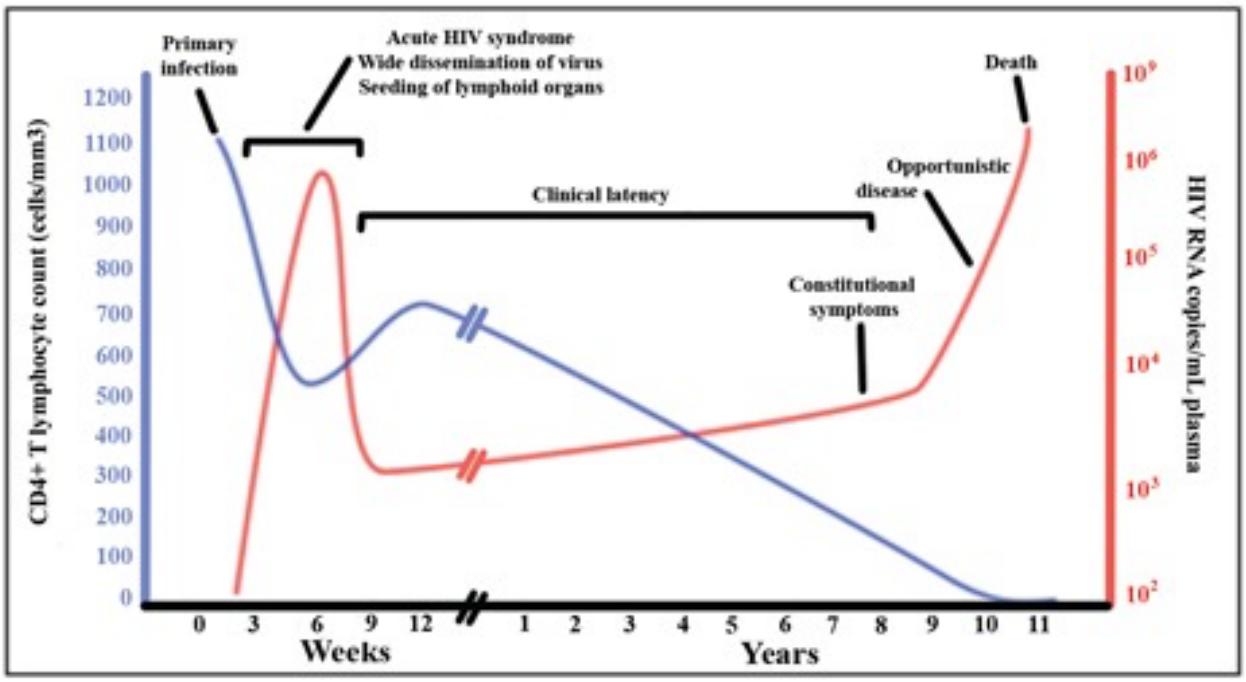

Figure 6. HIV course of infection. Adapted from Naif HM. Pathogenesis of HIV infection. Infect Dis Rep 2013; 5:s1.e6.

Anti-HIV immune responses are induced that include anti-HIV Abs and CTLs. These responses bring the HIV VL to a set point and increase the CD4 count. However, if the infected person does not start ART, CD4 T cells will become exhausted and will die. Eventually, this stage advances to the final stage of the disease, the "AIDS phase". The host immune system has been weakened by the virus. It is no longer able to fight against opportunistic infections (CDC 1992. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Recommended Rep 41(RR-17):961-962). Untreated AIDS patients rarely survive more than 3 years.

IMMUNE RESPONSE TO HIV INFECTION

Following initial HIV infection, $CD8^+$ CTLs are induced, which lead to VL control to a VL set point (93). These effectors cells can inhibit viral replication. For example, they are activated by the recognition of viral peptide HLA complexes to kill HIV^+ target cells. Activated $CD8^+$ CTL release the contents of their cytotoxic granules. These granules contain perforin, which makes holes in the target cell membrane so granzymes, also present in cytotoxic granules can enter the target cells and induce the apoptosis pathways. $CD8^+$ T cells express Fas ligand, which upon binding Fas (CD95) on the surface of infected cells leads to apoptotic cell death.

$CD8^+$ T cells produce many soluble antiviral factors including interferon-γ (IFN-γ), which through a cascade of receptor-mediated binding and activation events, makes neighboring cells resistant to productive viral infection. Other antiviral factors produced by $CD8^+$ T cells are the beta chemokines macrophage inflammatory protein-1 α (MIP-1α), macrophage inflammatory protein-1 β (MIP-1β), and regulated on activation, normal T expressed and secreted (RANTES), that bind to CCR5. This results in internalization of this critical HIV co-receptor, blocking HIV from entering HIV susceptible $CD4^+$ cells (94). Other antiviral factors produced by $CD8^+$ T cells can control HIV replication via inhibition of viral transcriptional activation (95).

Despite the confirmed role of $CD8^+$ T cells in controlling of HIV, it is still not clear whether the magnitude of $CD8^+$ T-cell target recognition predicts disease outcome (96, 97).
Although $CD8^+$ T cell count does not decrease in HIV infection, sustained viral replication inhibition is not achieved. One explanation for this, is likely to be the accumulation of viral

escape mutations, which make the infected cells undetectable to recognition by the $CD8^+$ T cells that are present at the time escape mutations appear (98, 99).

Furthermore, lytic activity (100, 101), proliferation *in vitro* (102) and expression of signaling molecules involved in activation through TCRs (103) all decline in HIV-specific $CD8^+$ T cells. As a result, these cells become dysfunctional in HIV-infected individuals. It has been suggested that sustained exposure to high levels of viral antigens, lack of CD4 help and chronic inflammation cause $CD8^+$ T cell dysfunction, although the cause and effect relationship between these two phenomenon is unclear.

The $CD4^+$ T-cell response to HIV antigens is impaired in HIV infection (104-106). Except for $CD4^+$ T cells from long-term non-progressors, those from most untreated HIV^+ persons proliferate poorly to several stimuli (107). However, CD4 proliferation can be restored or preserved in HIV-infected people who initiate treatment right after infection (108) and in some chronically infected patients in whom viral replication is suppressed by ART (109, 110).

NATURALLY EFFICIENT IMMUNE RESPONSES AGAINST HIV INFECTION

There is a subset of individuals who, despite multiple exposures to HIV, remain persistently HIV seronegative. These persons have been called HIV exposed seronegative (HESN) (111). There are also HIV-infected individuals who progress to AIDS much more slowly than do typical HIV^+ persons. These are called slow progressors (SP). SPs include Elite Controllers (EC), whose VLs are undetectable by standard VL tests, Viral Controllers (VC) whose VL is <2000 copies/ml of

plasma and long-term non-progressors (LTNP) whose CD4 counts stay above 500 cells/mm^3 without treatment at least for 7 years (112). Although mechanisms have been proposed for protection from infection or slower progression to AIDS, more research on host immune resistance to HIV is needed to better understand these phenomena. Understanding what accounts for these phenotypes is important for the design of effective protection and therapeutic vaccines against HIV.

HESN

In the late 1980s, several HESN cohorts were described worldwide (113). Interestingly, the frequency of ΔCCR5 homozygotes (hmz) in HESN cohorts is very low suggesting other mechanisms such as genetic factors and/or unique immunological responses were involved in protection from infection in these cohorts (114). There were HESN cohorts described among individuals exposed sexually and parenterally. Sexually exposed HESN can be found among HIV discordant couples, people with high risk sexual behavior such as commercial sex workers (CSWs) and men who have sex with men (MSM). Non-sexually exposed HESN include IDUs, people who received contaminated blood products such as hemophilia patients and children born to HIV-infected mothers.

Discordant couple cohorts, in which one partner is infected, represent possibly the largest number of HESNs globally. Although much informative data has been learned from these cohorts regarding mechanisms used for protection from infection, there are some limitations to what can be learned by studying these cohorts as well. For example, the risk of HIV transmission per unprotected coital act differs depending on which partner is infected. Also, it has been

reported that HIV exposure differs in these type of cohorts ranging from 25 to 112 exposures per year (114). The ART status of the infected partner has an important impact on reducing the risk of HIV transmission. The VL of the infected partner, their disease status and circumcision all affect risk of transmission and need to be documented and considered when interpreting whether someone indeed meets the criteria for being classified as an HESN (46-48).

Despite the abovementioned limitations, the most abundant HESNs are those in HIV discordant couples. Such HESNs are found in many geographical regions worldwide. They are likely to be the most accessible and the most relevant individuals in which to identify factors contributing to HIV protection following sexual transmission.

Individuals with high risk sexual behavior such as CSWs and MSM also include subsets of sexually exposed HESNs. The best known HESN CSW cohorts are in Africa and Asia. The Pumwani CSW cohort in Nairobi, Kenya is one of the best characterized HESN cohorts (115). Of note, the HESN women who participated in this cohort remained uninfected despite having high rates of exposure with an average of 15 clients per day through unprotected sex (115, 116). This cohort has been intensively studied to provide information on mechanisms implicated in HIV prevention in women.

Interestingly, cohorts of MSM have confirmed high rates of HIV in resource-rich countries (117-119). Though MSM are exposed sexually their route of exposure differs from that of CSWs. MSM have a rate of infection that is approximately 10 times higher than do HIV susceptible CSWs.

Studying MSM and CSW cohorts has the potential to provide important information for the design of HIV vaccine strategies. CSWs are of special interest due to the exposure of their immune system to multiple viral isolates and to the high frequency of exposure to HIV positive clients. However, all abovementioned sexually exposed cohorts have the potential to elucidate factors contributing in protection against sexual transmission.

IDUs are individuals who are HIV exposed by sharing contaminated needles. As the route of exposure for these people is intravenous, they are at a higher risk of infection than sexually exposed individuals. This makes them a tremendous resource of useful information for vaccine design strategies (120). The initial route of exposure for IDUs is directly into the peripheral circulation with ready access to the immune system (121). Therefore, parenteral exposure can elicit immune responses that differ from those in sexually exposed individuals. Precisely defining the route of exposure in IDUs is complicated by the fact that most IDUs are also sexually exposed.

Children born from HIV-infected mothers are another group of non-sexually exposed individuals. If exposed children become infected it usually occurs *in utero* or through breast feeding (122). In the absence of any anti-HIV treatment the mother to child transmission rates range from 15%-45% (123-126). Since the introduction of ART to prevent vertical transmission, MTCT is rare.

Based on available data, CSWs and discordant couples are the most informative HESN cohorts. Of note, since different groups of exposed people have their own features such as mode of

exposure, lifestyle and concurrent infections, the results from each group needs to be recorded and considered separately because they may each have their own distinct protection mechanisms.

Specific T cell responses against HIV

Ranki et al. in 1989 reported HIV-specific T cell responses in HESN (113). Since then, several publications have reported these responses in high risk HESNs (127-129). Proliferation to HIV Env peptides and HIV-specific T cell responses to HIV have been defined (130-133). *Lacap et al.* found that HLA class II allotypes were associated with protection from HIV infection (135).

HIV specific T cell responses are divided into two categories: 1) direct CTL responses (cytotoxic responses) and 2) indirect T cell responses mediated by the secretion of antiviral factors such as some chemokines and cytokines. Between 30% and 60% of HESNs do not have HIV-specific T cell responses (136). Considering all these findings, the potential contribution of HIV-specific T cell responses to protection from infection is controversial (137). Although a substantial subset of HESNs may have HIV-specific T cell responses, these responses may be marker of prior exposure rather than mediators of HIV protection in HESNs. The efficacy of HIV-specific CTL in protection from HIV infection was called into question by the results of the failed Merck sponsored STEP vaccine trial, in which HIV-specific T cell responses were induced (138, 139).

In 2011, a study on discordant couple cohort in Zambia confirmed the lack of detectable HIV-specific T cell responses in HIV uninfected HESN partners (140). Another study showed that the magnitude of HIV specific T cell responses in HESNs are usually lower than those in infected subjects (131, 141).

Humoral anti-HIV responses

The presence of IgA as an anti-HIV humoral response in the mucosa and serum of HESN individuals has been confirmed (129, 142-146). Although in some cases IgA responses are lacking (147) in most cases this type of response has been reported in HESN individuals who were exposed through mucosal routes (136). In some ex-vivo assays, HIV specific IgA isolated from HESN individuals recognized and bound to epitopes in gp41 resulting in blocking of viral replication (148). Further, IgA produced in HESN subjects can block the transcytosis of HIV through epithelial cells resulting in protection from infection (149, 150). Furthermore, antibody-dependent cellular cytotoxicity (ADCC) can be elicited by HIV specific IgA to kill infected cells (151, 152).

Despite all these abovementioned anti-HIV IgA functions, the efficacy of these functions is under debate. As an example, anti-HIV IgA responses were undetectable or not consistent in an HESN CSW cohort in Tanzania (153). Another study showed that there is no association between anti-HIV IgA responses and protection from infection (154). Therefore, the contribution of anti-HIV IgA responses in protection from transmission in HESN cohorts is not certain yet and needs more investigation.

HIV-Ag derived factors

Some studies on immune responses against HIV in HESN found a role for MIP-1α and MIP-1β in protection from HIV (155, 156). These chemokines compete with HIV to enter target cells

through binding to the CCR5 co-receptor (157, 158). Both T cells and NK cells secrete these factors and may contribute to protection from HIV infection using this mechanism.

Mucosal Integrity

Mucosal integrity is another contributing factor in control of HIV transmission in HESNs (159). Generally, infection elicits immune activation, which consequently leads to systemic and local inflammation. Local inflammation destroys mucosal integrity leading to increased HIV transmission and subsequently increased availability of CD4 target cells (160).

NK cells

The role of NK cells in immune responses against HIV in HESN subjects will be discussed in the NK cell section.

Slow Progressors (SPs)

SPs represent up to 5% of HIV infected people. The disease progression of SP is much slower than that of other HIV infected subjects (161, 162).

Cellular Immunity

The low level of VL in SPs is due to the contribution of cellular immunity and broader Gag-specific $CD8^+$ T responses (163). Certain HLA-I allotypes such as HLA-B*57 and HLA-B*27 (both HLA-I) are overrepresented in SPs and are important in shaping an efficient anti-HIV

response in SPs (164-169). In HLA-B*57^{+} SPs, the dominant Gag epitope-specific CTL responses are restricted by this allotype (166). Another study found that the frequency of HIV-specific CD8 cells is higher in SPs compare to progressors. They also showed that cytolytic granules secreted by CD8 cells are necessary to kill infected target cells (170). Based on Genome-Wide Association Studies (GWAS), there is a remarkable association between the presence of single nucleotide polymorphism (SNP) at HLA-B*5701 and upstream of the HLA-C loci and low level of VL (169, 171).

Neutralizing Antibodies

Although still controversial, it was also shown that the presence of some specific neutralizing Abs are present in almost 23% of HIV infected long term non progressors (LTNPs) and may be associated with their long-term regression status. These neutralizing Abs bind to a highly conserved epitope of gp41 and elicit viral neutralization explaining their low viral load and high CD4 counts (172). However, the role of neutralizing antibodies in LTNPs is controversial and needs more investigation.

NK cells

The role of NK cells in SP status has been highlighted, which will be discussed more in NK section (173-178).

In conclusion, studying the different immune mechanisms that may contribute to SP status is important for the design of effective therapeutic vaccine and treatment strategies.

C-C chemokine receptor type 5 (CCR5) and its key roles in HIV control

CCR5 is expressed on several types of cells, including dendritic cells (DCs), memory T cells, NK cells, macrophages, etc. (179). The ligands for CCR5 include MIP-1α, MIP-1β and RANTES, which can attract target cells to the mucosa. HIV uses CCR5 and/or C-X-C chemokine receptor type 4 (CXCR4) as co-receptor for entry into HIV susceptible cells (184-188). The natural CCR5 ligands can block HIV infection of new target cells. R5 strains are the more common during acute and chronic phase infection; during the late stage of infection around the time AIDS is diagnosed the predominant HIV isolates are X4 using strains (189).

In vitro studies showed that CCR5 expression on the surface of some cell lines can influence the rate of HIV infection (190). In one study from 2000, they found a strong association between CCR5 expression levels and VL (191). CCR5 expression levels were not significantly different in HIV infected individuals and healthy controls. ART reduced HIV VL in infected persons without changing CCR5 expression levels. Together, these findings suggested that the strong association between CCR5 and VL was not due to HIV-infection dependent upregulation of CCR5. A threshold of 10,000 copies of cell surface CCR5/CD4 was proposed to be necessary to achieve infection. Therefore, CD4 cells with lower numbers of cell surface CCR5 have a reduced susceptibility to HIV infection (190).

Environmental factors can contribute to CCR5 expression. Infection with *Mycobacterium tuberculosis (MTb)* increases HIV infection risk through a CCR5 expression dependent process (192). Lipopolysaccharide (LPS) as an endotoxin that upregulates CCR5 expression (193). Thus, bacterial infections can facilitate HIV entry and infection. Some medications can influence

CCR5 expression levels. Statins reduce expression of both CCR5 and CXCR4 receptors *in vitro* (194). Persistent alcohol consumption is another factor suggested to reduce CCR5 expression (195).

The Δ32 CCR5 Mutation

Dean et al. found that the Δ32CCR5 mutation was rare or absent in HIV infected individuals (196). This mutant encodes a variant that cannot be expressed on the cell surface. The mutant allele is mostly limited to persons of European descent, although it has been found in some other populations such as in the Ashkenazi Jewish population, possibly due to European gene admixture (197). Interestingly, the Δ32CCR5 mutant allele is not present in African populations with an exception of African Americans, again due to admixture (196, 198).

Discovery of the Δ32CCR5 mutation raised hopes for complete protection for individuals who are homozygous for this mutant allele. However, HIV infection has been reported for some of these homozygotes (199, 200). Further analysis confirmed that these individuals were infected with X4 using strains, which use CXCR4 instead of CCR5 as a co-receptor for HIV entry (201).

Various small molecules have been constructed that act as CCR5 antagonists. Among these are Maraviroc (Pfizer), Vicriviroc (Schering-Plough) and Aplaviroc (GlaxoSmithKline), which bind to CCR5 and block HIV entry and new infections. Some “combination therapies” propose using an anti-HIV strategy in which a CCR5 directed medication such as a CCR5 antagonist is combined with an anti-CCR5 mAb to boost the efficiency of the treatment (202).

Hutter et al. in 2009 performed a bone marrow transplant (BMT) on an HIV-infected subject using cells from a donor who was a Δ32 CCR5 hmz (203). After the transplant, the recipient has been living without ART for 11 years. He converted to seronegative status and has an undetectable VL (203). A second patient who received a BMT from a Δ32 CCR5 hmz was recently reported to be living off ART with an undetectable HIV VL. The findings provide a proof of concept that eliminating CCR5 is a pathway to a procedure for HIV cure. But it should be noted that undergoing a BMT with cells from a Δ32 CCR5 hmz to achieve an HIV cure will not readily transferable to most HIV infected individuals.

In summary, CCR5 has a critical role in HIV control. However, more studies are required to develop efficient CCR5-based strategies against HIV infection.

HIV VACCINE TRIALS

Since the early 1980s, around 70 million people have been HIV infected and 35 million have died of AIDS related causes. Despite the significant success of HIV treatments, in 2017, 1.8 million new infections occurred. Therefore, having an effective HIV vaccine continues to be an important goal towards ending the ongoing HIV epidemic.

So far, only six vaccine trials have been completed (Table 3). Most of these vaccine trials used vaccines that induced Ab responses against HIV; a few induced cellular immune responses. Initial studies on non-human primates (NHPs) and humans confirmed that Env glycoproteins are immunogenic and safe to use in HIV vaccines (204-211). Therefore, the first few vaccines were

designed based on HIV Env epitopes. The first completed vaccine clinical trial, AIDSVAX B/E (VAX003), started in 1999 in Thailand as a randomized, double-blinded, placebo-controlled study on over 2500 IDUs. VAX003 had two gp120 recombinant antigens, one from CRF01-AE isolate A244, which corresponds to an R5 using subtype and another from an X4 using lab adapted clade B 6240 isolate (212). Despite the anti-gp120 responses elicited in this trial, the vaccine failed due to lack of efficacy (efficacy = 0.1) (212).

Trial	Vaccine	Phase	Number of participants	Year	Results
AIDSVAX B/E (VAX003)	Clade B gp120 and CRF01_AE gp120	III	2546	1999-2003	Not protective
AIDSVAX B/B (VAX004)	Clade B recombinant gp120 antigens in alum	III	5417	1998-2003	Not protective
HVTN502 (STEP)	MRKAd5HIV-1 Gag/Pol/Nef	IIb	3000	2014-2007	Infection increased (early transient) in vacinees
HVTN503 (Phambili)	MRKAd5 clade B Gag/Pol/Nef	IIB	801	2007-2007	Not protective, infection increased in male vacinees
RV144	ALVAC-HIV vCP1521, AIDSVAX B/E rgp120 in alum	III	16,402	2003-2006	31.2 % efficacy
HVTN505	DNA, rAD5 (A, B, C)	IIb	2504	2009-2017	Not protective

Table 3. HIV vaccine trials. Previous HIV vaccine clinical trials completed. All abbreviations are listed in the abbreviation section of this thesis. Information adapted from Gao Y (2018), made by Zahra Kiani.

The VAX004 trial was the next Env-based vaccine to be studied. The trial was conducted in the Netherlands and North America, beginning in 1998. The vaccine construct consisted of clade B

recombinant gp120 antigens tested on over 5000 MSM and women at a high risk for HIV acquisition (213). Although this vaccine generated CD4 blocking and some neutralizing Abs, it did not protect people who got the vaccine from HIV infection (213).

Disappointing results from Env-based vaccine trials drove the idea of designing a vaccine in which cellular immune responses could be triggered (214). Consequently, the international multicenter STEP trial (HIV Vaccine Trials Network 502, HVTN502) was started in 2004 on 3000 MSM and female CSWs in North America, South America, Australia and the Caribbean (139). This vaccine includes the adenovirus type 5 (Ad5) as a vector with the *gag, pol* and *nef* HIV genes, to induce T cell responses. Protection from infection and decreased VL in the participants who became infected were the main goals of this vaccine trial. This study was stopped three years after initiation when the interim efficacy results were released, showing an increased rate of infection in males who received the vaccine compared to the placebo group.

Another HIV vaccine trial, very similar to the STEP trial, was started in 2007. The HVTN503 (Phambili) trial consisted of an Ad5 vector with Clade B *gag, pol, nef*, although the dominant clade circulating in the geographical area where the trial was conducted was clade C. This vaccine was tested on 3000 men and women at high risk of HIV acquisition in South Africa. The trial was stopped in 2007 for the same reason as STEP trial (215).

The RV144 HIV vaccine trial was the first to show significant, though modest, efficacy. This trial was started in 2003 in Thailand in an area with high risk of infection, though the study did not target participants that were at a particularly high risk of HIV acquisition. Over 16,000

volunteers participated in this trial. The vaccine consisted of a “prime” and “boost”. The prime was an ALVAC-HIV vCP1521 vector and the boost was AIDSVAX B/E rgp120, which included two recombinant gp120 Envs from clade E and AE isolates. Administration of this vaccine reduced the rate of infection by 31.2% (216).

Analysis of the RV144 results has been intensive to understand the correlates of protection of this vaccine (217). The vaccine elicited the production of non-neutralizing IgG Abs against variable regions 1 and 2 of the Env glycoprotein (217). In secondary analyses, it was confirmed that anti-HIV Env V1/V2 loop IgG induced by this vaccine was ADCC competent. ADCC activity was proposed to be one of the mechanisms underlying this vaccine’s ability to mediate protection from infection (218, 219).

HVTN505 was another HIV vaccine trial started in 2009 and tested on over 2,500 men and transgender women who had sex with men. This vaccine consisted of *gag/pol/nef* DNA from a clade B HIV isolate and *env* from clade A, B and C isolates. This trial was stopped in 2017 due to lack of efficacy (220).

In conclusion, of the six HIV vaccine trials conducted to date, the RV144 trial was the only one found to be effective against HIV. An efficacy of 31.2% is insufficient to stop the HIV epidemic. Although no estimated degree of protection has been suggested, an effective HIV vaccine should be able to protect against infection by varied strains of HIV. More effective vaccines are needed to achieve this goal. Planned vaccine trials are building on the success of the RV144 trial and

what has been learned regarding the immune responses that correlated with protection from HIV infection.

NK Cells

NK cells were identified in 1975 by *Keissling et al.* who described the presence of cells in the spleen of adult mice, which were neither T nor B cells. These cells responded both *in vitro* and *in vivo* to mouse leukemia cells with remarkable cytolytic functions. Since these cells were activated and responded to the target cells without prior immunization, they were called "Natural Killer" or NK cells (221). In the same year, *Pross et al.* found that human non-T lymphocytes were activated and responded to mouse cell lines. This was called spontaneous lymphocyte-mediated cytotoxicity (SLMC). They also showed that NK cells from different donors varied in their potential cytotoxicity to target cells (222).

NK cells make up 10-15% of PBMCs. Although NK cells were found originally in mouse spleen and PBMCs, they are also found in multiple lymphoid and non-lymphoid tissues such as bone marrow (BM), thymus, lymph nodes (LNs), tonsils, liver, skin, gut and lungs, among others (223).

NK cells differ from T cells and NKT cells in several aspects. For example, they do not express T cell receptors (TCRs). Human and murine NK cell markers differ from each other. For instance, expression of CD56 (neural cell adhesion molecule [NCAM]) and lack of CD3 (224,

225) are the hallmarks of NK cells in humans while in mice expression of NK1.1, NCR1 and CD49b marks NK cells (226, 227).

In mice, NK cells develop and mature in BM niches. Hematopoietic stem cells (HSCs) are triggered upon humoral and cytokine stimulation to become common lymphoid progenitors (CLPs). CLPs then differentiate into NK cell progenitors (NKPs) upon stimulation with IL-7 and IL-15 produced by non-hematopoietic stromal cells. Subsequently, $CXCR4^{+}$ NKPs are stimulated by CXCL12, generated by CXCL12-abundant reticular cells to differentiate into mature NK cells (mNK). mNK cells are then transported to secondary lymphoid organs to carry out their functions.

Human NK cells can develop and mature in the BM and secondary lymphoid tissues (SLT) such as tonsils and LNs (228) in which they express a discrete panel of lineage specific surface markers at each stage of their differentiation (229) (Fig 7). In humans, HSCs, which are Lin-$CD34^{+}CD133^{+}CD244^{+}$ develop into lymphoid primed multipotent progenitor (LMPP) cells that express CD45RA. In the following stage of development, LMPPs differentiate into CLPs that express CD38, CD7, CD10 and CD127. CLPs then differentiate either into pro-B, pre-T, innate lymphoid cells (ILCs) or NKPs. Expression of CD122 marks NKPs that are committed to differentiate along the pathway towards becoming NK cells. This pathway includes becoming $CD56^{bright}$ then $CD56^{dim}$ NK cells. It has been proposed that NKPs can differentiate directly into $CD56^{dim}$ NK cells without going through the intermediate $CD56^{bright}$ stage. However, this idea needs to be investigated and validated with more evidence (230).

As mentioned earlier, in each stage in NK cell development, NK cells exhibit a panel of cell surface marker(s), which distinguish that stage from others (231). For instance, expression of CD244 is specific to stage 1, while expression of CD7 and CD127 defines stage 2 and CD122 expression identifies stage 2b NK cells. In stage 3, immature NK (iNK) cells express NKG2D. The hallmark of stage 4 NK cells is high level CD56 expression characteristic of $CD56^{bright}$ NK cells with expression of NKG2A, CD337, CD335 and NKp80. Stage 5 NK cells express lower levels of CD56 as seen on $CD56^{dim}$ NK cells and CD16a (FcRγIIIa) expression. Stage 6, as the final step of NK cells differentiation, is defined by expression of KIRs and the maturation marker CD57 (Fig 7).

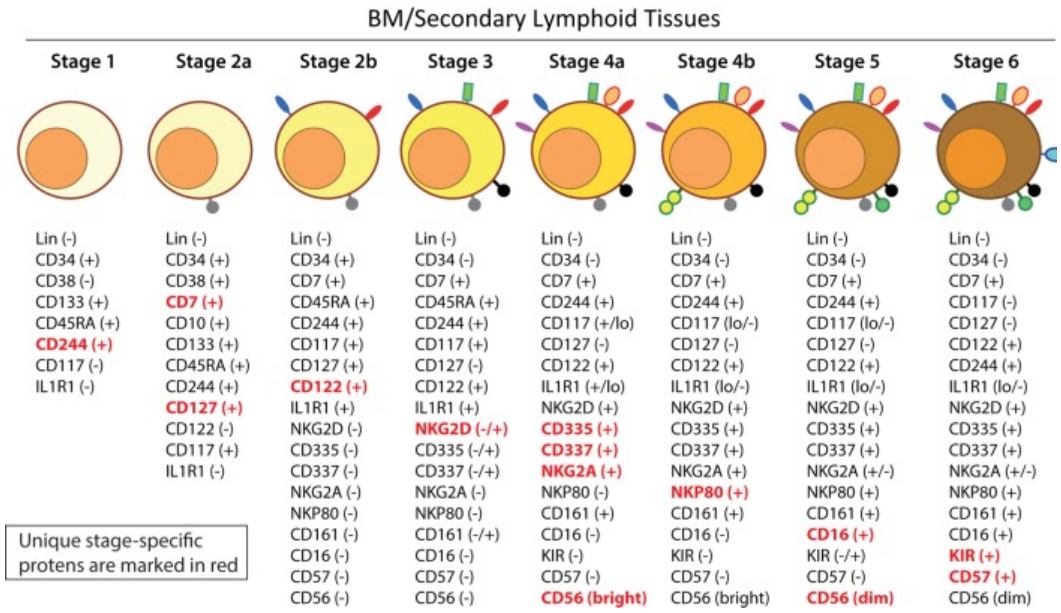

Figure 7. Schematic of different stages of human NK cell development and maturation in bone marrow and lymph nodes. Six different maturation stages are depicted with their

phenotypic markers in black. Markers highlighted in red are the ones considered to be the hallmark of each stage. Adapted from Abel, AM, (2018).

$CD56^{bright}$ cells are less mature than $CD56^{dim}$ NK cells and represent the minority of NK cells in PBMCs but the majority of NK cells in the SLTs (232). CD56dim NK cells have strong cytolytic activity, while their $CD56^{bright}$ counterparts are known for their regulatory activity due to their ability to secrete cytokines (232, 233).

NK RECEPTORS

NK cells express germ line encoded receptors, which belong to several families and which can be either activating or inhibitory (234). The integration of signals received from these receptors govern NK activation status and function.

CD16a (Fcγ RIIIa)

CD16a (FcγRIIIa) is an activating receptor, belonging to Ig superfamily of receptors, it is expressed as a transmembrane glycoprotein on the majority of mature human and mouse NK cells (235, 236). As mentioned earlier $CD56^{dim}$ NK cells express high level of CD16a, whereas $CD56^{bright}$ NK cells rarely express this activating receptor. CD16a uses adaptor proteins that include immunoreceptor tyrosine-based activation motif (ITAM) motifs such as CD3ζ and FcεRIγ to transmit activating signals (237). Upon ligation of CD16a with the fragment crystallizable (Fc) portion of IgG1 and/or IgG3 Abs, a series of downstream signals are induced

resulting in antibody-dependent cell cytotoxicity (ADCC) leading to killing of target cells and antibody-dependent NK activation (ADNKA), which activates NK cells to produce cytokines/chemokines and release the contents of cytotoxic granules that will kill the target cells (234, 238, 239). The involvement of NK cells in ADCC and subsequently killing of cells as a result CD16a ligation by target cell-bound Abs highlights the importance of these responses in viral infections such as HIV (240).

Natural Cytotoxicity Receptors (NCRs)

NCRs were discovered in the late 1990s and classified as activating receptors (Fig 8). They include NKp46, NKp44 and NKp30 (241-244). NCRs are members of Ig-like transmembrane receptor family, which are expressed on NK cells, some subsets of T cells and NK-like cells (241-243, 245). NCRs are quite different from each other structurally, despite their similar functions.

NKp46 (NCR1/CD335) has two extracellular Ig-like domains with a very short cytoplasmic tail. In contrast to NKp46, NKp44 (NCR2/CD336) and NKp30 (NCR3/CD337) have one extracellular Ig-like domain. NCRs are expressed assembled with adaptor molecules with ITAM motifs that transmit activating signals to the effector cells upon binding their ligands. Although NKp44 cell surface expression depends on the association of this receptor with DAP12, an adaptor protein containing ITAMs, its cytoplasmic tail has a tyrosine sequence, which resembles an immunoreceptor tyrosine-based inhibitory motif (ITIM) able to transmit signals that inhibit cytolysis and IFN-γ secretion (242, 243, 246).

NCRs target and kill transformed tumor cells (247). The expression level of NCRs on NK cells and the presence of NCR ligands on tumor cells can affect the ability of effector cells to kill tumor cells through activation of these receptors (248). In addition to NCR's potential to kill tumor cells, they have been implicated in the clearance of cells infected with viruses such as HIV, influenza, human CMV (HCMV) and bacteria such as *streptococcus pneumonia* (249-253). NCRs are also expressed on a high frequency (up to 40%) of decidual NK cells in human uterine tissue (254). The interaction of decidual NCRs with their cognate ligand expressed on trophoblasts leads to the expression of proteins such as vascular endothelial growth factor (VEGF), placental growth factor (PLGF), stromal cell derived factor-1 (SDF-1). These proteins stimulate angiogenesis in the decidua and orchestrate trophoblast invasion, which is critical for supplying an adequate blood supply to the fetus as pregnancy progresses (254).

In addition to the abovementioned beneficial roles of NCRs, these receptors also contribute to pathogenesis of some autoimmune diseases. For example, in type 1 diabetes, the ligand for NKp46 is upregulated on the surface of pancreatic β cells leading to migration of many of $NKp46^+$ NK cells to pancreatic islets. This results in NK cell degranulation and the killing of pancreatic cells, which are the source of the insulin hormone needed to regulate blood glucose levels (255).

The ligands for NCRs are not as well described as the ligands for other NKRs, though this is currently an active area of research. They include non-HLA self and pathogen-derived molecules. Some examples of ligands for NCRs are HLA -B-Associated Transcript 3 (BAT3) and B7-H6. These are non-HLA self-ligands expressed on the surface of tumor cells, which bind

the NKp30 receptor to elicit anti-tumor cell responses (256, 257). Heparin sulfate proteoglycan (HSPG) is another non-HLA ligand, expressed on the surface of tumor cells, which binds both NKp30 and NKp46 resulting in anti-tumor NK cell responses (258). Proliferating cell nuclear antigen (PCNA) on cancer cells, binds NKp44. In this case NK cell cytotoxicity and cytokine production is inhibited due to the presence of the ITIM motif in the intracellular domain of this receptor (259). Influenza hemagglutinin (HA) is an example of a pathogen-derived molecule, which binds to NKp46 to provoke NK cell responses against influenza infected cells (260). In addition to virus derived peptides, NCRs bind to parasite, fungus and intracellular bacteria-derived molecules as well, such as antigens on *Plasmodium falciparum* (malaria) and *Cryptococcus neoformans* (261).

In conclusion, the identification of NCR ligands has raised their profile as potential targets for clinical applications.

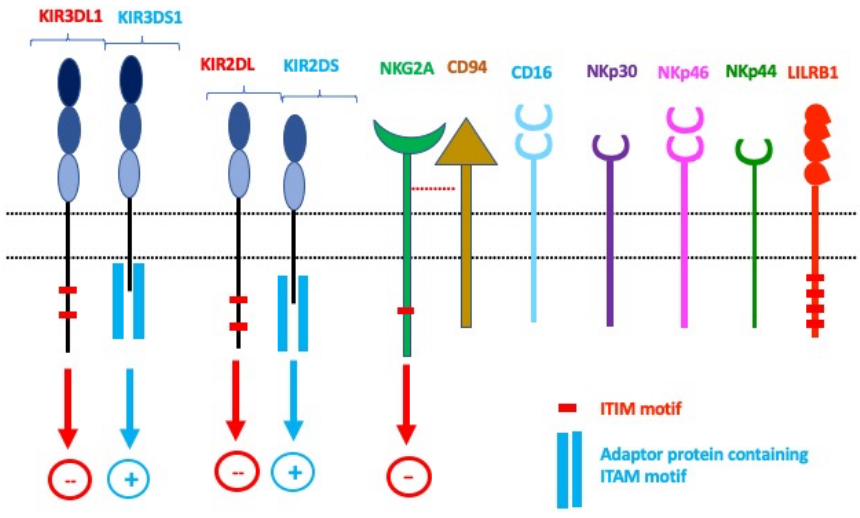

Figure 8. NK cell receptors. Motifs in red are ITIMs on the intracellular domain of inhibitory receptors. As activating KIRs do not have ITAMs, a positively charged aa located in their

transmembrane (TM) region is associated with adaptor proteins containing ITAMs in blue. Made by Zahra Kiani.

Activating and inhibitory leukocyte immunoglobulin-like receptors (LILRs)

LILRs belong to Ig superfamily of receptors. Other names used for LILRs include CD85 and ILT receptors. These receptors are expressed on various cells types including, T, B, NK cells and professional antigen presenting cells (APCs) such as DCs (262). In humans, genes encoding these receptors are located on chromosome 19q13.4 within the leukocyte receptor complex (LCR), which encodes many Ig-like receptors (263). LILRs are divided into two subsets, LILRBs (LILRB1-LILRB5) are inhibitory and LILRAs (LILRA1-LILRA6) are activating receptors (264). LILRBs have either 2 or 4 Ig-like extracellular domain with long cytoplasmic tails containing 2 or 4 ITIMs (262) whereas LILRA possess short cytoplasmic tails with an Arg in their transmembrane domain that recruits adaptor proteins such as the FcεRIγ chain containing ITAMs that transmit activating signals to effector cells bearing these receptors (265).

LILR ligands are classified into two groups: 1) membrane-bound proteins, including MHC class I molecules and 2) soluble proteins. HLA-B, -C free heavy chains and HLA-F are examples of these MHC class I ligands, whereas angiopoietin-like proteins is an example of a soluble ligand for LILRs (266).

LILRs play an important role in bacterial and viral infections. Individuals infected with *Plasmodium falciparum* have a higher frequency of LILRB1^{+} (ILT2^{+}) apoptotic B cells compared to uninfected controls (267). Also, upregulation of LILRB2 (ILT4) and LILRB4

(ILT3) has been reported on the surface of Salmonella infected *in vitro* cultured macrophages (268).

A role of LILRs in HIV-1 disease pathogenesis has been described. Some studies showed that MHC class I polymorphism can influence the binding strength of LILRB1 (ILT2) or LILRB2 (ILT4) to HLA molecules. HLA-B*35 can be divided into two subtypes based on the aa position 9 of the epitope restricted by HLA-B*35 Px and Py allotypes. Px and Py allotypes have an opposite effect on HIV infection outcomes as Px is associated with rapid HIV progression while Py allotypes are associated with neutral effects on HIV disease progression (269, 270). *Huang et al.* showed that Px allotypes have a stronger affinity for LILRB2 (ILT4) expressed on DCs than Py allotypes, which may translate into more potent inhibition that impairs antigen presentation and cytokine production by DCs in HIV infected individuals carrying Px alleles (271). Early after HIV infection, the functional features of DCs are compromised in HIV progressors while ECs maintain functional DCs that successfully present viral antigens to $CD8^+$ T cells and produce cytokines/chemokines to orchestrate immune responses (272, 273). Therefore, LILRs receptors contribute to regulating DCs functions, which are important mediators of HIV disease outcomes. Furthermore, LILRB2 (ILT4) is upregulated in HIV progressors, which is correlated with an elevated level of soluble HLA-G in the plasma of infected individuals (274, 275). HLA-G, a non-classical MHC class Ib antigen, is the ligand for LILRB2 (276, 277). Their interaction results in APC dysfunction in HIV-infected individuals. HLA-B*57 and HLA-B*27 are associated with favorable HIV disease outcomes. The interaction of LILRB2 (ILT4) with these two MHC class I molecules may have beneficial outcomes for HIV progression because LILRB2 (ILT4) binds to these two molecules very weakly resulting in minimal DC inhibition (278).

In addition to classical MHC class I, LILRB1 (ILT2) binds to peptide loaded β2-microglobulin (β2-m)-HLA-F. HLA-F is a non-classical MHC class Ib molecule. By contrast, HLA-F open conformers (OC) do not bind to this inhibitory NK cell receptor (iNKR) (279). In chapter 3 of my thesis I will show that a higher frequency of $ILT2^+$ than ILT2- NK cells responded to HIV-1 infected $CD4^+$ T cells (iCD4) by eliciting several functions, possibly due to the presence of HLA-F OC on the surface of iCD4 cells, which are unable to bind $ILT2^+$. These results suggest that educated $ILT2^+$ NK cells are recognizing autologous HIV-infected sorted $CD4^-$ cells as non-self and through this missing-self recognition are activated to produce CCL4, IFN-γ and CD107a. Each of these factors have anti-HIV activity, suggesting a beneficial role of $ILT2^+$ NK cells against HIV-infected cells.

In summary, LILRs are immunomodulatory receptors modulating immune cell responses such as those of NK cells and professional APCs against various pathogens, which result in tuning immune activation in the context of innate and adaptive immunity.

NKG2 receptors

NKG2 receptors are members of the C-type lectin receptor family expressed on the surface of human and mouse NK cells and a subset of $CD8^+$ T cells. In humans, genes encoding these receptors are found in the natural killer complex (NKC) on chromosome 12 (280). NKG2 receptors are expressed as type II transmembrane glycoproteins that form heterodimers with CD94 via disulfide bonds (281) (Fig 8). The NKG2/CD94 receptor family includes NKG2A, B, C, D, E, F and H, which are categorized into inhibitory and activating receptors with diverse extracellular and intracellular domains (Fig 8). They recognize different ligands and transmit

varied signals to NK cells (282). NKG2A and its splice variant NKG2B are iNKR containing two ITIMs in their cytoplasmic tail (283) whereas NKG2C and NKG2D are activating NK receptors (aNKRs) (284) transmitting their activating signals through adaptor proteins such as DNAX activating protein of 10kb (DAP10) and DNAX activating protein of 12kb (DAP12) (285). Non-classical HLA-I molecules are ligands for some of the NKG2/CD94 receptors (286). HLA-E, is the ligand for NKG2A and NKG2C. HLA-E, only has two variants in humans (287). HLA-E is expressed on almost all human cells with the highest expression levels on immune cells. The HLA-E heavy chain forms a complex with β2-m and 9-mer peptides derived from leader sequence of many HLA-A, -B, -C and -G allotypes (288). Although HLA-I (A, B and C) molecules are downmodulated in some viral infections including HIV, in some cases such as in HCMV infection, HLA-E expression is maintained on the surface of infected cells due to binding peptides derived from HCMV encoded UL40 in order to avoid NK cell recognition through NKG2A; this favors NK cell cytolysis (289).

Interestingly, NKG2D is an exception to the NKG2 family of receptors. It is not dimerized with CD94 and instead is expressed as a homodimer coupled to the DAP10 adaptor protein, which transmits activating signals resulting in activation of cytotoxicity and cytokine/chemokine production. In contrast to other members of NKG2 receptor family, NKG2D does not bind to HLA-I antigens. Instead, its ligands are stress-induced molecules, such as MHC class I-chain-related protein A (MICA) and MHC class I-chain-related protein B (MICB), and UL binding protein-1-6 (ULBP-1-6) (290). NKG2D ligands are not structurally similar to classical HLA-I (291); however, they are highly polymorphic, a feature shared with classical HLA-I molecules.

Following ligand binding to inhibitory NKG2 receptors, the Tyrosine aa in their ITIM becomes phosphorylated possibly by a Rous sarcoma oncogene cellular homolog (Src) family kinase, resulting in the engagement of the lipid phosphatase Src homology (SH2)-containing inositol phosphatase 1 (SHIP-1) and SH2-containing phosphatases 1 and 2 (SHP-1/2), which dephosphorylate proteins contain ITAMs associated with activating receptors. Consequently, the inflow of Ca^{2+}, degranulation of NK cells and production of cytokine/chemokine is inhibited (234).

Due to the lack of ITAMs in the cytoplasmic domain of activating NKG2 receptors, the downstream activating signaling pathways are dependent on the presence and function of adaptor proteins such as ITAM containing DAP10 and DAP12. A negatively charged aa (Lysine [Lys] or Arg) within adaptor proteins interacts with activating receptors through a positively charged aa in their transmembrane domain (Aspartate). Following ligation of the activating NKG2 receptors, tyrosine residues in the transmembrane region of the adaptor protein become phosphorylated, possibly by a Src family kinase, resulting in binding of the phosphorylated adaptor protein to tyrosine kinases such as Spleen tyrosine kinase (Syk) and/or Zeta-chain associated tyrosine kinase-70 (ZAP-70), leading to initiation of various downstream signaling cascades, which result in degranulation and cytokine/chemokine production by NK cells (234).

Several studies have confirmed the contribution of NKG2 receptors to the regulating of immune responses such as NK responses against variety of pathogens. *Thomas et al.* demonstrated that the presence of the gene encoding NKG2C is associated with protection

from HIV infection and slower time to AIDS (292). NK cells bearing inhibitory NKG2A responded more robustly than those negative for this receptor against autologous HIV infected cells (293).

In conclusion, both inhibitory and activating NKG2 receptors are involved in orchestrating innate and adaptive immune responses. Therefore, a better understanding of NKG2 ligands and NKG2 downstream signaling pathways should aid the design of treatment strategies for variety of immune-related diseases.

Killer Immunoglobulin-like receptors (KIRs)

KIRs are a large family of Ig-like receptors, expressed stochastically on NK cells as well as other lymphocyte subsets (294). So far, fourteen KIRs have been identified, which are encoded by genes mapping to the chromosome 19q13.4 region (295). KIR protein nomenclature is based on their structure. The number following "KIR" refers to the number of extracellular Ig-like domains they have, while the "D" stands for domain. The next letter in the protein's name is either "L" for a long cytoplasmic tail or "S" for a short one. The next number indicates an individual KIR protein. KIRs with a long intracellular chain (i.e. 3DL1-3, 2DL1-3, 2DL5) are generally inhibitory KIRs (iKIRs) while those with short intracellular domains (3DS1, 2DS1-5) are activating KIRs (aKIRs) (296) (Fig 8).

IKIRs have 1 or 2 ITIMs in their cytoplasmic tail that transmit NK cell inhibitory signals, whereas aKIRs lack ITAMs in their cytoplasmic tail. A positively charged aa in the

transmembrane region of aKIRs interacts with the adaptor protein DAP12 containing ITAM, which transmit activating signals to NK cells.

In contrast to other NKRs, KIRs are highly polymorphic, with the iKIRs being more so than the aKIRs. The highest allelic polymorphism has been reported for KIR3DL1, with 84 allotypes described to date (297). This high level of allelic polymorphism is thought to have arisen from pathogen pressure on NK cells. Allotype differences can affect KIR cell surface expression levels, their binding affinity to their ligands, as well as their functions upon stimulation (298).

Telomeric and centromeric *KIR* genes are in linkage disequilibrium with each other. These 2 regions are separated from each other by a recombination hot spot. Due to the high level of linkage disequilibrium (LD) among the KIR genes, they can be classified into two haplotype groups, A and B. Haplotype group A consists mostly of genes encoding *iKIRs*, while haplotype group B contains these *iKIR* genes with various additional numbers of genes encoding aKIRs (299).

A subset of MHC class I molecules are cognate ligands for iKIRs. The genetic area encoding MHC class I antigens in humans is located on chromosome 6p21.3, which is distant from the *KIR* genes (300). This means that *KIR* and *MHC* class I genes segregate independently, resulting in huge KIR/HLA combination diversity. Among the MHC class I antigens are the classical (HLA-A, -B and -C) and non-classical (HLA-E, -F and -G) antigens. Like KIRs, all classical MHC class Ia molecules are highly polymorphic, whereas non-classical MHC class Ib antigens are more conserved (301).

HLA-B allotypes can be classified as either Bw4 or Bw6, based on the aas at positions 77 to 83 in the α1 domain of these molecules (302). Bw4 antigens are KIR3DL1 ligands, which can differ from each other in terms of affinity for different KIR3DL1 allotypes, whereas Bw6 allotypes do not interact with any of the KIRs (303, 304). There is also a dimorphism at position 80 of Bw4 HLA heavy (H) chain, which divides Bw4 allotypes into those with an Isoleucine (80I) or a Threonine (80T) at this position. Bw4*80I molecules are higher affinity ligands for KIRs than Bw4*80T allotypes (305). However, the Bw4*80T HLA-B*27:05 antigen is a high affinity ligand for KIR3DL1 and is a protective allele in the context of HIV infection (306, 307).

HLA-C molecules are ligands for KIR2D receptors. HLA-C antigens also have a dimorphism at position 80 of their HLA H chains that split them into the C1 group, which have an Asparagine at position 80 and the C2 group, which have a Lysine at position 80. The C1 and C2 group antigens are ligands for different KIR2D receptors. C1 allotypes bind to KIR2DL2 and KIR2DL3, while C2 allotypes are ligands for KIR2DL1 receptors. The affinity each HLA-C antigen for its KIR2D allotype varies. Binding of C2 to KIR2DL1 has been reported to trigger stronger inhibitory signals than inhibitory signals generated through binding of C1 to KIR2DL3 (308-310).

The extracellular Ig-like domain amino acid sequences of aKIRs are highly homologous to their iKIR counterparts. The highest similarity is between KIR3DS1 with KIR3DL1, which exceeds 97% sequence similarity. It was expected that aKIRs would bind the same ligands as their corresponding iKIR, though possibly with a lower affinity. This has been found to not be the case, with a few exceptions. C2 antigens interact with KIR2DL1 and with a lower affinity to

KIR2DS1 (311). Many attempts to demonstrate that KIR3DS1 binds HLA-Bw4 ligands as does KIR3DL1 have failed (312). There is one exception to this where KIR3DS1 was found to bind HLA-B*5701, which is a Bw4*80I allotype, but only in the presence of particular HIV peptides (313).

As mentioned earlier, KIRs are stochastically expressed on NK cells, so that there exists a variety of NK cell clones expressing different KIRs, each with its own ligand specificity. This ensures that some NK cells will be able to rapidly recognize transformed cells with downmodulated levels of HLA-I ligands.

Several studies have shown that certain KIR-HLA combinations are associated with outcomes that can affect human health (307, 314, 315). In the context of HIV infection, co-expression of HLA-A and B ligands containing Bw4 motifs with their interacting KIR, is associated protection from infection and slower time to AIDS (134, 316). Homozygosity for KIR2DL3 and its HLA-C ligand is associated with protection from chronic HCV infection (317).

In conclusion, co-carriage of genes encoding KIRs and their cognate MHC-I ligands, is associated with outcomes in the context of a variety of human diseases. Thus, identifying the unknown ligands for KIRs and understanding how these NKR-ligand interactions affect NK cell function and disease outcomes may provide crucial information required to develop more efficient vaccines and therapies for a variety of human health complications.

NK EDUCATION

NK cells acquire their effector functions through a process called “education”, “licensing” or “arming”. In this process, both iNKRs and aNKRs contribute in shaping NK cell responsiveness by interacting with self MHC class I ligands. NK cells bearing iNKRs to self MHC are hyperresponsive when they enter the periphery and encounter target cells lacking self MHC molecules. The role of aNKRs in NK education is controversial. Based on the initial principals of NK cell education, binding of aNKRs to self MHC ligands tunes down NK functionality. However, recent studies have shown that this is not always the case and the opposite may occur as has been described in chapter 3 of my thesis (312).

As mentioned earlier, NK cells express a variety of iNKRs such as iKIRs, NKG2A and LILRBs, which all bind to self-classical or non-classical HLA-I ligands. The interaction between iNKRs and self HLA-I during NK cell development educates NK cells. If an NK cell has no iNKRs to self HLA, the NK cell will be hyporesponsive. If their iNKRs interact with ligands they will develop the potential to become functional when they encounter self-cells that have downmodulated their HLA ligands due to stress, viral infection or transformation. Of note, NK cell education is a tunable process, as it can be adjusted by copy number and cell surface density of iNKRs, by the expression level of self HLA-I and by the binding affinity of receptor/ligand interactions. Allelic variation of both receptors and ligands influences the strength of receptor/ligand binding and consequently NK education.

HLA-Bw4*80I and Bw4*80T can differ in their cell surface density and affinity for their ligands. Accordingly, KIR3DL1^{+} NK cell functional potential can range from low in NK cells

from subjects who are Bw6 hmz to potent in NK cells from subjects who express KIR3DL1high/Bw4*80I combinations (318). NK cells from KIR3DL1*h/*y + *HLA-B*57* donors are able to inhibit viral replication in autologous CD4 T cells better than those from KIR3DL1 *l/*x + *HLA-B*B57* and *Bw6* hmz donors (314). The *KIR3DL1*h/*y* carriers encode high expression allotypes while the *KIR3DL1*l/*x* carriers express their gene products at a more modest density (307). Carriers of the *KIR3DL1*h/*y* gene combination were also shown to have a 5-fold lower risk of HIV infection compared to *Bw6* hmzs (134).

Although NK cell education has been highlighted mainly through the binding of iKIRs to self HLA-I ligands, other iNKRs can influence NK education, such as NKG2A and LILRBs. Ligation of these two iNKRs to self HLA-I ligands leads to NK education and thereby elevated NK cell responsiveness to target cells that have downmodulated their ligands for iNKR (293, 319).

In addition to the presence of educated NK cells, there are NK cell subsets that are uneducated or unlicensed due to lack of interaction between iNKRs and self HLA ligands. These NK cells are hyporesponsive. This is important in maintaining tolerance and avoids NK cell mediated autoimmune responses.

NK cell education has been well-studied in the context of HIV infection. HIV down modulates the expression of HLA-I molecules on the surface of infected cells to evade CTL responses (303, 320-322). However, this downmodulation makes infected cells more susceptible NK cell recognition and killing through “missing-self” mechanisms. The potency of NK responses to

HIV infected cells is directly linked to the potency of NK cell education (318). This observation has been confirmed by epidemiological studies in which infected individuals carrying the *KIR3DL1/Bw4*80I* genetic combination showed slower time to AIDS than carriers of other genotypes (316).

In contrast to iNKRs, the interaction of aNKRs with their ligands tunes down NK cell responsiveness. KIR2DS1 is an aKIR able to bind HLA-C2 group antigens. KIR2DS1^{+} NK cells from *HLA-C2* hmz donors become self-tolerant by developing reduced functionality to target cells expressing HLA-C2 *in vivo* as well as when co-cultured with target cells expressing HLA-C2 (311, 323).

Altogether, NK cell education is a tunable and resettable process determined by engagement of both iNKRs and aNKRs that orchestrate the balance between generating hyperresponsive and self-tolerant NK cells.

NK CELL FUNCTIONS

NK cells recognize transformed, virally infected and stressed cells (324). NK cell activation leads to the production of cytokine/chemokines (such as IFN-γ, TNF-α, CCL3/4/5) and/or degranulation measured by CD107a expression, which is a surrogate marker for target cell cytolysis. ADCC is another major function of NK cells, in which NK cells, activated by the engagement of CD16 by the Fc portion of IgG release the contents of their cytotoxic granules containing perforin and proteases such as granzymes, resulting in lysis of target cells. In the

context of HIV infected cells, opsonized with anti-HIV Env-specific Abs (anti-gp120) are recognized and lysed by NK cells. In the literature, ADCC has frequently been incorrectly used to refer to ADNKA, which, as its name implies, is the activation of NK cells through CD16 engagement to produce cytokines and chemokines (325). These two Ab dependent NK cell functions may not correlate with each other (326). NK cell education appears to be important for ADNKA while ADCC activity appears not to be (325, 326).

Besides the important role of NK cells in innate immunity, NK cells are involved in shaping adaptive immune responses as well via their "cross talk" with other immune cells such as DCs and T cells. Mature DCs (mDCs) produce IL-12, IL-15, IL-18 and Type I IFNs leading to activation, proliferation and survival of NK cells. In turn, activated NK cells produce cytokines/chemokines, which are important for the differentiation of immature DCs (iDCs) into mDCs (327, 328). NK cells, activated by DCs, are able to lyse iDCs leaving mature myeloid DC untouched.

In addition to DCs, NK cells influence T cell responses by producing IL-10 and IFN-γ, which inhibit or promote Th1 mediated immune responses, respectively, thus playing a role in clearance of virally infected cells mediated by $CD8^+$ CTL responses.

MEMORY-LIKE NK CELLS

Immunological memory is one of the important features of immune cells such as T cells and B cells. Memory cells recall antigens encountered previously and mount faster and more robust

immune responses upon subsequent exposures. NK cells were thought to be members of the innate immune system with no immunological memory. In 2006, *O'Leary et al.* showed that liver NK cells from recombinant-activating gene (*RAG*)-deficient mice had memory-like features to haptens that they were previously exposed to (329). In 2009, *Sun et al.* reported that in C57Bl/6 mice infected with murine CMV (MCMV), the aNKR Ly49H, binds to m157, an MHC class I homolog encoded by MCMV (330). $Ly49H^+$ NK cells clonally expanded in response to MCMV, then underwent a contraction phase, as MCMV infection resolved. The remaining $Ly49H^+$ NK cells were long lived, had a heightened response to re-challenge with MCMV and transferred protective anti-MCMV responses to murine strains that lack $Ly49H^+$. These are all features of memory cells. Furthermore, the immunological memory responses from NK cells are virus-specific as memory-like NK cells from mice immunized with influenza virus-like particles were only protected against influenza virus and not against other viral infections (331).

There is an analogous situation in humans where memory-like NK cells have been reported in HCMV infected individuals (332-334). Approximately 50% of individuals who are HCMV seropositive, possess higher frequencies of $CD56^{dim}NKG2A^-NKG2C^+CD57^+$ NK cells with memory-like characteristics (long-lived, terminally differentiated) than HCMV seronegative subjects (335). These expanded, memory-like NK cells have elevated ADCC responses to Ab opsonized HSV-1, influenza viruses and HCMV infected cells (336, 337). Of note, these NK cells exhibit persistent and inheritable epigenetic imprints similar to memory T cells (338). Moreover, it has been shown that memory-like NK cells transferred from donors to recipients, who are HCMV seropositive but clinically HCMV DNA undetectable (i,e, latently infected), by transplantation of adult donor allogeneic hematopoietic cells expand following HCMV

reactivation in recipients (339). This can also occur in recipients of CMV naïve umbilical cord transplants to recipients following CMV reactivation (340). Blocking of interaction between NKG2C and HLA-E results in abrogation of $NKG2C^+$ NK cell expansion, implicating a requirement for receptor/ligand interactions in $NKG2C^+$ NK expansion. NKG2C binds to a peptide derived from HCMV encoded UL40 presented by HLA-E (334, 341).

Gondois-Rey et al. studied $NKG2C^+$ memory-like NK cells in individuals undergoing HIV primary infection (PI), who started cART within 2 months of infection. Subjects were studied at the start of cART and after 3 months on cART. They found that the frequency of $NKG2C^+CD57^+$ memory-like NK cells did not change on cART and was inversely associated with HIV VL and positively associated with plasmacytoid DCs (pDCs) in this population (342).

Reeves et al. reported on memory-like NK cells in the spleen and liver of RMs infected with SIV_{mac251} or $SHIV_{SF162P3}$ that lysed Gag and Env pulsed DCs that were absent in uninfected animals (343). They also identified a long-lived subset of splenic and hepatic NK cells in RMs vaccinated with adenovirus serotype 26 (Ad26) based vaccines as these vaccinated animals killed antigen-matched but not mismatched antigen pulsed DCs for up to 5 years after initiation of vaccination. These findings confirmed that antigen-specific, potent and durable NK memory-like responses could be elicited after either SIV infection or vaccination.

Memory-like NK cells have been found in other viral infections, such as Hantavirus, Chikungunya virus and SIV/SHIV infection in RMs (343-345). However, in each of these cases

NK cells with markers for memory-like activity are only found in individuals who are also HCMV co-infected.

In summary, although historically, NK cells were thought to be innate immune cells, an increasing number of studies have demonstrated that there are unique subsets of NK cells known as "memory-like or adaptive NK cells" that have memory/adaptive-like characteristics that expand rapidly upon re-encountering antigen they have recognized before and that can transfer immunity to an antigen to naïve hosts. Therefore, NK cells are either directly or indirectly involved in both innate and adaptive immune responses against tumor cells or virally infected cells.

NK CELLS IN VIRAL INFECTIONS

NK cells in HCMV infection

CMV is a dsDNA virus belonging to *herpesviridae* family. CMV infects eukaryotic cells from mice (MCMV), humans (HCMV) and various species of NHPs, among others. CMV infection is life-long as after the resolution of acute infection, the virus frequently establishes a persistent latent infection with occasional periods of reactivation characterized by re-expression of viral genes leading to the lytic viral life cycle (346). As for other types of viral infections, CMV infection results in the generation of effector/memory $CD8^+$ T cells. As seen in the previous section, CMV infection can leave a long-lasting imprint on NK cells, phenotypically and functionally, which can influence NK cell responses to other pathogens as well. HCMV provides viral peptides to stabilize the expression of HLA-E on the surface of infected cells in order to

maintain inhibitory signals through NKG2A expressed on NK cells (347). HLA-E is also the ligand for the aNKR, NKG2C. The affinity of HLA-E for NKG2C is lower than that for NKG2A, such that inhibitory signals through NKG2A can dominate those activating signals through NKG2C, resulting in NK cell inhibition (348). HCMV encodes decoy molecules such as UL18, which binds to the inhibitory receptor ILT-2, expressed on NK cells, leading to inhibition of ILT-2^+ NK cells but activation of ILT-2- NK cells (349). As mentioned before, MCMV encodes a viral peptide, m157, that can bind to both iNKR and aNKR to either inhibit or activate NK cells (350) (351). CMV contributes to the downmodulation of ligands for aNKR, which allows these cells to escape NK cell responses (352). CMV infection also downmodulates the expression of MHC class I molecules on the surface of infected cells, making CMV infected cells poor targets for $CD8^+$ T cell responses. Reduced MHC class I expression can result in "missing-self" recognition and cytolysis of CMV infected cells by NK cells.

NK cells in influenza infection

Influenza virus, which belongs to Orthomyxoviridae family, is an enveloped virus with eight single stranded RNA segments. This virus causes seasonal epidemics leading to 250,000-500,000 deaths annually world-wide (http://www.who.int/mediacentre/factsheets/fs211/en/). Due to antigenic drift, different viral variants predominate each season. For this reason, vaccines against this virus need to be selected and constructed for each seasonal epidemic. Neutralizing Abs to the cell surface glycoprotein, hemagglutinin (HA) and specific CTL responses are two main immune responses with important roles in controlling influenza infection (353, 354). In addition to T cells, NK cells also contribute to immune responses against this virus. For example, KIR2DL3$^+$ NK cells educated through HLA-C1 interactions mediate potent anti-influenza responses (355).

Further, *in vitro* studies indicate that NKp46, expressed on NK cells, can recognize and kill HA expressing infected cells (356).

Infected DCs are proposed to play an important role in activating NK cells to respond to influenza infection by expressing ligands for aNKRs such as NKp46 and NKG2D. NK cell activation via this pathway leads to the production of cytokines such as IFN-α and IL-12 (357).

Like other viruses, influenza has developed strategies to avoid NK cells responses. Among these are changing the glycosylation pattern of HA preventing binding to aNKRs (358). There is evidence that influenza infection results in the accumulation of MHC class I molecules on the surface of infected cells, which increases binding to iNKRs and consequently NK cell inhibition (359).

NK cells in HIV infection

NK cells can recognize and respond to virally infected cells without prior exposure to viral antigens. In addition to direct target cell recognition, anti-HIV Abs produced during infection can activate NK cells via ADNKA assessed by cytokine/chemokine production and degranulation. Anti-HIV Abs can also opsonize HIV Env expressing target cells for ADCC measured by delivery of granzyme B to target cells as an early marker of apoptosis (360) and/or positivity for Annexin V as a later marker of apoptosis (Dupuy et al. unpublished results). Although both ADNKA and ADCC have the potential to mediate anti-HIV activity, the former results in activation of NK cells to secrete IFN-γ and CC-chemokines, which have anti- HIV functions and express CD107a, which leads to direct lysis of infected cells (325). The RV144

HIV vaccine trial found that the vaccine regimen induced non-neutralizing Abs (nNAbs) that mediated ADCC against HIV Env coated target cells (361). These nNAbs also induced ADNKA for IFN-γ, CCL4 secretion and expression of CD107a (219). Furthermore, *Ackerman et al.* showed that the polyfunctional Abs in ECs mediate innate immune responses such as ADCC, ADNKA and phagocytosis by monocytes and neutrophils, which may be a factor in the ability of this rare group of HIV-infected individuals to spontaneously control HIV infection (362). One critique of this work is the use of HIV Env gp120 coated CEM.NKr.CCR5 cells as target cells for the ADCC assay. The monomeric Env used to coat these cells is in a conformation that is quite different from that found on CD4 T cells infected with wild-type HIV. Env on infected cells is trimeric and in a closed conformation (363). Coated and infected target cells differ in the epitopes they present to the anti-HIV Env-specific Abs in plasma from infected individuals.

Some studies have reported poor NK responses against HIV infected CD4 cells (364). NK cell phenotype and function can change during the course of HIV infection. This may be the reason that NK cells are not effective at controlling infection in those infected. HIV infection modulates the phenotype and frequency of NK cell subsets, differentially, based on the phase of infection (365). During the acute phase of HIV infection, there is an elevated frequency of $CD56^{dim}CD16^{+}$ NK cells, whereas the frequency of $CD56^{bright}CD16^{-}$ NK cells starts to decrease during this phase of infection. As the infection passes from the acute to the chronic phase with persistent viral replication, the frequency of $CD56^{dim}CD16^{+}$ NK cells declines and the number of "anergic" $CD56^{-}CD16^{+}$ cells increases (365). These anergic NK cells have lower expression levels of NCRs such as NKp30 and NKp46 and they also have poor antiviral functions, including decreased antiviral cytokine production, cytotoxicity and ADCC activity (366). *Shah et al.*

demonstrated that co-activating signals are necessary for NK cell degranulation in addition to downmodulation of ligands for iNKRs and upregulation of ligands for aNKRs (367). He showed that HIV infected cells are able to induce NK activation but they are incapable of triggering maximal degranulation due to downmodulation of NTB-A (co-activating molecules on the surface of infected cells) through Vpu, an accessory HIV protein (367). Of note, all the above-mentioned phenotypic and functional changes in the NK cell repertoire are in the favor of HIV replication.

The protective role of NK cells in the context of HIV infection (protection from infection)

The role of NK cells in protection from HIV infection is partially understood. Immune quiescence is proposed to be an important reason for HIV resistance in HESN from the Nairobi, Kenya CSW cohort. More efficient killing of autologous iDCs and a higher level of IFN-γ secretion by activated NK cells could have a role in HIV resistance in this cohort (368).

In line with the proposed protective role for NK cells, *Scott-Algara et al.* showed that NK cells from HESN IDUs from Vietnam were superior, compared to NK cells from uninfected subjects and HIV susceptible persons before they became infected, at killing target cells and at secreting antiviral cytokines in response to HLA-null K562 and Daudi cells (369). The findings are consistent with NK cells playing a role in protection from HIV infection.

The hypothesis that HESNs may be more likely than HIV susceptible/infected persons to carry genetic *KIR/HLA* combinations encoding molecules that educate NK cells more potently was tested by the Bernard laboratory. They used KIR3DL1 and MHC class I geno- and allotyping to

show that HESNs, compared to HIV susceptible individuals, had higher frequencies of two genotype combinations: the high expression KIR3DL1*h/*y with HLA-B*57 (*h/*y/B*57) and homozygosity for KIR3DS1 (134, 370). NK cells from HIV seronegative subjects carrying the **h/*y/+B*57* combination was compared to those from *Bw6* hmzs (whose KIR3DL1^{+} NK cells were uneducated) or who did not express the HLA-B*57 ligand or the high expression KIR3DL1 receptors. When these NK cells were tested for functional potential by stimulation with HLA null cells those from carriers of **h/*y/+B*57* were significantly more functional than those from carriers of the other *KIR/HLA* combinations (176). This was the first demonstration that HESNs are more likely to carry *KIR/HLA* combinations associated with protection from HIV infection, and that these KIR/HLA combinations were more potent at educating NK cells for higher functionality. The functions they elicited included IFN-γ, TNF-α, CD107a and CCL4, all of which have anti-HIV activity. NK cells from carriers of the **h/*y/+B*57* combination were also more potent than NK cells from *Bw6* hmzs and other control genotype combinations at inhibiting HIV replication in autologous HIV infected cells (314). This inhibition was due in part to the secretion of higher levels of CC-chemokines by NK cells from **h/*y/+B*57* carriers than those from subjects with control *KIR/HLA* combinations (314). Further, NK cells from carriers of *KIR/HLA* combinations that supported, versus not, NK cell education responded more potently to autologous HIV infected CD4^{+} T cells (371).

Previous work from the Bernard laboratory also showed that KIR3DS1 homozygosity was associated with protection from HIV infection and slower time to HIV seroconversion in HESNs (370, 372). KIR3DS1 is an aNKR encoded by a gene within the KIR haplotype B telomeric region. This gene is in tight LD with other KIR genes in this region. A functional approach was

used to show that *KIR3DS1* and not gene products in LD with *KIR3DS1* was responsible for functional response to both HLA-null and autologous HIV infected cells (373, 374). In chapter 2 and 3 of this thesis, I will present results describing the mechanism underlying the association between carriage of the *KIR3DS1* hmz genotype and protection from HIV infection.

Role of NK cells in the context of HIV control (slower time to AIDS)

There are several studies demonstrating the role of NK cells in progression to AIDS. *Martin et al.* showed that carriage of the *KIR3DS1/HLA-Bw4*80I* genotype was associated with slower time to AIDS in infected individuals (316). They also reported that *HLA-Bw4*80I* was not linked with slower time to AIDS in the absence of *KIR3DS*1. Surprisingly, the presence of *KIR3DS1* alone was associated with faster disease progression. The protective effect of *HLA-Bw4*80I* in *KIR3DS1* carriers was not due to the presence of *HLA-B*57* as this allotype is protective in the presence and absence of *KIR3DS1*. Also, the protective effect of *HLA-Bw4*80I* carriage was not due to the co-carriage of *HLA-B*27* as the most common allele of *HLA-B*27 (B*27:05)* is a *Bw4*80T* allele, whose gene product does not interact with KIR3DS1. *Martin et al.*'s findings suggest a strong synergistic effect of carriage of *KIR3DS1* with *HLA-Bw4*80I* on CD4 cell loss, emphasizing the protective role of KIR/HLA combination on NK cell activity at early stages of HIV infection (316). *Qi et al.* reported that carriage of *KIR3DS1* in combination with *HLA-Bw4*80I* was associated with reduced VL and protection from opportunistic infections compared that seen in the control group who were *Bw6* hmzs (375). *Alter et al.* confirmed experimentally the protective effect of the *KIR3DS1/Bw4*80I* gene combination in controlling HIV. They demonstrated that NK cells from carriers of this genotype inhibited HIV viral replication in

autologous HIV infected CD4 cells more potently than NK cells from carriers of genes encoding the KIR receptor or HLA ligand alone or neither (376).

HLA-B*57 is one of the most protective HLA allotypes in HIV infection. HIV-infected SPs have a higher frequency of this allotype compared with HIV-infected typical progressors or unexposed healthy individuals (164, 166, 171). The protective effect associated to this allotype is mediated through $CD8^+$ T cells recognizing HLA-B*57 HIV restricted epitopes (166, 377-379). However, HLA-B*57 is also a high affinity ligand for KIR3DL1*h/*y allotypes. *Martin et al.* demonstrated that individuals who are *KIR3DL1*h/*y+B*57* carriers exhibit a slower rate of HIV disease progression with a lower VL than *Bw6* hmzs (307). Therefore, HLA*-B*57 mediates HIV control through both $CD8^+$ T cell and NK cell responses. The protective effect of this allele may be achieved through stronger binding to KIR3DL1 during development, which educates these $KIR3DL1^+$ NK cells for superior function when they encounter HIV infected targets with downmodulated HLA-Bw4 (175, 176, 307). Of note, the presence of low expression KIR3DL1 iKIRs encoded by carriers of the *KIR3DL1*l/*x*, genetic combination co-carried with *HLA-Bw4*80T* was associated with weak protection against disease progression similar to that of the iKIR/HLA gene products encoded by *Bw6* hmzs. My host laboratory was the first to show that carriage of the *KIR3DL1*h/*y+B*57* combination was protective against HIV infection, not just against disease progression in those infected (134). They showed that NK cells from carriers of *KIR3DL1*h/*y+B*57* controlled viral replication more potently than NK cells from carriers of other *KIR/HLA* combinations through secretion of higher levels of CCL3/4/5 chemokines (314). In addition, my host laboratory showed that NK cells from SPs bearing the *KIR3DL1*h/*y+B*57* combination had the highest frequency of trifunctional $CD107a^+$IFN-

γ^+TNF-α^+ NK cells compared to subjects carrying all other *KIR/HLA* genotypes (175). The findings can be explained by the KIR3DL1**h/*y+B*57* combination supporting the strongest educational signal for NK cells during development. This potent education translates directly into these NK cells having the highest functional potential when they encounter autologous HIV infected CD4 cells, which have downmodulated the level of HLA-Bw4 expression on the cell surface by HIV Nef mediated function. This leads to the strong inhibitory signal through KIR3DL1/HLA-B*57 being abrogated so that NK cells become activated and respond to infected cells.

The HLA-B*27 allele is another protective isoform belonging to the Bw4-80T group. Similar to HLA-B*57, HLA-B*27 is a protective HLA allotype in terms its association with slow time to AIDS. Slowed HIV progression is due to its restricting HIV epitope that are well recognized by CTL responses. HLA-B*27 also binds to KIR3DL1, though with lower affinity compared to HLA allotypes belonging to the Bw4*80I group. Unexpectedly, carriage of the *KIR3DL1*l/*x+HLA-B*27* combination is associated with slower HIV disease progression and lower VL than carriers of *KIR3DL1*h/*y+HLA-B*27* genotypes (307). This could be due to stronger binding between HLA-B*27 and KIR3DL1*l/*x than KIR3DL1*h/*y allotypes, leading to more potent education and higher functional potential (380).

GWAS identified single nucleotide polymorphisms (SNPs) associated with HIV disease progression. Many did not achieve significance due to the large number of comparisons in these studies (169, 381, 382). There was a strong association between the rs2395029 SNP and HIV control. This SNP is close to the HLA complex P5 (HCP5) located in a gene, which is physically

close but not in the MHC class I region (381). Another SNP associated with VL control was located 35 kb upstream of the HLA-C region (rs9264942) (169). This SNP is associated with differential HLA-C expression levels; rs9264942^{+} genotypes are linked with higher expression of HLA-C, more potent HLA-C restricted anti-HIV CTL responses and more efficient HIV inhibition (383). A role for NK cells, which when educated can also respond to HIV infected cells with downmodulated HLA-C has not been excluded. *Korner et al.* showed that educated KIR2DL1^{+} and KIR2DL2/L3^{+} NK cells (through interactions with their C1 and C2 group ligands, respectively) from HIV infected individuals respond more potently to HLA null cells than uneducated NK cells (384). This work did not address whether educated KIR2D^{+} NK cells contribute to protection from HIV infection or slower disease progression.

NK CELLS AS A POTENTIAL CANDIDATE IN HIV VACCINE

Over the last 30 years, numerous HIV vaccine trials have been conducted. The only one to show efficacy was the RV144 trial (385). HIV's high level of diversity, its high mutation rate that promotes immune evasion and its capacity to suppress immune responses are barriers to the design of effective vaccines for this virus. An effective vaccine not only should inhibit free virions it also needs to prevent cell to cell transmission.

As mentioned earlier, interactions between NK cells and DCs could influence the development of both innate and adaptive immune responses against variety of pathogens. In line with this, *Cummings et al.* found that NK cells co-cultured with autologous HIV infected DCs became activated and responded to infected cells by degranulation and cytokine production.

Additionally, these primed NK cells proliferated robustly and modulated their NKG2D and NKp46 aNKR making them more active against HIV infection (386). Interestingly, these activated NK cells inhibited viral replication in an HIV-specific manner as they were not responsive to other viral infections or to tumor cells. Such NK cells may be of interest to test as a vaccine candidate.

Interest in using ADCC activity to kill tumor cells and HIV infected cells is a vaccine strategy that has gained interest recently for therapeutic approaches against cancers and HIV infection. Such approaches have shown promising results in both pre-clinical and clinical trials. *Lu et al.* showed that both bNAbs and nNAbs mediate anti-HIV directed ADCC activity, which was correlated negatively with HIV replication and positively with protection from infection (387). Investigation of the correlates of protection for the RV144 vaccine trial found that anti-HIV-Env specific IgG3 nNAbs that were ADCC-competent correlated with protection from infection in individuals who received the vaccine (217, 388). In another study, RMs were immunized with an ALVAC/Pentavalent vaccine recognizing 5 subtypes of HIV gp120. Plasma Abs from vaccinees recognized HIV-infected cells. The level of Abs mediating ADCC, ADCC potency and production of MIP-1β through ADNKA were significantly higher in the vaccinated compared with the control group and were associated with protecting these animals from acquisition of SHIV infection with an efficacy of 55% (389).

In the context of treatment, adoptive transfer of activated and more functional NK cells would be another alternative to cure HIV-infected individuals. In line with this hypothesis, chimeric-antigen receptor (CAR) T cells and NK cells expressing modified antigen receptors against

tumor cells showed promising results in cancer therapy (390). Regarding HIV infection, it has been shown that adoptive transfer of genetically modified hematopoietic stem cells (HSC) to humanized mice results in the differentiation of these cells into human T cells and NK cells, which are resistant to acquisition of HIV infection and able to inhibit viral replication (391).

In conclusion, NK cells recognize and kill HIV infected cells either directly or by ADCC. Their activation leads to the secretion of antiviral chemokines that block the entry of HIV to new cells and cytokines with other anti-viral activities and activation of cytolytic functions. NK cells contribute to generating adaptive immune responses that in turn inhibit viral replication. Altogether, NK cells should be considered and evaluated further as an important effector immune cells in vaccine and treatment strategies against HIV.

CHAPTER 2

HLA-F on HLA-null 721.221 cells activates primary NK cells expressing the activating killer immunoglobulin-like receptor (KIR) 3DS1

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This chapter was adapted from an article published in the Journal of Immunology, 201. DOI10.4049/jimmunol.1701370

Authors contributions:

Z.K. designed the study, performed the experiments and data analysis and prepared the manuscript; F.P.D. performed some of the experiments and edited the manuscript; J.B. and B.L. provided clinical samples; E.J., C.X.Z. and I.L. performed the experiments, analyzed data and edited the manuscript; S.D.F edited the manuscript; DEG provided the anti-HLA-F mAbs and edited the manuscript; N.F.B. designed the study and prepared and revised the manuscript. The authors declared no conflict of interest.

RATIONALE AND RESEARCH OBJECTIVES

Previous work from the Bernard laboratory demonstrated a higher frequency of KIR3DS1 homozygosity in HESN individuals compared with HIV infected subjects. It was recently shown that HLA-F, expressed on activated lymphocytes and some cell lines, is a cognate ligand for KIR3DS1. This prompted us to investigate the responses of KIR3DS1$^+$ NK cells to HLA null cells as a model of HIV infected cells that downmodulate their MHC class I antigen levels. To ensure that the responsiveness of KIR3DS1$^+$ NK cells was not due to co-expression of other a/iNKR, we designed a flow cytometry Ab panel and a strategy that gated out KIR3DS1$^+$ NK cells that co-expressed other NKRs that may contribute to KIR3DS1$^+$ NK cell functionality.

Publication status

This work has been published in the J Immunol May 9, 2018, ji1701370; DOI: https://doi.org/10.4049/jimmunol.1701370

ABSTRACT

NK cells elicit important responses against transformed and virally infected cells. Carriage of the gene encoding the activating Killer Immunoglobulin-like Receptor (KIR)3DS1 is associated with slower time to AIDS and protection from HIV infection. Recently, open conformers of the non-classical major histocompatibility complex class I (MHC-1b**)** antigen HLA-F were identified as KIR3DS1 ligands. Here, we investigated whether the interaction of KIR3DS1 on primary NK cells with HLA-F on the HLA-null cell line 721.221 (221) stimulated KIR3DS1$^+$ NK cells. We used a panel of antibodies to detect KIR3DS1$^+$CD56^{dim} NK cells that co-expressed the inhibitory NK cell receptors (iNKR) KIR2DL1/L2/L3, 3DL2, NKG2A and ILT2, the activating NK cell receptors (aNKR) KIR2DS1/S2/S3/S5 and CCL4, IFN-γ and CD107a functions. We showed that both untreated and acid-pulsed 221 cells induced a similar frequency of KIR3DS1$^+$ cells to secrete CCL4/IFN-γ and express CD107a with a similar intensity. A higher percentage of KIR3DS1$^+$ than KIR3DS1$^-$ NK cells responded to 221 cells when either inclusive or exclusive (i.e co-expressing none of the other iNKR and aNKR detected by the antibody panel) gating strategies were employed to identify these NK cell populations. Blocking the interaction of HLA-F on 221 cells with KIR3DS1-Fc chimeric protein or anti-HLA-F antibodies on exclusively gated KIR3DS1$^+$ cells reduced the frequency of functional cells compared to that of unblocked conditions for stimulated KIR3DS1$^+$ NK cells. Thus, ligation of KIR3DS1 activates primary NK cells for several anti-viral functions.

INTRODUCTION

NK cells are effectors of the innate immune system that are particularly well adapted for anti-tumor and anti-viral activity (1). They can control viral replication at early stages of infection by secreting cytokines and chemokines and by killing virally infected cells (1-4). NK cells can also link the innate and adaptive arms of the immune response leading to NK cell activation of multiple functions including cytotoxicity (5, 6)

The Killer Immunoglobulin-like Receptors (KIRs) are a large family of receptors found on subsets of lymphocytes, including NK cells (7). They are encoded by genes within the KIR genetic region that maps to chromosome 19q 13.4, within the leukocyte receptor complex (8). The KIR region is polygenic and the KIR genes within this region are polymorphic (9-13). KIR nomenclature is based on whether they have 2 (2D) or 3 (3D) extracellular immunoglobulin (Ig)-like domains, and whether they have long (L) or short (S) cytoplasmic tails. The KIR-L are generally inhibitory while KIR-S are activating receptors (14). NK cell activation status is determined by the integration of activating and inhibitory signals received from cell surface receptors binding their ligands on neighboring cells (15).

The highly polymorphic *KIR3DL1/S1* locus is unique among KIR region genes in that it encodes both activating (KIR3DS1) and inhibitory (KIR3DL1) receptors. The 84 unique proteins encoded by this locus identified to date can be classified into 4 subgroups based on their cell surface density and sequence similarity: KIR3DL1, inhibitory receptors that are not expressed on the cell surface (KIR3DL1-null), those expressed at a low density (KIR3DL1-

low) those expressed at a high density (KIR3DL1-high) and KIR3DS1 receptors (16-19). The ligand for KIR3DL1-low and KIR3DL1-high receptors are a subset of HLA-B and -A isotypes belonging to the HLA-Bw4 group, which is defined by amino acids present at positions 77-83 of the HLA heavy chain (20-22). A dimorphism present at position 80 of HLA-Bw4 heavy chains dichotomizes these isotypes into those with an isoleucine (*80I) or threonine (*80T) at this position. This dimorphism differentially affects receptor-ligand affinity, inhibitory strength and NK cell education potency (23-26). The remaining HLA-B alleles belonging to the Bw6 group do not interact with KIR3DL1. KIR3DS1 and KIR3DL1-null receptors have not been reported to interact with *HLA*Bw4* antigens (27, 28). However, the presence of 2 epitopes, one derived from HIV Pol and the other from HIV Nef have been shown to enable interactions between KIR3DS1 and one of the *HLA Bw4*80I* antigen, HLA*B57 (29).

Epidemiological studies implicate a role for KIR3DS1 in HIV outcomes. HIV seropositive individuals who are carriers of at least 1 copy of *KIR3DS1* and *HLA-Bw4*80I* have a slower time to AIDS than those expressing the receptor or ligand alone or neither (30). NK cells from carriers of the *KIR3DS1+Bw4*80I* genotype also inhibit viral replication more potently than those from carriers of the receptor or ligand alone (31, 32). HIV infected $CD4^+$ T cells from carriers of this *KIR/HLA* genotype stimulate autologous $KIR3DS1^+$ NK cells, but not those expressing inhibitory KIR3DL1, to degranulate (31). Furthermore, there is an association between carriage of the *KIR3DS1* homozygous (hmz) genotype and protection from HIV infection since a higher frequency of HIV exposed seronegative (HESN) subjects than recently HIV infected individuals are *KIR3DS1* homozygotes and HIV exposed carriers of this genotype seroconvert more slowly than do carriers of other *KIR3DL1/S1* genotypes (33, 34).

KIR3DS1 has also been associated with outcomes of viral infections other than HIV (30, 35-37), cancer (38-40), transplantation (41, 42) and autoimmune diseases (43).

Garcia-Beltran et al. reported that open conformers (OC) of HLA-F are high affinity ligands for KIR3DS1 (28). They found that the interaction of KIR3DS1 with HLA-F was functional and led to activation of cell lines expressing KIR3DS1. Here, we extended these findings by investigating the effects of HLA-F ligation expressed on the 221cell line with KIR3DS1 on the activation of primary KIR3DS1$^+$ NK cells. We employed a comprehensive antibody panel able to detect several activating and inhibitory NK cell receptors (aNKR and iNKR) to gate on KIR3DS1$^+$ NK cells that either co-expressed (inclusive gating) or not (exclusive gating) other aNKR and iNKR. The novel exclusive gating strategy enabled us to study the function of stimulated KIR3DS1$^+$ NK cells without the contribution of signalling through other i/aNKR. Antibodies able to detect CCL4/IFN-γ secretion, and CD107a expression were included in the antibody panel to measure the frequency of functional NK cell in phenotypically defined NK cell populations. By comparing these 2 gating strategies and blocking the interaction of KIR3DS1 and HLA-F we showed that KIR3DS1$^+$ ligation by HLA-F on the 221 cell line, activated primary NK cells expressing this receptor.

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 Recherche du Centre Hospitalier de l'Université de Montréal and the Research Ethics Committee of the McGill University Health Centre. All subjects provided written informed consent for the collection of samples and subsequent analysis.

Study population and KIR3DL1/S1 genotyping

The study included 10 KIR3DS1 hmz, 2 KIR3DL1 hmz and 2 KIR3DL1/S1 heterozygous donors. Genomic DNA was extracted from PBMCs using QIAamp DNA blood kits (Qiagen Inc., Mississauga, ON, Canada) as per manufacturer's directions. *KIR3DL1/S1* generic genotyping was performed by PCR, as described previously (33).

Cells

PBMCs were isolated from leukophoresis samples or from blood draws into vacutainer tubes containing ethylenediaminetetraacetic acid anticoagulant by density gradient centrifugation (Lymphocyte Separation Medium, Wisent Bioproducts, St-Bruno, QC, Canada) and cryopreserved in 10% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO); 90% FBS (Wisent).

The HLA-null cell line 221 was a kind gift from Dr. Galit Alter (Ragon Institute, Harvard University, Cambridge, MA). 221 cells were cultured in RPMI 1640 medium supplemented with 10% FBS; 2mM L-glutamine; 50 IU/ml penicillin; 50 mg/ml streptomycin (R10, all from Wisent).

Preparing acid-pulsed 221 cells

Two million 221 cells were resuspended and incubated in 1 ml R10, pH 2.2 for 90 sec. Acid pulsing was stopped by adding 13 ml of R10, pH 7.4 to the cells (44). Cells were then washed and resuspended in R10 before use as acid pulsed 221 cells.

Detection of HLA-F on untreated and acid pulsed 221 cells

To detect HLA-F on untreated and acid-pulsed 221 cells we used the monoclonal antibody (mAb) 3D11(45-48). One million 221 cells were resuspended in 100 µl of phosphate buffered saline (PBS, Wisent); 5% FBS (FACS buffer). 3D11 mAb or mouse immunoglobulin G1 (IgG1) isotype control (MOPC-21, BioLegend, San Diego, CA) was added to the cell suspension for 40 min at 4ºC. Primary antibody binding was detected by incubating washed cells with 1µg/ml of F(ab')2 goat anti-mouse IgG-eFluor660 (eBioscience, San Diego, CA) secondary antibody for 25 min at 4ºC. A recombinant KIR3DS1-Fc chimeric protein (R & D Systems Minneapolis MN) was also used to detect HLA-F on untreated and acid pulsed 221 cells. One million 221 cells were resuspended in 100 µl of FACS buffer and incubated with KIR3DS1-Fc or with human IgG1 (ET901, BioLegend) as a negative isotype control for 40 min at 4ºC. Binding of the KIR3DS1-Fc chimera protein and control were detected by adding goat anti-human IgG Fc-PE secondary antibody (eBioscience) for 25 min at 4ºC. To verify that acid pulsing of 221 cells

produced HLA cell surface OCs, the mAbs W6/32, 3D12 and 2M2 (Biolegend), were used to stain untreated and acid pulsed 221 cells. While 221 cells are HLA-A, B and C null they do express HLA-E, which is detected by the pan-HLA specific mAb W6/32, the HLA-E specific mAb 3D12 and the β_2M specific mAb 2M2. These mAbs do not recognize OCs of HLA-E on acid pulsed cells. One million 221 cells were resuspended in 100 µl of FACS buffer with W6/32, 3D12, 2M2 or a mouse IgG2a, κ isotype control for W6/32 (MOPC-173, BioLegend) or a mouse IgG1, κ isotype control for 3D12 and 2M2 (MOPC-21, BioLegend) for 30 min at 4ºC. Primary antibody binding was detected using F(ab')2-goat anti-mouse IgG-PE secondary antibody (eBioscience). Cells were then washed twice and fixed in 2% paraformaldehyde (PFA, Santa Cruz Biotechnology, Santa Cruz, CA) until acquisition within 2 hrs.

NK cell activation and staining for phenotype and function

Cryopreserved PBMCs were thawed and rested in R10 for 3 hrs in a humidified 5% CO_2 incubator. Rested PBMCs (E) were co-cultured with either untreated or acid-pulsed 221 cells (T) at an E:T ratio of 5:1 in 200 µl of R10 in U-bottomed 96-well plates for 6 hrs at 37°C in a humidified 5% CO_2 incubator. PBMCs cultured alone in R10 served as an unstimulated negative control and PBMCs stimulated with 5.8 µg/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich); 1.2 µg/ml ionomycin (Sigma-Aldrich) (P/I) in R10 served as a positive control to ensure that the NK cells being stimulated were viable and functional. Brefeldin A (24 µg/ml; Sigma-Aldrich) and monensin (3 µg/ml, Golgi Stop; BD Biosciences, Mississauga, ON, Canada) were added 30 min after the initiation of the co-culture. All cells responded to P/I stimulation.

After stimulation, cells were stained using a fluorochrome conjugated antibody panel that was validated with single-stained control beads (CompBead, BD Biosciences). Cells were stained for viability using the UV Live/Dead staining kit (Invitrogen, Carlsbad, CA), as per manufacturer's instructions. Cells were surface stained with anti-CD3-BV785 (OKT3), anti-CD56-BV711 (HDC56), anti-KIR2DL1/S1/S3/S5-FITC (HP-MA4) and anti-CD107a-BV421 (H4A3) (all from BioLegend), anti-KIR3DL1/S1-PE (REA168), anti-NKG2A-Biotin (REA110) (both from Miltenyi Biotec, Inc. Auburn, CA), anti-KIR2DL2/L3/S2-FITC (CH-L, BD Biosciences), anti-KIR3DL2-APC (539304) and anti-ILT-2-APC (292305) (both from R & D Systems) for 30 min at 4^{o}C. After washing, binding of anti-NKG2A was detected by incubating cells with Qdot^{TM} 655 Streptavidin Conjugate (Life Technologies, Burlington, ON, Canada) for 20 min at 4^{o}C. Cells were then washed and fixed with 2% PFA, permeabilized with Permeabilization Medium B (Invitrogen) and stained intracellularly with anti-CCL4-AF700 (D21-1351) and anti-IFN-γ-BV510 (B27) (both from BD Biosciences). Samples were then washed twice and fixed with 2% PFA until acquisition.

To investigate whether HLA-F on 221 cells can interact with ILT-2 on NK cells to mediate inhibitory signals affecting NK cell function, the anti-ILT-2-APC antibody in the cell surface antibody panel described in the previous paragraph was changed to one conjugated with PE/Cy7 (clone GH1/75, BioLegend) so that ILT-2$^{+/-}$ NK cells could be exclusively gated on and assessed for the frequency of functional cells. To verify that 221 stimulation activated NK cells, we measured the frequency of CD69^{+} NK cells in unstimulated and stimulated exclusively gated KIR3DS1^{+} NK cells by adding anti-CD69-BV605 (clone FN50, BioLegend) to the antibody cocktail used for cell surface staining described in the previous paragraph.

For some experiments PBMC were stimulated with Epstein-Barr Virus (EBV) transformed B lymphoblastoid cell lines instead of 221 cells using the same conditions and antibody panels as described above. The EBV lines were HLA-F^+ as determined by staining with 3D11 as described above for staining 221 cells.

For some experiments, the interaction between KIR3DS1 on NK cells and HLA-F on 221 cells was blocked by incubating 221 cells in R10 with 25 µg/ml of KIR3DS1-Fc chimera protein (R & D Systems), 25 µg/ml of anti-HLA-F specific mAbs 3D11, 4A11 or 6A4 for 50 min at 37ºC in a humidified 5% CO_2 incubator. For these experiments, PBMC were also incubated with anti-human CD16 (FcγRIII) F(ab')2 (3G8, Ancell, Bayport, MN) at 10 µg/ml final concentration for 45 min prior to co-incubation with 221 cells to minimize the effect of signalling through this Fc receptor (FcR) on NK cell activation. FcR blocked PBMCs were co-cultured with 221 cells for 6 hrs at 37ºC in a humidified 5% CO_2 incubator. The control conditions for these experiments were unstimulated PBMCs, PBMCs stimulated with 221 cells that were not pre-incubated with KIR3DS1-Fc chimera protein or anti-HLA-F specific mAbs and 221 cells pre-incubated with human IgG1 isotype control (ET901, Biolegend) at a concentration of 25 µg/ml as a control for KIR3DS1-Fc or 25 µg/ml or MOPC-21 as a control for anti-HLA-F specific mAbs 3D11, 4A11 and 6A4. Following co-culture, cells were stained for cell surface and intracellular markers as described above. The percent reduction in 221 KIR3DS1$^+$ or KIR3DL1$^+$ NK cell stimulation in the presence of blocking reagents was calculated using the following equation: ((frequency of functional cells in the 221 stimulated NK cell population in the presence of a blocking reagent – frequency of functional cells in the unstimulated NK cell population) / (frequency of functional

cells in the 221 stimulated NK cell population - frequency of functional cells in the unstimulated NK cell population) *100).

Flow cytometry analysis

Between 7 x 10^5 and 1.5 x 10^6 total events were acquired for each sample using an LSR Fortessa X-20 flow cytometer (BD Biosciences). Results obtained from flow cytometry data were analyzed by FlowJo software (V10.2, TreeStar, Ashland, OR). We measured the frequency of KIR3DS1$^{+/-}$CD3$^-$CD56^{dim} NK cells exhibiting the 7 possible combinations of CCL4, IFN-γ and CD107a function (i.e. tri-functional, 3 combinations of bi-functional and 3 combinations of mono-functional response patterns). We also assessed the sum of the frequencies all functions tested (total), the sum of the frequencies of functional subsets secreting CCL4 (total CCL4), IFN-γ (total IFN-γ) and expressing CD107a (total CD107a). These functional subsets were assessed for KIR3DS1$^+$ and KIR3DS1$^-$ NK cell populations using gating strategies that either included or excluded NK cell populations co-expressing the NKRs KIR2DL1/L2/L3, KIR2DS1/S2/S3/S5, KIR3DL2, ILT-2 and NKG2A. The data presented are background subtracted using results for matched unstimulated control conditions.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used to perform data analysis and graphical presentation. Wilcoxon matched-pairs signed rank and Friedman tests with Dunn's post tests were used to assess the significance of comparisons of 2 and more than 2 groups, respectively for within individual matched data sets. P-values <0.05 were considered significant.

RESULTS

Expression level of HLA-F on untreated and acid pulsed 221 cells

Acid pulsing favors OCs of cell surface classical and non-classical MHC class I (MHC-I) antigens, including HLA-F. HLA-F OCs are the preferred conformation for interactions with KIR3DS1 (28, 49). Fig. 1A and B show that 3D11 and KIR3DS1-Fc bound to untreated (top panel) and acid pulsed (second panel) 221 cells with a similar MFI (mean Fluorescent intensity). Isotype controls for these HLA-F-specific reagents bound untreated (third panel) and acid pulsed (bottom panel) 221 cells with lower MFIs than did 3D11 and KIR3DS1-Fc and with MFIs that did not substantially differ from each other. To ensure that the acid pulsing regimen produced MHC-I OCs, we stained untreated and acid-pulsed 221 cells with the mAbs W6/32, 3D12 and 2M2. W6/32 recognizes correctly folded classical MHC-1a and non-classical MHC-1b HLA-E and F antigens, but not their OC counterparts (28). 3D12 is specific for correctly folded HLA-E, which is expressed at a low intensity of 221 cells. 2M2 is specific for β_2M, which is present on correctly folded HLA-E but not OCs on 221 cells. W6/32 bound untreated 221 cells (top panel) with a higher MFI that that of acid pulsed 221 cells (second panel), which was in the range of the MFI with which the isotope control for this mAb bound untreated (third panel) and acid pulsed (bottom panel) 221 cells (Fig. 1C). 3D12 bound untreated 221 cells (top panel) with a marginally higher MFI than that of acid pulsed 221 cells (second panel). The MFI with which the isotope control for this mAb bound untreated (third panel) and acid pulsed (bottom panel) 221 cells was similar (Fig. 1D). 2M2 bound untreated 221 cells (top panel) with a higher MFI than that of acid

pulsed 221 cells (second panel). The MFI with which the isotope control for this mAb bound untreated (third panel) and acid pulsed (bottom panel) 221 cells was similar (Fig. 1E).

Untreated and acid pulsed 221 cells stimulate KIR3DS1^{+} NK cells to exhibit similar functional profiles.

We next compared the flow cytometry plots generated for total CCL4 and total IFN-γ secretion and total CD107a expression for KIR3DS1^{+}CD56^{dim} NK cells stimulated with untreated or acid pulsed 221 cells and for KIR3DS1^{+}CD56^{dim} NK cells left unstimulated. The strategy used to gate on functional KIR3DS1^{+}CD56^{dim} NK cells is shown in Figs. 2A, C. The histograms for these 3 functional profiles overlapped in KIR3DS1^{+}CD56^{dim} NK cells from a representative individual stimulated with untreated 221 cells (top panel) and acid pulsed 221 cells (middle panel) and had a higher MFI than that seen in unstimulated cells (bottom panel) (Fig. 3A-C). The frequency of KIR3DS1^{+}CD56^{dim} NK cells from 10 KIR3DS1 hmz responding to untreated and acid pulsed 221 cells was similar for the functional profiles characterized by the sum of all function, total CCL4 secretion, total IFN-γ secretion and total CD107a expression (Fig. 3D-G, respectively).

Collectively, these results show that acid pulsing 221 cells did not improve HLA-F recognition by either an HLA-F-specific mAb or the KIR3DS1-Fc chimera protein and did not enhance the ability of 221 cells to stimulate KIR3DS1^{+}CD56^{dim} NK cells. Given these results, we used untreated 221 cells in subsequent experiments.

A higher frequency of KIR3DS1^{+} than KIR3DS1^{-} NK cells responded to stimulation with 221 cells

PBMCs were stimulated with 221 cells and then stained with the antibody panel described in the methods section that included fluorochrome conjugated antibodies able to recognize KIR2DL1/L2/L3/S1/S2/S3/S5, KIR3DL2, ILT-2 and NKG2A on KIR3DS1$^{+/-}$CD56^{dim} NK cells. As seen in Fig. 2A, lymphocyte singlet CD3^{-}CD56^{dim} NK cells that inclusively expressed, or not, KIR3DS1 were gated on. Boolean gating was used to assess the frequency of KIR3DS1^{+} and KIR3DS1^{-} cells that expressed the various combinations of the three functions tested. The frequency of cells exhibiting the sum of all functions tested, total CCL4 and total IFN-γ secretion and total CD107a expression was higher among KIR3DS1^{+} than KIR3DS1^{-} NK cells ($p<0.0001$, for all, Wilcoxon matched-pairs signed rank test) (Fig. 4A-D).

For 10 subjects, we assessed the frequency of tri-, 3 combinations of bi- and 3 combinations of mono-functional KIR3DS1$^{+/-}$ NK cells. A higher frequency of inclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells secreted CCL4 only and both CCL4 and IFN-γ ($p=0.027$ and $p=0.002$, respectively, Wilcoxon) (Fig. S1A/B) while a lower frequency of KIR3DS1^{+} than KIR3DS1^{-} NK cells expressed CD107a only ($p=0.039$) (Fig. S1C).

Inclusively gated KIR3DS1^{+} and KIR3DS1^{-} NK cells can express various stochastic combinations of other iKIR, aKIR and NKG2A depending on the immunogenetic profile of the study subject. NK cells bearing iKIR to self HLA and NKG2A would be educated through the interaction of these receptors with their HLA ligands. This would make them responsive to stimulation by 221 cells. The level of stimulation resulting from missing self-recognition of

HLA-null cells by educated NK cell subsets can differ from one person to another and may confound differences in the stimulation levels of inclusively gated KIR3DS1^{+} and KIR3DS1^{-} NK cells. This prompted us to minimize this possibility by using an exclusive gating strategy to gate out NK cell populations bearing KIR2DL1/L2/L3/S1/S2/S3/S5, KIR3DL2, ILT-2 and NKG2A from KIR3DS1$^{+/-}$CD56^{dim} NK cells. The gating strategy used for exclusive KIR3DS1^{+} and KIR3DS1^{-} NK cell gating is shown in Fig. 2B.

Fig. S2 shows that 221 stimulation increased the frequency of CD69^{+} exclusively gated KIR3DS1^{+} and KIR3DL1^{+} NK cells ($p=0.04$ and $p=0.057$, respectively, Wilcoxon). Fig. 5 shows for the 10 KIR3DS1 hmz that even after excluding NK cells expressing the NKR recognized by the antibody panel other than KIR3DS1, a higher frequency of KIR3DS1^{+} than KIR3DS1^{-} NK cells responded to 221 stimulation. This was the case when all functions tested were considered ($p=0.005$, Wilcoxon) as well as when all cells positive for CCL4, IFN-γ and CD107a were considered ($p<0.04$ for all, Wilcoxon) (Fig. 5B-D). A higher frequency of exclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells also secreted CCL4 only and both CCL4 and IFN-γ ($p=0.027$ and $p=0.009$, respectively, Wilcoxon) (Fig. S1 D, E), while the frequency of KIR3DS1^{+} and KIR3DS1^{-} NK cells expressing CD107a only did not differ significantly ($p<0.05$) (Fig. S1 F).

The effect of HLA-F-ILT-2 interactions on the function of ILT-2^{+} NK cells.

Dulberger et al. recently showed that HLA-F-β_2M complexes loaded with peptide can interact with the iNKR ILT-2 (50). We reasoned that if HLA-F-β_2M complexes on 221 cells were interacting with ILT-2 on NK cells that a lower percentage of exclusively gated ILT-2^{+} than ILT-

2^- NK cells would be functional. Fig. S3 shows that there were no significant differences in the frequency of CCL4 and IFN-γ secreting cells in the exclusively gated ILT-2^+ versus ILT-2^- populations ($p>0.05$, Wilcoxon test, Fig. S3 B, C). The frequency of ILT-2^+ with total functionality was significantly lower than that of ILT-2^- cells ($p=0.004$, Fig. S3 A), while the frequency of CD107a expressing cells was marginally though significantly higher in the ILT-2^+ than ILT-2^- populations ($p=0.04$, Fig. S3 D).

EBV lines express HLA-F and stimulate KIR3DS1$^+$ NK cells

EBV transformed B cells also express HLA-F (51). We confirmed this expression by staining with 3D11 and used an HLA-F^+ EBV transformed cell line to stimulate PBMC using the same conditions as described above. A higher frequency of exclusively gated KIR3DS1$^+$ cells exhibited the sum of all functions tested, total CCL4 and IFN-γ secretion and total CD107a expression following stimulation. Blocking with KIR3DS1-Fc chimera protein reduced the frequency CCL4 and IFN-γ secreting cells to the level of unstimulated PBMC, while total functionality and CD107a expression were reduced by 27.5% and 26.6%, respectively, of that of 221 stimulated KIR3DS1$^+$ NK cells (not shown).

KIR3DS1$^+$ NK cell responses to 221 are due to the interaction of KIR3DS1 with HLA-F

To determine whether the responsiveness of KIR3DS1$^+$ NK cells was due to the interaction of this receptor with HLA-F on 221 cells, we blocked this interaction by pre-incubating 221 cells with KIR3DS1-Fc chimera protein before using them to stimulate PBMCs. As shown in Fig. 6, this treatment diminished the frequency of exclusively gated KIR3DS1$^+$ NK cells responding to

221 cells compared to that observed for responses to 221 cells that were not pre-treated with KIR3DS1-Fc or were pre-treated with control human IgG1. The frequency of functional KIR3DS1$^+$ NK cells under KIR3DS1-Fc blocking conditions was significantly lower than that for either the no blocking or blocking with human IgG1 control conditions for total responsiveness, total CCL4 and total IFN-γ secretion ($p<0.05$ for all, Friedman tests with Dunn's post-tests) (Fig. 6A-C). The frequency of functional KIR3DS1$^+$ NK cells under KIR3DS1-Fc blocking conditions was also lower than that for the blocking with human IgG1 control conditions for total CD107a expression ($p<0.05$, Dunn's) (Fig. 6D). By contrast, differences in the frequency functional cells stimulated with KIR3DS1-Fc blocked 221 cells was as low as that of unstimulated cells for each of these functional subsets (Fig. 6A-D).

Three HLA-F specific mAbs were also used to block the interaction of HLA-F with KIR3DS1. Blocking with 3D11 was tested on 5 *KIR3DS1* hmz subjects, while blocking with 4A11 and 6A4 was tested on 3 subjects each. Each of these mAbs reduced the frequency of functional cells compared to that seen for 221 stimulated cells in the presence or absence of mouse IgG1.The differences between stimulated cell with or without blocking with 3D11 all trended towards but did not achieve significance (p=values between 0.0625 and 0.12). Pre-coating 221 cells with these anti-HLA-F mAbs did not reduce the frequency of functional cells to the levels seen for unstimulated PBMC (Fig. 6 E-H).

Blocking experiments with KIR3DS1-Fc were also performed on exclusively gated KIR3DS1$^+$ NK cells from 2 *KIR3DL1/S1* heterozygotes (Fig. S4) Blocking the KIR3DS1-HLA-F interaction

reduced the frequency of functional cells to 37.8%, 0%, 16.4% and 74% of that of stimulated PBMC for total, CCL4, IFN-γ and CD107a functions (Fig. S4 A-D). Garcia-Beltram et al. reported that HLA-F is a ligand for KIR3DL1 (28). Burian et al. did not confirm this finding (49). KIR3DL1-Fc was used to block interactions between KIR3DL1 and HLA-F using PBMC responder cells from 2 *KIR3DL1* hmz and 2 *KIR3DL1/S1* heterozygotes. The frequency of functional KIR3DL1$^+$ NK cells averaged 74.3%, 56.3%, 46.3% and 99.1% of that of 221 stimulated PBMC for total function, CCL4, IFN-γ and CD107a, respectively (Fig. S4 E-H). None of these differences achieved statistical significance, likely due to the small number of subjects tested.

Together, these results show that blocking the interaction of KIR3DS1 on NK cells bearing this receptor with HLA-F on 221 cells reduced the functionality of these cells to a level that was not significantly different from that of unstimulated KIR3DS1$^+$ NK cells and that was significantly lower than that seen in conditions where this interaction was not blocked. Furthermore, the exclusive gating strategy used to detect KIR3DS1$^+$ NK cells minimized the possibility that the activation of these cells was due to missing self-recognition of HLA-null cells lacking ligands for iNKRs. Thus, the interaction of KIR3DS1 with HLA-F activated primary KIR3DS1$^+$ NK cell.

DISCUSSION

KIR3DS1 is an activating NKR, whose presence is associated with beneficial HIV outcomes such as slow time to AIDS and protection from HIV infection (30, 33, 52). The ligand for KIR3DS1 was recently shown to be the non-classical MHC-Ib molecule, HLA-F (28, 49). Here, we confirmed that HLA-F is present on the surface of the HLA-null cell line, 221. Co-culture of 221 cells with PBMC activates a higher frequency of $CD3^{-}CD56^{dim}$ $KIR3DS1^{+}$ than $KIR3DS1^{-}$ NK cells to express CD69 and to secrete CCL4 and IFN-γ and to express CD107a. We showed that the activation of KIR3DS1 expressing cells by 221 cells occurred in the absence of interrupted signaling through iKIR due to missing-self recognition or by activation through several co-expressed aNKR by using a gating strategy that excluded $KIR3DS1^{+/-}$ NK cells co-expressing a panel of iNKR and aNKR. We showed that the ligation of KIR3DS1 by HLA-F on 221 cells was responsible for the higher frequency of stimulated exclusively gated $CD56^{dim}$ $KIR3DS1^{+}$ than $KIR3DS1^{-}$ NK cells by demonstrating that blocking the interaction of KIR3DS1 with HLA-F reduced the frequency of 221 responsive $KIR3DS1^{+}$ NK cells.

The nature of the ligand for KIR3DS1 has been elusive until recently. Epidemiological studies found that carriers of *KIR3DS1* and *HLA-Bw4*80I* combinations progress to AIDS more slowly than carriers of the receptor of ligand alone (30). However, in the absence of *HLA-Bw4-80I* alleles, *KIR3DS1* homozygosity is associated with more rapid progression to AIDS (30). Studies investigating NK cell mediated inhibition of HIV replication found that effector cells from carriers of this *KIR/HLA* genotype combination suppressed HIV replication in autologous infected $CD4^{+}$ T cells more effectively than carriers of the receptor of ligand alone (31, 32).

Despite this, attempts to demonstrate a direct interaction between KIR3DS1 and HLA-Bw4*80I antigens have failed (27, 28). An exception to this was the observation that KIR3DS1 could bind the HLA-Bw4*80I antigen, HLA*B57, if certain HIV derived peptides were present to facilitate this interaction (53). Garcia-Beltran *et al.* used fusion proteins of the extracellular domain of several KIR gene products with the Fc portion of IgG1 to screen a panel of 100 recombinant MHC-I proteins. KIR3DS1-Fc bound no HLA-A, B or C antigens. Rather, the preferred ligand for KIR3DS1 was HLA-F OCs (28). Several other KIR3D receptors shared with KIR3DS1 the ability to bind HLA-F OCs (28, 54). Surface Plasmon Resonance pull down experiments, heterodimerization experiments and the use of HLA-F tetramers were used to show that KIR3DS1 binds to OCs of HLA-F (28, 49).

HLA-F differs from classical MHC-Ia proteins in several ways. MHC-Ia proteins are highly polymorphic, form complexes with β_2M and short peptides and are expressed at the cell surface of most human cells (55). HLA-F is monomorphic, binds weakly with β_2M and peptide, is retained intracellularly in resting cells and can be expressed on the cell surface of activated cells as an OC without peptide or β_2M through it cell surface expression may increase as MHC-1a is up-regulated (44, 56-59). HLA-F is present on the surface of B lymphoblastoid cell lines such as 221 and on HIV infected cells (28). Goodridge *et al.* reported that HLA-F can be expressed independently of the ER-peptide binding pathways, due to its altered cytoplasmic domain (44). Based on differential antibody staining for HLA-F, they concluded that at least 3 different forms of HLA-F may be expressed over the course of lymphocyte activation. They confirmed that HLA-F is expressed on activated lymphocytes independently of TAP and Tapasin. Thus, HLA-F can be spontaneously expressed as OCs (59).

Mild acid treatment of cells dissociates β_2M and peptide from HLA heavy chains producing OCs (51). We compared the ability of the HLA-F-specific mAb 3D11 and a KIR3DS1-Fc chimera protein to recognize HLA-F on untreated and acid pulsed 221 cells and found that both reagents bound HLA-F on untreated and acid pulsed 221 cells with a similar MFI. We also showed that untreated and acid pulsed 221 cells stimulated a similar frequency of KIR3DS1$^+$ NK cells to produce CCL4, IFN-γ and express CD107a with similar intensities. We verified that the procedure used for acid pulsing produced OCs by showing that the mAbs W6/32 and 3D12 recognized HLA-E on untreated but not on acid pulsed 221 cells. W6/32 and 3D12 only bind correctly folded HLA-E. Furthermore, the β_2M specific 2M2 antibody served as a control for acid pulsing stripping β_2M from HLA-E on 221 cells since it only recognized unpulsed cells. These findings contrast with what has been shown by Garcia-Beltran *et al.* They showed that Jurkat cells expressing KIR3DS1 were triggered more potently by acid pulsed than untreated 221 cells. Despite this, the same group showed that cell-sized coated beads loaded with HLA-F activated KIR3DS1 expressing Jurkat cells to a similar extent whether acid pulsed or not (28). Discrepant results may be due to disparate sensitivities of the cell types responding to stimulatory cells expressing HLA-F (primary NK cells versus KIR3DS1 transduced Jurkat cells) or to the nature of the stimulatory signal (untreated and acid pulsed 221 cells versus HLA-F coated beads). A least in our hands, the HLA-F present on the surface of 221 cells is in a conformation able to interact with KIR3DS1 present on primary NK cells isolated from *KIR3DS1* hmz and acid pulsing improves neither HLA-F's stimulatory capability nor its recognition by HLA-F specific reagents.

The interaction of iNKR with their HLA ligands is necessary for NK cell education, which in turn confers NK cell with the functional potential to become activated if they encounter transformed, stressed or virus infected self-cells with down-modulated ligands for these iNKR (60, 61). Some activating KIRs may also participate in NK cell education but these interactions should tune down NK cells responsiveness to altered self-cells (62, 63). HLA-null cells such as 221 are well known to stimulate educated NK cells because they lack the ligands for several iNKR, which transmit inhibitory signals (64-68). Reducing the effect of missing-self recognition on NK cell activation was the impetus for excluding KIR3DS1^{+} and KIR3DS1^{-} NK cells co-expressing a panel of iNKR and aNKR. Antibodies to KIR2DL5 were not included in this panel because we found that co-expression of KIR2DL5 with KIR3DS1 did not modulate the responsiveness of NK cells expressing KIR3DS1 without KIR2DL5 to 221 stimulation (69). Antibodies to KIR2DS4 were not included in the panel because none of the 10 KIR3DS1 hmz study subjects carried *KIR2DS4,* as the gene encoding this receptor is in negative linkage disequilibrium with KIR2DS1 frequently found on telomeric KIR region group B haplotypes (70, 71). The observation that exclusively gated KIR3DS1^{+} NK cells responded better to 221 stimulation than KIR3DS1^{-} NK cells narrowed the possibilities for the identity of the NKR responsible for 221 dependent KIR3DS1^{+} activation. The loss of exclusively gated KIR3DS1^{+} NK cell activation when the interaction between KIR3DS1 and HLA-F was blocked confirmed that the interaction between this receptor/ligand pair is functional and responsible for the activation of primary KIR3DS1^{+} NK cells. On the other hand, mAbs to HLA-F were less effective at blocking the activation of KIR3DS1^{+} NK cells. The reason for this is unclear as the concentrations of Abs used were saturating in binding assays. It is possible that these Abs have a higher off rate than the KIR3DS1-Fc chimera protein, exposing HLA-F to KIR3DS1.

Alternately, this result may suggest that KIR3DS1 recognizes other ligands on 221 cells whose interaction with KIR3DS1 is not blocked by these Abs. Combining more than 1 Abs for blocking did not further reduce KIR3DS1$^+$ NK cell activation. We cannot formally exclude the possibility that receptors other than KIR3DS1 are involved in the activation of KIR3DS1$^+$ NK cells. However, the reduction in the frequency of activated KIR3DS1$^+$ NK cells to levels at or below that seen for unstimulated PBMC when the interaction between KIR3DS1 and HLA-F is blocked by KIR3DS1-Fc chimera protein suggests that the contribution of other NK receptors to activation is unlikely or minimal.

221 cells that were not acid pulsed did induce a significantly lower frequency of exclusively gated ILT-2$^+$ than ILT-2$^-$ NK cells exhibiting the sum of all functions. However, differences in the frequency of functional cells was small, i.e. 34 ± 12.4% versus 36.4 ± 12.3% for ILT-2$^+$ vs ILT-2$^-$ cells, respectively. Between-group differences were not significant for the frequency of total CCL4 and total IFN-γ secretion. A higher frequency of ILT-2$^+$ than ILT-2$^-$ cells expressed CD107a indicating that the ILT-2 receptor was not mediating inhibitory signals measured by CD107a expression. Thus, there is some evidence for inhibition of the function of ILT-2$^+$ cells by HLA-F on 221. The low level of inhibition and the absence in differences in 3D11 and KIR3DS1-Fc staining in acid pulsed versus unpulsed cells would support the conclusion that much of the HLA-F on 221 cells are OCs and not in complexes with β2M and peptide, which if the form that can interact with HLA-F (50).

Activated KIR3DS1$^+$ NK cells secrete CCL4, IFN-γ and express CD107a. It is notable that CCL4, is a chemokine able to bind the CCR5 co-receptor for HIV entry, blocking HIV infection

of new target cells (72). IFN-γ is a critical cytokine for innate and adaptive immune responses against viral infections (73) and CD107a is a marker of NK cell degranulation, a surrogate marker for target cell lysis (74). Production of these molecules by stimulated KIR3DS1^{+} NK cells may be a mechanism underlying the association of KIR3DS1 homozygosity with a reduced risk of HIV infection (33, 34, 52). Others have shown that HIV infected cells express HLA-F and that KIR3DS1^{+} NK cells suppress HIV replication in autologous CD4^{+} T cells (28, 31, 32). The frequency of KIR3DS1^{+} cells in *KIR3DS1* hmz was reported to be nearly twice as high as that in *KIR3DL1/S1* heterozygotes with a median (range) of 61% (range 41, 81) in a population of 5 *KIR3DS1* hmz (75). For the 10 *KIR3DS1* hmz subjects studied here the mean ± standard deviation of KIR3DS1^{+}CD56^{dim} NK cell1as 46.0 ± 20.2% (range 7.5 to 75.9%). It is interesting to speculate that the high frequency of KIR3DS1^{+} cells in *KIR3DS1* hmz donors may be a factor in the association of this genotype with protection from infection. Mature NK cells express pre-stored IFN-γ transcripts, granzyme and perforin and are ready to lyse targets within minutes of activation, which would be a desirable characteristic of cells mediating early responses to HIV that may prevent the establishment of infection (73). Secretion of the chemokines CCL3, CCL4 and CCL5 by activated NK cells also mediate anti-viral effects by blocking HIV entry (72). KIR3DS1 is also maintained on the surface of NK cells in HIV infected individuals (75). The high frequency of NK cells bearing activating KIR3DS1 receptors in individuals carrying the gene encoding this receptor is consistent with the epidemiological data suggesting a critical role for this receptor in controlling HIV-1 pathogenesis and contributing to the prevention of HIV infection (28, 30-34, 52).

FIGURES AND LEGENDS

Figure 1

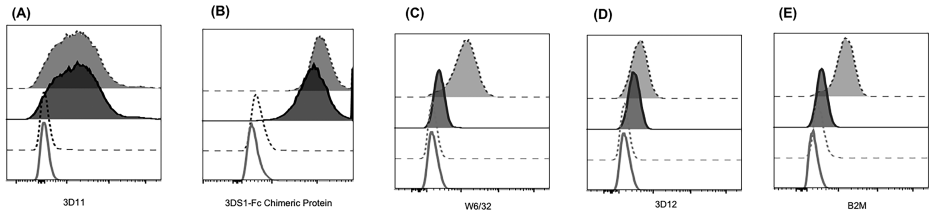


Figure 1. Untreated and acid pulsed 721.221 (221) cells express cell surface open conformers (OCs) of HLA-F. The histograms in panels A and B show the fluorescence pattern generated by staining untreated (top and third histograms) or acid pulsed 221 cells (second and bottom histograms) with mAb 3D11 (A) or KIR3DS1-Fc chimera protein (B) (top and second) or with an isotype control (third and bottom). Panels C-E show the fluorescence pattern generated by staining untreated (top and third histograms) or acid pulsed (second and bottom histograms) 221 cells with mAbs W6/32 (C), 3D12 (D) or 2M2 (E) (top and second), or with isotype control (third and bottom). Binding of 3D11, KIR3DS1-Fc, W6/32, 3D12, 2M2 and isotype controls was detected using fluorochrome conjugated secondary antibodies.

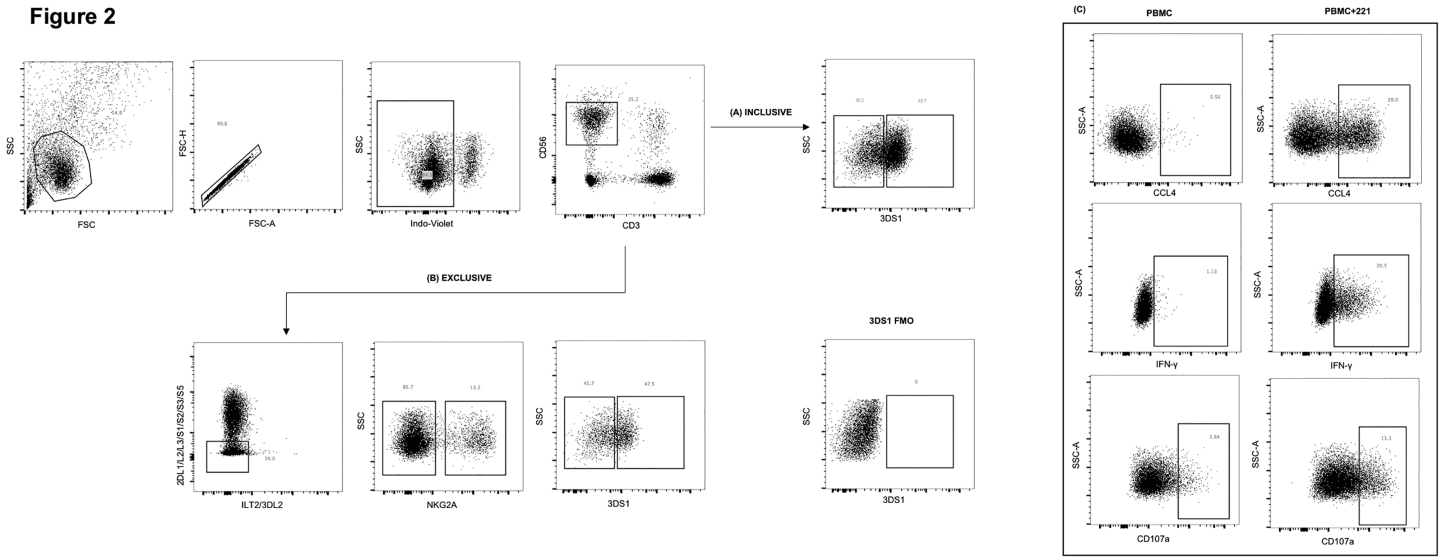


Figure 2. Gating strategy for detecting inclusively and exclusively gated functional KIR3DS1^{+}CD56^{dim} NK cells. Live, singlet lymphocytes were gated on from which CD3^{-} CD56^{dim} cells were selected. (A) For inclusive gating, KIR3DS1^{+} (3DS1^{+}) and KIR3DS1^{-} (3DS1^{-}) NK cells were selected for examination of their unstimulated and 221 stimulated functional profiles. (B) For exclusive gating, CD3^{-}CD56^{dim} NK cells negative for KIR2DL1/L2/L3/S1/S2/S3/S5, ILT-2 and KIR3DL2 were selected. From these cells, those negative for NKG2A were gated on to identify KIR3DS1^{+} and KIR3DS1^{-} cells. (C) Inclusively and exclusively gated KIR3DS1^{+} and KIR3DS1^{-} NK cells were then examined for all possible combinations of CCL4, IFN-γ and CD107a expression by Boolean gating. Functional gates were set on gated total CD3^{-}CD56$^{\mathbf{dim}}$ cells (NK cells) in unstimulated PBMCs. Unstimulated conditions were used to background subtract 221 stimulated conditions.

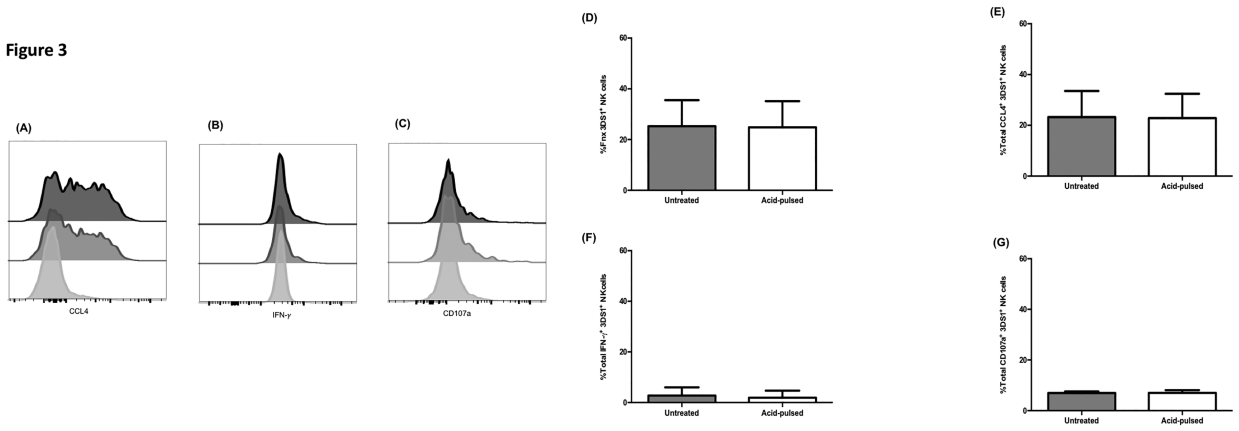
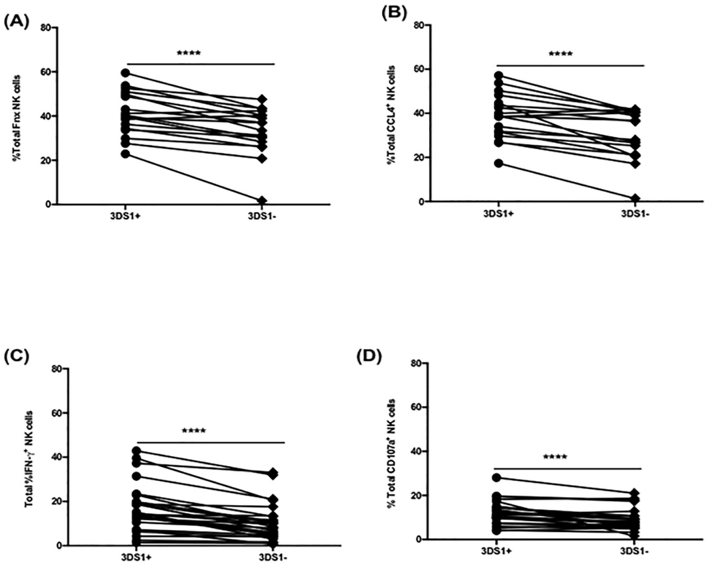


Figure 3. Untreated and acid pulsed 221 cells stimulate KIR3DS1^{+} NK cells that exhibit similar functional profiles. The histograms in panels A-C show the fluorescence profile of CCL4 secretion (A), IFN-γ secretion (B) and CD107a expression (C) following stimulation of KIR3DS1^{+}CD56^{dim} NK cells with untreated (top) or acid pulsed (middle) 221 cells. The fluorescence profile of unstimulated KIR3DS1^{+}CD56^{dim} NK cells is shown in the bottom panels. These results are a representative example of experiments performed on 10 KIR3DS1 homozygotes. In panels D-G the y-axes show the frequency of KIR3DS1^{+}CD56^{dim} NK cells (3DS1^{+}) positive for the sum of the three functions tested (% Fxn 3DS1^{+} NK cells) (D), total CCL4 secretion (% Total CCL4^{+} 3DS1^{+} NK cells) (E), total IFN-γ secretion (% Total IFN-γ^{+} 3DS1^{+} NK cells) (F) and total CD107a expression (% Total CD107a 3DS1^{+} NK cells (G). The frequency of exclusively gated KIR3DS1^{+}CD56^{dim} NK cells stimulated by untreated and acid pulsed 221 cells are shown in the right and left-hand bars, respectively, of each panel. Bar height and error bars represent the median and interquartile range of the background corrected values generated from NK cells from 10 KIR3DS1 homozygotes.

Figure 4



Figures 4. A higher frequency of inclusively gated KIR3DS1$^+$ than KIR3DS1$^-$ NK cells responded to stimulation with the HLA-null cell line 221. Shown on the y-axis is the frequency of inclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ NK cells exhibiting (A) the sum of all functions tested (% Total Fxn NK cells), (B) the sum of cells secreting CCL4 (% Total CCL4$^+$ NK cells), (C) the sum of cells secreting IFN-γ (% Total IFN-γ$^+$ NK cells) and (D) the sum of cells expressing CD107a (% Total CD107a$^+$ NK cells). Each data point represents the results of experiments performed using cells from one individual. Panels A and B include results for 10 KIR3DS1 homozygotes for whom 8 were tested twice for a total of 18 observations. Panels C and D include results for 10 KIR3DS1 homozygotes for whom 8 were tested on three occasions for a total of 26 observations. The significance of between-group results was assessed using Wilcoxon matched pairs tests. Significant between-group differences are shown by **** - $p \leq 0.0001$.

Figure 5

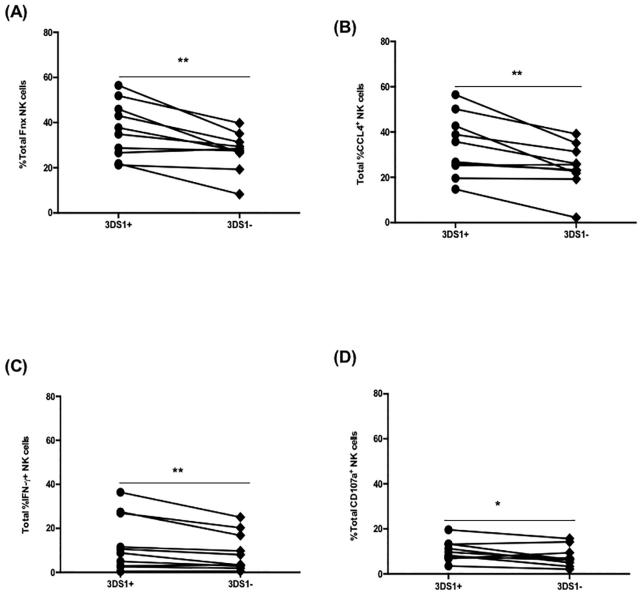


Figure 5. A higher frequency of exclusively gated KIR3DS1$^+$ than KIR3DS1$^-$ NK cells responded to stimulation with the HLA-null cell line 221. Shown on the y-axis is the frequency of exclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ NK cells exhibiting (A) the sum of all functions tested (% Total Fxn NK cells), (B) the sum of cells secreting CCL4 (% Total CCL4$^+$ NK cells), (C) the sum of cells secreting IFN-γ (% Total IFN-γ$^+$ NK cells) and (D) the sum of cells expressing CD107a (% Total CD107a$^+$ NK cells). Each data point represents the results of experiments performed using cells from 1 of 10 KIR3DS1 homozygotes. The significance of between-group results was assessed using Wilcoxon matched pairs tests. Significant between-group differences are shown by * - $p \leq 0.05$, and ** -$p \leq 0.01$.

Figure 6

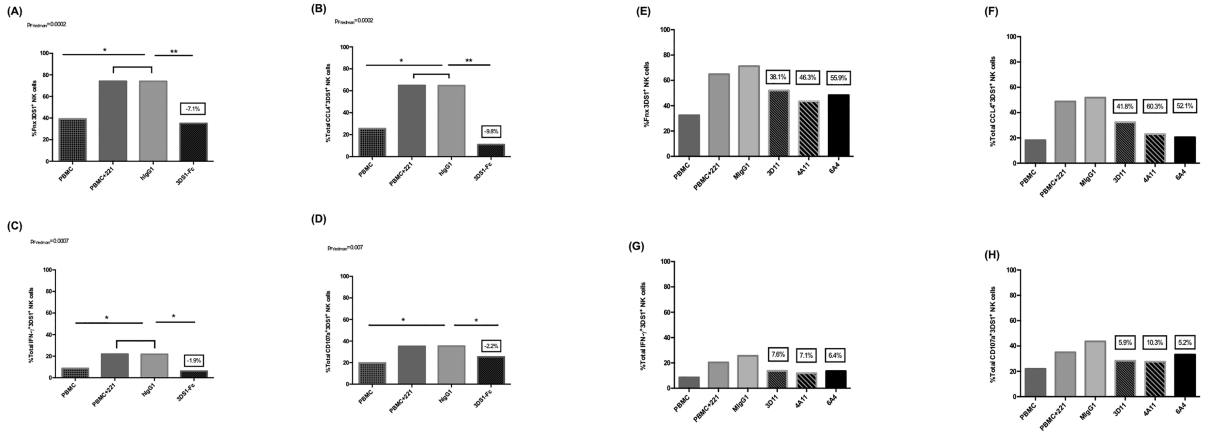


Figure 6. Blocking KIR3DS1 on 221 cells suppresses the activation of KIR3DS1$^+$ NK cells stimulated by 221 cells. Shown on the y-axis is the frequency of exclusively gated KIR3DS1$^+$CD3$^-$CD56^{dim} NK cells exhibiting (A, E) the sum of all functions tested (% Total Fxn NK cells), (B, F) the sum of cells secreting CCL4 (% Total CCL4$^+$ NK cells), (C, G) the sum of cells secreting IFN-γ (% Total IFN-γ$^+$ NK cells) and (D, H) the sum of cells expressing CD107a (% Total CD107a$^+$ NK cells) following no stimulation (PBMC), stimulation with 221 cells (PBMC + 221), stimulation with 221 cells pre-coated with human IgG1 (hIgG1) antibody (A-D) or mouse IgG1 (E-H) and stimulated with 221 cells pre-coated with KIR3DS1-Fc chimera protein (3DS1-Fc) (A-D) or the anti-HLA-F monoclonal antibodies 3D11, 4A11 or 6A4 (E-H). For panels A-D, bar heights and error bars represent the median and interquartile range of results generated using cells from 8 KIR3DS1 homozygotes. The significance of between-group results was assessed using Friedman tests with Dunn's post tests. The boxes over the 3DS1-Fc blocking conditions show the percent reduction in the frequency of functional cells from that seen for cells stimulated with 221 cells with no blocking. Statistically significant between-group comparisons are shown by a line linking groups. Significant between-group differences are shown by * - $p \leq 0.05$ and ** - $p \leq 0.01$. For panels E-H, bar heights represent the mean of 5 observations for

PBMC, PBMC+221, MIgG1 and the blocking condition using mAb 3D11; 3 observations were assessed for blocking with the mAbs 4A11 and 6A4. The boxes over the mAbs blocking conditions shows the percent reduction in the frequency of functional cells from that seen for cells stimulated with 221 cells in the same experiment.

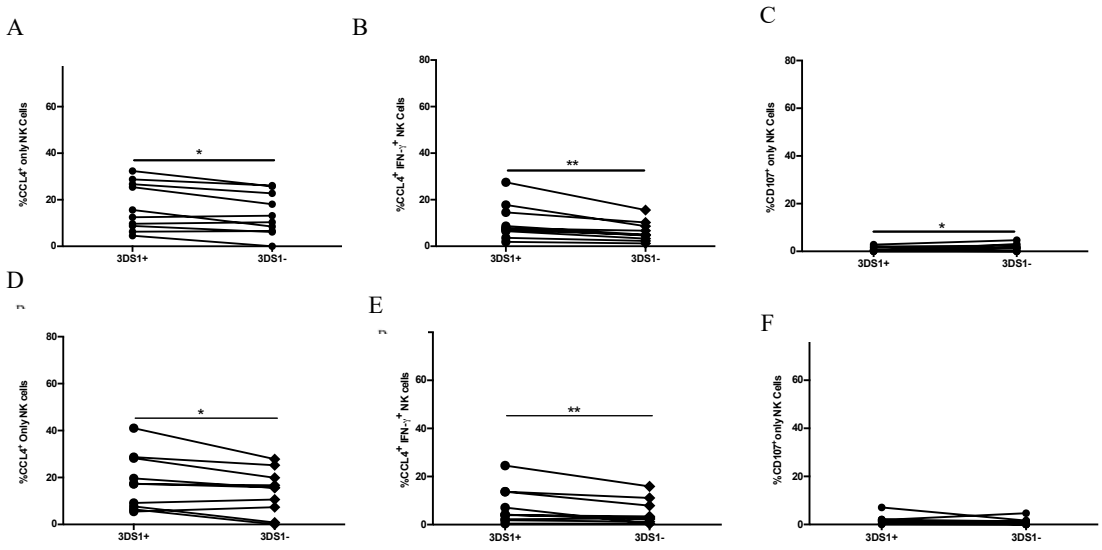


Figure S1. Differences in the frequency of 221 stimulated KIR3DS1$^+$ than KIR3DS1$^-$ NK cells secreting CCL4 only, secreting both CCL4 and IFN-γ and expressing CD107a only. Shown on the y-axis is the frequency of inclusively gated (A-C) and exclusively gated (D-F) KIR3DS1$^+$ (3DS1$^+$) and KIR3DS1$^-$ (3DS1$^-$) cells positive for CCL4 secretion only (% CCL4$^+$ only NK cells) (A, D), positive for CCL4 and IFN-γ secretion (% CCL4$^+$IFN-γ$^+$ NK cells) (B, E) and positive for CD107a expression only (% CD107a$^+$ only NK cells) (C, F). Each data point represents results from 1 of a total of 10 KIR3DS1 hmz. The significance of between-group results was assessed using Wilcoxon matched pairs tests. Significant between-group differences are shown by * - $p \leq 0.05$ and ** - $p \leq 0.01$.

Figure S2

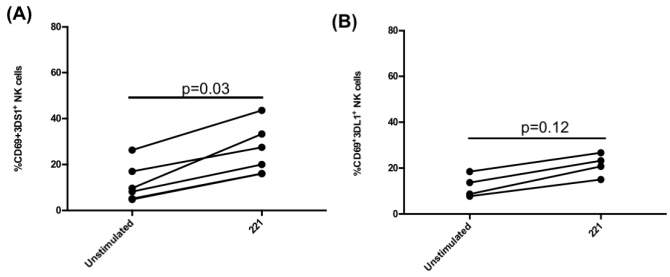


Figure S2. 221 stimulation induces an increased the frequency of CD69$^+$ NK cells. Shown on the y-axis is the frequency of exclusively gated CD69$^+$KIR3DS1$^+$ CD56^{dim} NK cells (left panel) and CD69$^+$KIR3DL1$^+$CD56^{dim} NK cells (right panel) on unstimulated and 221 stimulated cells. Results from 5 and 4 observations were used to generate results for KIR3DS1$^+$ and KIR3DL1$^+$ NK cells, respectively. A Wilcoxon matched pars test was used to assess the significance of differences between unstimulated and stimulated conditions. P-values for these comparisons are shown over the lines linking the 2 test conditions.

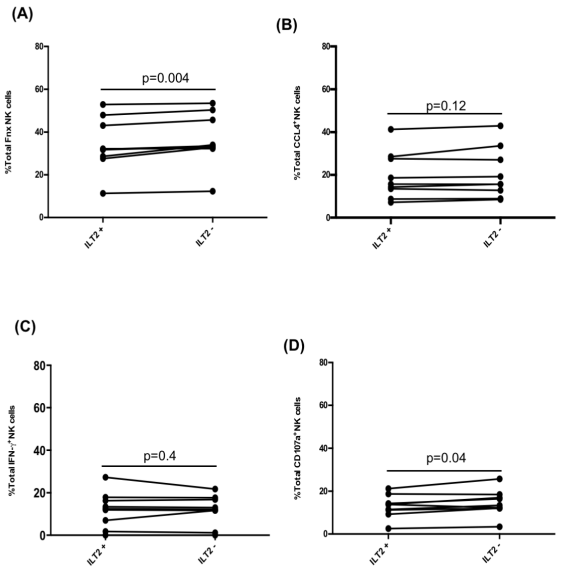


Figure S3. Comparisons of the frequency of functional ILT-2$^+$ versus ILT-2$^-$ cells following stimulation with 221 cells. Shown on the y-axis is the frequency of exclusively gated ILT-2$^+$ and ILT-2$^-$ NK cells exhibiting (A) the sum of all functions tested (% Total Fxn NK cells), (B) the sum of cells secreting CCL4 (% Total CCL4$^+$ NK cells), (C) the sum of cells secreting IFN-γ (% Total IFN-γ$^+$ NK cells) and (D) the sum of cells expressing CD107a (% Total CD107a$^+$ NK cells). Each data point represents results from 1 of a total of 9 KIR3DS1 hmz. The significance of between-group results was assessed using Wilcoxon matched pairs tests. P-values for comparisons between exclusively gated ILT-2$^+$ and ILT-2$^-$ NK cells are shown over the lines linking the 2 populations in each panel.

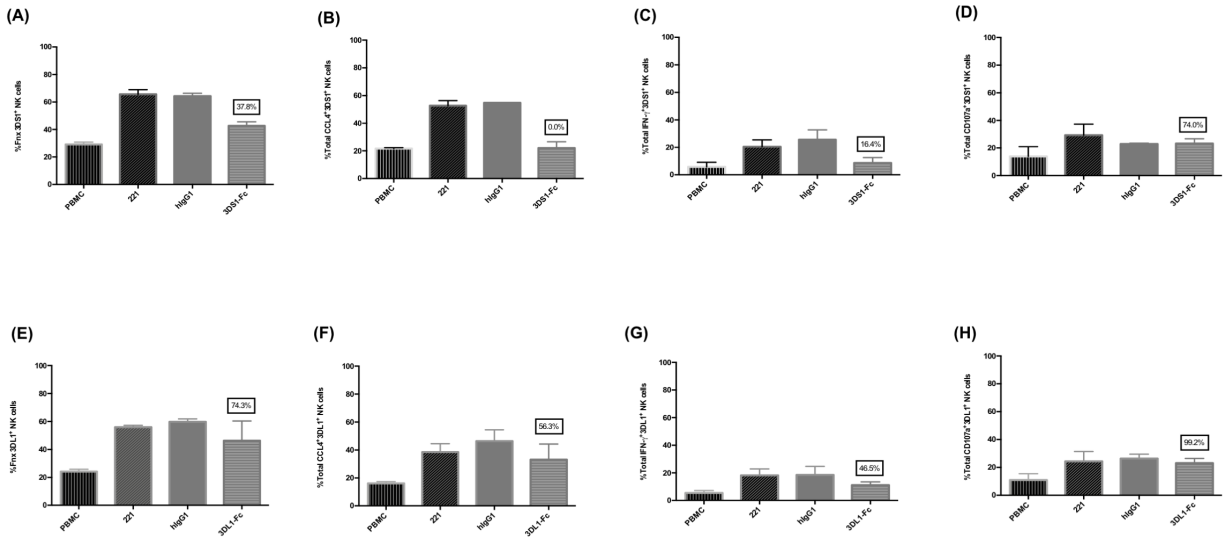


Figure S4. The effect of blocking KIR3DS1 or KIR3DL1 on 221 cells from *KIR3DL1/S1* heterozygotes and KIR3DL1 on NK cells from KIR3DL1 homozygotes stimulated by 221 cells. Shown on the y-axis is the frequency of exclusively gated KIR3DS1^{+}CD3^{-}CD56^{dim} NK cells (A-D) or exclusively gates KIR3DL1^{+}CD3^{-}CD56^{dim} NK cells (E-H) exhibiting (A, E) the sum of all functions tested (% Total Fxn NK cells), (B, F) the sum of cells secreting CCL4 (% Total CCL4^{+} NK cells), (C, G) the sum of cells secreting IFN-γ (% Total IFN-γ^{+} NK cells) and (D, H) the sum of cells expressing CD107a (% Total CD107a^{+} NK cells) following no stimulation (PBMC), stimulation with 221 cells (221), stimulation with 221 cells pre-coated with human IgG1 antibody (hIgG1) and stimulated with 221 cells pre-coated with KIR3DS1-Fc chimera protein (3DS1-Fc) (A-D) or the KIR3DL1-Fc (E-H). Bar heights represent the mean of results for KIR3DS1^{+} cells from 2 *KIR3DL1/S1* heterozygotes carrying no *HLA-Bw4* alleles (A-D) and KIR 3DL1^{+} NK cells from 2 *KIR3DL1/S1* heterozygotes and *2 KIR3DL1* homozygotes carrying no *HLA-Bw4* alleles. The boxes over the 3DS1-Fc and 3DL1-Fc blocking conditions show the percent reduction in the frequency of functional cells from that seen for cells stimulated with 221 cells with no blocking.

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CHAPTER 3

HLA-F on Autologous HIV Infected Cells Activates Primary NK Cells Expressing the Activating Killer Immunoglobulin-like Receptor (KIR) 3DS1

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Author contributions:

Z.K. designed the study, performed the experiments and data analysis, and prepared the manuscript; J.B. provided clinical samples and edited the manuscript; D.E.G. provided the anti-HLA-F mAbs and edited the manuscript; N.F.B designed the study, participated in data analysis and prepared the manuscript.

RATIONALE AND RESEARCH OBJECTIVES

In the previous chapter, we showed that a higher frequency of KIR3DS1^{+} that KIR3DS1^{-} NK cells responded to HLA null cells. We also, demonstrated that the functionality of KIR3DS1^{+} NK cells was due to the interaction of KIR3DS1 with HLA-F on the surface of HLA null cells. By using our Ab panel for flow cytometry and our exclusive gating strategy, we showed that the higher responsiveness of KIR3DS1^{+} than KIR3DS1^{-} NK cells, was not due to co-expression of other i/aNKR. In chapter 2, we used HLA null cells as a model for HIV infected cells with downmodulated MHC class Ia antigens that maintained the expression of HLA-F, which is a ligand for the aKIR KIR3DS1. Previous work showed that HIV exposed KIR3DS1 hmzs were at a lower risk of HIV infection than HIV exposed individuals with other KIR/HLA genotypes. In chapter 3 we wished to explore whether KIR3DS1^{+} NK cells from KIR3DS1 hmzs could be activated by autologous HIV infected cells. We used primary NK cells and sorted HIV infected cells to question whether the later maintained enough HLA-F to stimulate KIR3DS1^{+} NK cells to elicit anti-HIV functions.

Publication status:

This work has been published in Journal of Virology Jul 2019, JVI.00933-19; DOI: 10.1128/JVI.00933-1

ABSTRACT

KIR3DS1 homozygotes have a reduced risk of HIV infection. HLA-F is the ligand for the activating NK cell receptor (NKR) KIR3DS1. HLA-F is expressed on HIV-infected CD4 T cells. Co-culture of sorted, HIV-infected $CD4^-$ ($siCD4^-$) T cells with NK cells activated a higher frequency of $KIR3DS1^+$ than $KIR3DS1^-$ NK cells from *KIR3DS1* homozygotes to elicit anti-HIV functions such as CCL4, IFN-γ and CD107a expression. This was the case whether $KIR3DS1^{+/-}$ NK cells were analyzed inclusively or exclusively by gating out those that co-expressed the NKRs, KIR2DL1/L2/L3, 3DL2, KIR2DS1/S2/S3/S5, NKG2A and ILT2. Pre-treatment of $siCD4^-$ cells with KIR3DS1-Fc chimeric protein or an HLA-F specific monoclonal antibody, reduced the frequency of activated exclusively gated $KIR3DS1^+$ cells compared to control conditions. $KIR3DS1^+$ NK cell activation by HIV-infected CD4 cells may underlie the reduced risk of KIR3DS1 homozygotes to HIV infection.

INTRODUCTION

Natural Killer (NK) cells, an important component of the innate immune system, play a role in anti-tumor and anti-viral activities (1). They can respond to viruses at early stages of infection by secreting cytokines and chemokines and by degranulating, which can result in target cell killing (1-4). In addition to their role in innate immunity, NK cells also contribute to shaping adaptive immune responses through their interactions with dendritic cells (5). NK cells have been implicated in host responses to several viral infections such as human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), hepatitis B virus (HBV), hepatitis C virus (HCV) and influenza (6-9). In the context of HIV infection, the importance of NK cells is illustrated by the development of HIV sequence polymorphisms due to escape from NK cell-mediated immune pressure (10).

NK cell functional status depends on the integration of signals received from inhibitory and activating NK receptors (NKRs) (11). Among the NKRs, is a large family of Killer-Immunoglobulin (Ig)-like receptors (KIRs), which are expressed on NK cells as well as on other lymphocyte subsets (12). KIRs are encoded by genes mapping to the chromosome 19q 13.4 region (13). KIR protein nomenclature is based on the structure of these antigens. For example, KIR3DS1 has three extracellular Ig-like domains (i.e. 3D in this protein's name) and a short cytoplasmic domain (S) characteristic of activating KIR receptors (aKIR); KIR3DL1 has a long cytoplasmic domain typical of inhibitory KIR (iKIR) (14).

The *KIR3DL1/S1* locus is a unique among *KIR* region genes in that it encodes both inhibitory and activating NKRs (i/aNKRs). Unlike *KIR3DS1*, which is a fairly conserved allelic

variant, *KIR3DL1* is highly polymorphic with up to 84 named alleles encoding unique proteins identified at this locus to date (15). The ligands for KIR3DL1 are the HLA-Bw4 allotypes, which are a subset of HLA-A and –B antigens defined by amino acids present at positions 77-83 of the HLA heavy (H) chain (16-18). A dimorphism at position 80 of the Bw4 H chain divides these isotypes into those with an isoleucine (*80I) or threonine (*80T) at this position (19-21). On the other hand, the ligand for KIR3DS1 is HLA-F, a non-classical major histocompatibility complex (MHC) class Ib antigen that also binds to KIR3DL2, KIR3DL1 and possibly to KIR2DS4(22) (23, 24). The cytoplasmic tail of KIR3DL1 has immunoreceptor transmembrane inhibitory motifs (ITIM), which are phosphorylated when this receptor binds its ligand (25). This leads to the recruitment of Src homology 2 domain containing proteins and the generation of inhibitory signals (26, 27). KIR3DS1 possesses a positively charged amino acid in its transmembrane domain, which enables this receptor to recruit the immunoreceptor transmembrane activating motif (ITAM)-bearing adaptor protein, DAP12, to transmit activating signal (28).

A growing number of studies have implicated a role for KIR3DS1 in several disease outcomes. These include autoimmune diseases, cancer, transplantation and viral infections (6, 29-37). In the context of HIV infection, carriage of *KIR3DS1* and *HLA-Bw4*80I* alleles was reported to be associated with slower time to AIDS (6). KIR3DS1$^+$ NK cells had a superior ability to suppress HIV replication in autologous HIV infected CD4$^+$ T cells when from carriers of *KIR3DS1/HLA-Bw4*80I* combined genotypes compared to carriers of *KIR3DS1* or *HLA-Bw4*80I* alone or neither (38). In these studies, KIR3DS1$^+$ NK cells exhibited higher degranulation capacity than KIR3DL1$^+$ NK cells in response to autologous HIV infected CD4$^+$ T cells (38). Despite this,

direct evidence for an interaction between KIR3DS1 and HLA-Bw4*80I has not been found (24, 39). We previously reported a higher frequency of *KIR3DS1* homozygotes (hmzs) among HIV exposed seronegative (HESN) subjects than among HIV susceptible individuals (40). *KIR3DS1* homozygosity was associated with a 2.1-fold reduced risk of HIV infection, which was not modified by co-carriage of an *HLA-Bw4*80I* allele (40, 41). The results of a screen to detect soluble KIR3DS1-Fc chimeric protein binding to beads coated individually with each of 97 HLA-A, -B and -C ligands found no binding to any of these MHC class Ia antigens, whether bound HLA was left untreated or acid pulsed, which produces HLA H chain open conformers (OC). However, KIR3DS1-Fc did bind to beads expressing HLA-F (24).

HLA-F is preferentially expressed as an OC independently of β2-microglobulin (β2-m) or bound peptide on the surface of most of activated lymphocyte subsets (42-44). However, *Dulberger et al.* showed that it is possible to produce peptide loaded β2-m-HLA-F complexes resembling conventional MHC class I antigens (45). The binding characteristics of HLA-F OC and peptide loaded β2-m-HLA-F have important differences (45). The peptide loaded β2-m-HLA-F binds to Ig-like transcript 2 (ILT2) whereas HLA-F OC does not bind this iNKR (45).

Given that carriage of the *KIR3DS1* homozygous genotype is associated with protection from HIV infection, we hypothesized that HLA-F on iCD4 would interact with KIR3DS1 on primary NK cells to activate them for anti-viral functions. Here, we demonstrate that KIR3DS1^{+} NK cells are indeed activated by autologous iCD4 cells. Using an antibody (Ab) panel able to detect several a/iNKRs allowed us to gate on KIR3DS1^{+} NK cells inclusively as well as exclusively, we showed that the interaction of HLA-F on iCD4 with KIR3DS1 on NK cells positive for this

receptor was sufficient to activate NK cells. Since the panel included Abs recognizing CCL4, IFN-γ and CD107a, we were able to measure the frequency of the KIR3DS1$^+$ NK cell responding to this stimulus. We also found that blocking the interaction of HLA-F with a KIR3DS1-Fc chimeric protein or an HLA-F specific monoclonal Ab (mAb), reduced the ability of iCD4 to activate exclusively gated KIR3DS1$^+$ NK cells. Our findings show that KIR3DS1 binds to HLA-F on infected cells, an interaction which induces primary KIR3DS1$^+$ NK cells to elicit anti-HIV functions.

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 Researche du Centre Hospitalier de I'Université de Montreal and the Research Ethics Board of the McGill University Health Center. All subjects were adults and provided written informed consent for the collection of samples and subsequent analysis.

Study population and KIR3DL1/S1 genotyping

This study included five HIV-1 uninfected subjects who were KIR3DS1 hmzs, tested twice for a total of ten observations. One was identified from among volunteers who were at low risk for HIV infection. Four were from the St Luc cohort of injection drug users. None of the four study subjects from the St Luc cohort met the criteria to be categorized as HESN (i.e. sharing needles or injection equipment with partners known to be HIV infected). Genomic DNA was extracted

from peripheral blood mononuclear cells (PBMCs) using QIAamp DNA Blood Mini Kits (Qiagen, Mississauga, ON, Canada) as per manufacturer's instructions. *KIR3DL1/S1* generic genotyping was performed by PCR, as described previously (40).

Cells

PBMCs were isolated from leukophoresis samples by density gradient centrifugation (Lymphocytes Separation Medium, Wisent Bioproducts, St-Bruno, QC, Canada) and cryopreserved in 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO); 90% fetal bovine serum (FBS, Wisent). CD4 and NK cells were isolated from PBMCs by negative selection using EasySep™ human $CD4^+$ T cell isolation kits for CD4 and EasySep™ human NK cell enrichment kits for NK cells (Stemcell Technologies, Inc., Vancouver, BC, Canada). Purity was verified by flow cytometry and was an average of 99.1% and 96.3% for CD4 and NK cells, respectively.

Preparation of HIV infected CD4 T cell stimulators

An average of 100 x 10^6 $CD4^+$ T cells, isolated by negative selection, were stimulated with anti-CD3/CD28 tetramers (Immunocult Human CD3/CD28 T cell activator, Stemcell Technologies, Inc.) and 100 international units (IU)/ml of recombinant human Interleukin-2 (hrIL-2, Cedarlane, Burlington, ON, Canada) in RPMI 1640 medium; 10% FBS; 2 mM L-glutamine; 50 IU/ml penicillin; 50 mg/ml streptomycin (R10) (all from Wisent) at a concentration of 8 x 10^5 cells/ml in 24 well plates (Fischer Scientific, Mississauga, ON, Canada) for 4 days at 37ºC in a humidified 5% CO_2 incubator. On day 4, stimulated $CD4^+$ T cells were infected with HIV-$1_{JR\text{-}CSF\text{-}VSVG}$ in R10; 100 IU/ml hrIL-2; 10 μg/ml polybrene (Sigma-Aldrich, Oakville, ON, Canada) for 2 hrs by spinoculation at 1200g. Cells were then washed

and cultured in R10; 100 IU/ml hrIL-2 for 2 days. These HIV-infected $CD4^+$ (iCD4) cells were stained with UV Live/Dead fixable dead cell stain kit (Invitrogen, Carlsbad, CA) as per manufacturer's directions and fluorochrome conjugated Abs to the following cell surface markers: CD3-APC (REA613), CD4-Vioblue (REA623) and CD8-PE-Vio770 (REA734) (all from Miltenyi Biotec, Auburn, CA). The iCD4 were then sorted for live, singlet, $CD3^+CD8^-CD4^-$ cells (i.e. $siCD4^-$) using a FACSAria cell sorter (BD Biosciences, Mississauga, ON, Canada). After sorting, $siCD4^-$ cells were resuspended in R10. The purity of the sorted $siCD4^-$ cells was 95%. The percentage of $siCD4^-$ cells that were HIV-infected was determined pre- and post-sorting by permeabilization and intracellular staining with anti-p24-FITC (KC57; Beckman Coulter, Atlanta, GA). After sorting, 96.7% of the $siCD4^-$ cells were $p24^+$.

Detection of HLA-F on the surface of iCD4 cells

Cell surface HLA-F was detected by staining with the HLA-F specific mAb, 3D11 (46). One million cells were resuspended in 100 µl of PBS (Wisent); 5% FBS (FACS Buffer). 3D11 mAb or mIgG1 isotype control (MOPC-21; Biolegend) was added to the cell suspension for 40 min at 4ºC. Primary Ab binding was detected by incubating washed cells with 1µg/ml F(ab')2 goat anti-mouse IgG-PE (eBioscience, San Diego, CA) secondary Ab for 25 min at 4ºC. Cells then were washed and fixed in 2% paraformaldehyde (PFA) (Santa Cruz Biotechnology, Santa Cruz, CA). Results were acquired within 1 hr.

NK cell stimulation

Isolated NK cells were co-cultured with either uninfected CD4 or iCD4 at a ratio of 1:1 in R10 for 6 hours at 37ºC in a humidified 5% CO_2 incubator. Brefeldin A (24 ug/ml: Sigma-Aldrich)

and monensin (3 ug/ml, GolgiStop; BD Biosciences) were added one hr after the initiation of the co-culture. NK cells cultured in R10 alone served as an unstimulated negative control. An aliquot of NK cells from each study subject was tested for their ability to respond to 5.8 ug/ml PMA and 1.2 ug/ml Ionomycin stimulation. All cells were responsive to this positive control stimulus. After 6 hrs stimulation, cells were stained for viability using UV Live/Dead staining and surface-stained with Abs to CD3-BV785 (OKT3), CD56-BV711 (HDC56), KIR2DL1/S1/S3/S5-FITC (HP-MA4), ILT2-PC7 (GHI/75), CD107a-BV421 (H4A3) (all from Biolegend), KIR3DL1/S1-PE (Z27; Beckman Coulter), NKG2A-FITC (REA110; Miltenyi Biotec), KIR2DL1/L2/L3-FITC (CH-L; BD Biosciences) and KIR3DL2-FITC (539304; R&D Systems, Minneapolis, MN) for 30 min at 4º C. Cells then were washed and fixed with 2% PFA, permeabilized with Permeabilization Medium B (Invitrogen) and stained intracellularly with anti-CCL4-AF700 (D21-1351) and anti-IFN-γ-BV510 (B27) (both from BD Biosciences). Samples were then washed twice and fixed with 2% PFA and acquired within 1 hour.

In parallel experiments, we blocked the interaction of KIR3DS1 on NK cells with HLA-F on siCD4$^-$ cells by pre-incubating siCD4$^-$ cells with either 25 μg/ml of KIR3DS1-Fc chimeric protein (R&D Systems) or anti-HLA-F specific mAb 3D11 (46) for 50 min at 37ºC in a humidified 5% CO_2 incubator prior to co-culture with NK cells. The control condition for blocking with KIR3DS1-Fc was pre-incubation of siCD4$^-$ cells with hIgG1 isotype control (ET901, Biolegend). The control for blocking with 3D11 was pre-incubation of siCD4$^-$ cells with mIgG1 (MOPC-21, Biolegend). Both isotype controls were used at a concentration of 25 μg/ml. Following co-culture of siCD4$^-$ with NK cells, NK cells were stained for cell surface and

intracellular markers as described above. The percent reduction in the function of KIR3DS1^{+} NK cells stimulation in the presence of blocking reagents was calculated using the following equation: 100 - ((frequency of functional cells in the siCD4^{-}-stimulated NK cell population in the presence of a blocking reagent - frequency of functional cells in the unstimulated NK cell population) / (frequency of functional cells in the siCD4^{-}-stimulated NK cell population - frequency of functional cells in the unstimulated NK cell population) X 100).

Flow cytometry analysis

Between 1 x 10^6 and 1.8 x 10^6 total events were acquired for each sample using an LSR Fortessa X-20 flow cytometer (BD Biosciences). Results were analyzed using FlowJo software (version 10.3; Tree Star, Ashland, OR). We measured the frequency of KIR3DS1$^{+/-}$ CD3^{-}CD56^{dim} NK cells exhibiting the seven possible combinations of CCL4, IFN-γ and CD107a functions (i.e., tri-functional, three bi-functional and three mono-functional response patterns). We also assessed the sum of frequencies of all functions tested (total) and the sum of the frequencies of functional subsets secreting CCL4 (total CCL4), secreting IFN-γ (total IFN-γ) and expressing CD107a (total CD107a). These functional subsets were assessed for KIR3DS1^{+} and KIR3DS1^{-} NK cell populations using gating strategies that either included or excluded NK cell populations co-expressing the NKRs, KIR2DL1/L2/L3, KIR2DS1/S2/S3/S5, KIR3DL2, ILT2 and NKG2A. The data presented were background subtracted using results for matched unstimulated control conditions.

Statistical analysis

GraphPad prism 6 (GraphPad Software, La Jolla, CA) was used for data analysis and graphical presentation. Wilcoxon matched-pairs signed rank tests were used to assess the significance of comparisons of inclusively and exclusively gated KIR3DS1^{+} versus KIR3DS1^{-} NK cells. P-values <0.05 were considered significant. Friedman tests with Dunn's post tests were used to assess the significance of differences between matched groups of unstimulated NK cells, NK cells stimulated with autologous siCD4^{-} in the presence of reagents that blocked the interaction of HLA-F with KIR3DS1 or isotype controls for these blocking reagents.

RESULTS

HLA-F expression levels on activated and HIV-infected CD4 cell subsets

Garcia-Beltran et al. reported that HIV-infected CD4^{+} T cells that were p24^{hi}CD4^{-}, which they termed late infected cells, had an MFI of HLA-F expression that was lower than that on p24^{lo}CD4^{+} cells or on IL-2 or IL-2 and anti-CD3^{+}/CD28^{+} stimulated CD4^{+} cells (24). To verify this, we activated purified CD4^{+} T cells for 4 days, infected them with HIV-1$_{JRCSF-VSVG}$ for 2 days and sorted them into CD4^{hi} and CD4^{-} populations. Fig. 1 shows that HLA-F was expressed at a lower MFI on sorted HIV-infected CD4^{-} cells (hereafter called siCD4^{-}) (average MFI 351) than on siCD4^{hi} cells (average MFI 531) at 2 days post infection. Four day activated uninfected CD4^{+} (unCD4^{+}) T cells had an average MFI of HLA-F staining of 1834.

Sorting for iCD4^{-} cells

HIV infection of CD4^{+} T cells resulted in an average infection rate of 8% as determined by either downmodulation of cell surface CD4 expression and/or by the presence of intracellular p24.

Using polybrene and spinoculation during the infection process increased the average infection rate to 23%, while maintaining an average viability of 95%. *Richard et al.* reported that $p24^{lo}CD4^{hi}$ cells present post infection of isolated $CD4^+$ T cells and which have a higher MFI of HLA-F expression that $p24^{hi}CD4^-$ cells, are not infected with replication competent HIV (47). $CD4^+$ T cells infected with wild type HIV, downmodulate CD4 through Nef and Vpu dependent mechanisms (48, 49). We next examined sorted $iCD4^{hi}$ and $iCD4^-$ cells for intracellular p24, as a marker of HIV infection. The gating strategy used for sorting is shown in Fig. 2. The bulk of live, singlet, $CD3^+CD8^-CD4^-$ cells originating from purified activated, HIV-infected $CD4^+$ T cells was $p24^{hi}$, whereas a high frequency of those that were $CD4^{hi}$ were $p24^{lo}$. As activated unCD4 and $p24^{lo}CD4^{hi}$ cells express HLA-F at a higher MFI than $p24^{hi}CD4^-$ iCD4 cells, it was necessary minimize the frequency of unCD4 and $siCD4^{hi}$ subsets from the population of cells used to stimulate $KIR3DS1^{+/-}$ NK cells from *KIR3DS1* hmzs. This was done by sorting for $CD3^+CD8^-CD4^-$ cells from the parental iCD4 population. This strategy generated an $siCD4^-$ population that was on average 96.7% $p24^{hi}$ and 95% $CD3^+CD8^-CD4^-$, which was used to stimulate NK cells.

A higher frequency of $KIR3DS1^+$ than $KIR3DS1^-$ NK cells responded to stimulation with $siCD4^-$ cells

NK cells stimulated with $siCD4^-$ cells were stained with the Ab panel shown in Table 1. Fig. 3 shows the inclusive and exclusive gating strategies used to gate on $KIR3DS1^+$ and $KIR3DS1^-$ NK cells (although NKG2D and NCRs were not included in the exclusive channels). Boolean gating was used to assess the frequency of $KIR3DS1^{+/-}$ cells expressing various combinations of the three functions tested. The frequency of cells exhibiting the sum of all functions tested, total

CCL4 and total IFN-γ secretion and total CD107a expression was higher in KIR3DS1^{+} than KIR3DS1^{-} NK cells ($p< 0.002$ for all, Wilcoxon matched-pairs signed rank test) (Fig. 4A-D).

We also assessed the frequency of tri-functional, three combinations of bi-functional and three combinations of mono-functional KIR3DS1$^{+/-}$ NK cells. A higher frequency of inclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells characterized by the following functional profiles responded to autologous siCD4^{-} cells: tri-functional CCL4^{+}IFN-γ^{+}CD107a^{+}, bi-functional CCL4^{+}CD107a^{+} and IFN-γ^{+}CD107a^{+} and mono-functional CCL4^{+} and IFN-γ^{+} only NK cells ($p< 0.03$ for all, Wilcoxon tests) (Fig. S1A-E).

Depending on the genetic profile of the study subjects, KIR3DS1$^{+/-}$ NK cells can co-express various stochastic combinations of other a/iNKRs. It is expected that NK cells co-expressing iKIRs, ILT2 and NKG2A to self HLA would be educated through these receptor-ligand interactions. HIV-infection downmodulates HLA-A, B and C expression on iCD4 (50, 51). Thus, stimulation of NK cells with siCD4^{-} cells may affect the responsiveness of KIR3DS1$^{+/-}$ NK cells to these cells by differentially abrogating inhibitory signaling through iNKRs. To minimize such effects on the responses of KIR3DS1$^{+/-}$ NK cells to siCD4^{-} cells, we used a gating strategy that excluded NK cell populations bearing KIR2DL1/L2/L3/S1/S2/S3/S5, KIR3DL2, ILT2 and NKG2A from KIR3DS1$^{+/-}$ CD56^{dim} NK cells (Fig. 3). A higher frequency of exclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells responded stimulation by siCD4^{-} cells. This was the case for the sum of all functions tested, total CCL4 and total IFN-γ secretion and total CD107a expression ($p<0.002$ for all, Wilcoxon tests) (Fig. 5A-D). Further analysis showed that a higher frequency of exclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells characterized by the

following functional profiles responded to siCD4$^-$ cell stimulation: tri-functional CCL4$^+$IFN-γ$^+$CD107a$^+$, bi-functional CCL4$^+$ CD107a$^+$ and IFN-γ$^+$CD107a$^+$ and mono-functional CCL4$^+$ and IFN-γ$^+$ only NK cells ($p<0.03$ for all, Wilcoxon tests) (Fig. S2A-E).

The effect of HLA-F-ILT2 interactions on the function of ILT2$^+$ NK cells

Dulberger et al. showed that HLA-F OCs do not interact with the iNKR ILT2 while peptide loaded β2-m-HLA-F does (45). We hypothesized that if HLA-F on siCD4$^-$ cells bound to ILT2 on NK cells, then a lower frequency of exclusively gated ILT2$^+$ than ILT2$^-$ NK cells would respond to siCD4$^-$ stimulation. Rather, we found a higher frequency of ILT2$^+$ than ILT2$^-$ NK cells responded to siCD4$^-$ stimulation, which was the case for the sum of all functions, total CCL4, total IFN-γ and total CD107a expression ($p<0.03$ for all, Wilcoxon tests) (Fig. S3A-D).

The interaction between HLA-F on siCD4$^-$ with KIR3DS1 on NK cells is required to stimulate KIR3DS1$^+$ NK cells

To confirm that the interaction of HLA-F with KIR3DS1 was required to activate KIR3DS1$^+$ NK cells, we blocked this interaction by pre-incubating HLA-F on siCD4$^-$ cells with KIR3DS1-Fc chimeric protein or 3D11 mAb prior to co-culture with NK cells. Fig. 6 shows that blocking HLA-F-KIR3DS1 interactions with KIR3DS1-Fc generated a lower frequency of functional, exclusively gated KIR3DS1$^+$ NK cells responding to siCD4$^-$ cells than when these stimulatory cells were pre-treated with a human IgG1 (hIgG1) isotype control. This was the case for total responses, total CCL4 and total CD107a expression ($p>0.001$, Friedman with Dunn's post-tests). While the frequency of IFN-γ secreting KIR3DS1$^+$ NK cells stimulated by autologous siCD4$^-$ cells was also lower in the presence of KIR3DS1-Fc chimeric protein than its isotype control, differences did not

achieve statistical significance. Blocking the interaction of HLA-F with KIR3DS1 using mAb 3D11 also decreased the frequency of functional exclusively gated KIR3DS1^{+} NK cells characterized by the sum of all function, total CCL4^{+}, total IFN-γ^{+} and total CD107a^{+} NK cells compared with that observed for responses to siCD4^{-} cells pre-treated with mouse IgG1 (mIgG1) isotype control. However, the differences for these four functional subsets failed to achieve statistical significance (Fig. 6A-D). Furthermore, blocking HLA-F-KIR3DS1 interactions with either KIR3DS1-Fc or 3D11 reduced the frequency of exclusively gated KIR3DS1^{+} exhibiting these four functional profiles to levels that were not significantly different from that of unstimulated KIR3DS1^{+} NK cells.

DISCUSSION

Recently, it was shown that the non-classical MHC-Ib antigen, HLA-F, is a high affinity ligand for KIR3DS1(24). In this study, we confirmed that HLA-F is expressed on the surface of activated unCD4 as well as on CD4 T cells exposed to HIV and sorted for p24^{lo}CD4^{+} and siCD4^{-} subsets. Although HIV infection down-modulated the expression of HLA-F on siCD4^{-} cells compared to activated unCD4 and p24^{lo}CD4^{+}, siCD4^{-} cells retained sufficient amounts of cell surface HLA-F expression to stimulate KIR3DS1^{+} NK cells. Co-culture of siCD4^{-} cells with isolated primary NK cells from KIR3DS1 hmzs activated a higher frequency of CD3^{-}CD56^{dim} KIR3DS1^{+} than KIR3DS1^{-} NK cells to secrete CCL4, IFN-γ and express CD107a. By using a gating strategy that excluded KIR3DS1$^{+/-}$ NK cells co-expressing a panel of iNKR and aNKR, we confirmed that the activation of KIR3DS1^{+} NK cells is not due to the co-expression of these

receptors through mechanisms such as missing-self recognition or activation through several co-expressed aNKR. Blocking the interaction of HLA-F on siCD4$^-$ cells with KIR3DS1 on primary NK cells reduced the frequency of exclusively gated functional KIR3DS1$^+$ NK cells responding to autologous siCD4$^-$ stimulation to levels lower than that seen in the absence of blocking or when blocking with HLA-F specific reagents was replaced by isotype controls and to levels that did not differ significantly from those of unstimulated KIR3DS1$^+$ NK cells.

A role for KIR3DS1 in HIV outcomes was first implicated based on results from epidemiological studies that reported that the co-carriage of *KIR3DS1* and *HLA-Bw4*80I* combined genotypes was associated with slower time to AIDS (6). In the absence of *HLA-Bw4*80I*, KIR3DS1 hmzs progressed to AIDS more rapidly (6). In contrast, *Boulet et al.* showed that the proportion of KIR3DS1 hmzs was higher in HESN than in HIV susceptible individuals enrolled in a primary infection (PI) cohort whereas the frequency of *KIR3DL1/S1* heterozygotes and *KIR3DL1* hmzs was similar in HESN and PI subjects (40). Co-expression of *HLA-Bw4*80I* did not modulate the effect of *KIR3DS1* homozygosity on HIV infection risk (40). These findings highlight that *KIR/HLA* genotypes influencing NK cell function may play distinct roles at the level of protection from HIV infection versus HIV control in those already infected.

Several attempts to demonstrate interactions between KIR3DS1 and HLA-Bw4*80I have failed (24, 39). An exception to this was noted when KIR3DS1 was found to interact with the HLA-Bw4*80I allotype, HLA-B*57, in the presence of certain HIV-derived peptides (52). More recently, HLA-F was shown to be a high affinity ligand for KIR3DS1 and other KIR3D based receptors, including KIR3DL1, KIR3DL2 and possibly KIR2DS4(23) (22, 24). In this study, the

effect of these receptors on NK cell responses to siCD4- cells was minimized by including a mAb specific for KIR3DL2 in our exclusive gating strategy. None of the *KIR3DS1* hmzs expressed KIR3DL1 or KIR2DS4 (53). The genes encoding KIR3DL1 and KIR2DS4 are in linkage disequilibrium with each other on the telomeric KIR region haplotype A and in negative linkage disequilibrium with *KIR3DS1*, a gene found on the telomeric KIR region haplotype B (54, 55).

HLA-F is expressed on the surface of Epstein-Barr virus-transformed B cells such as 721.221, activated lymphocytes and HIV infected CD4 T cells (44, 46, 56). HIV infection modulated the expression of HLA-F on the surface of iCD4 cells (24). In this study, we confirmed that HLA-F was present on the surface of the $siCD4^{-}$ cells, though at a lower MFI than present on activated $unCD4^{+}$ T cells or on $siCD4^{hi}$ cells two days after HIV exposure to isolated activated $CD4^{+}$ cells. Two days after HIV infection, $p24^{lo}CD4^{hi}$ cells were shown by others to be essentially negative for intracellular HIV *gag-pol mRNA* using a sensitive RNA-flow fluorescence *in situ* hybridization assay, which is 1000-fold more sensitive for detecting HIV-infected cells than intracellular staining for Gag protein alone (47, 57). These $p24^{lo}CD4^{hi}$ cells were deemed not to be in an early phase of HIV infection as an additional 5 days of culture failed to increase the frequency of infected cells to a frequency of more than 3% (47). Thus, it is unlikely that $p24^{lo}CD4^{hi}$ cells are HIV infected. Given that $siCD4^{-}$ cells activated autologous $KIR3DS1^{+}$ NK cells, HLA-F downmodulation on these cells was insufficient to prevent the activation of $KIR3DS1^{+}$ NK cells to elicit several anti-HIV functions. Activated $KIR3DS1^{+}$ NK cells secrete CCL4, IFN-γ and express CD107a. CCL4 is a chemokine able to bind to CCR5, the co-receptor for HIV entry, which results in blocking HIV infection of new target cells (58). IFN-γ is an

important antiviral cytokine for both innate and adaptive immune responses (59) and CD107a is a marker of NK cell degranulation, a surrogate marker for target cell lysis (60). Production of these molecules by activated KIR3DS1^{+} NK cells and the ability of NK cells to rapidly elicit these functions upon encountering HIV-infected CD4 cells may explain why *KIR3DS1* hmzs have a reduced risk of HIV infection (40, 41, 61).

The interaction of iNKR with self-HLA ligands is necessary for NK cell education, which in turn determines NK cell functional potential when they encounter transformed, stressed or virus-infected cells with downmodulated ligands for iNKR (62, 63). Some aNKR are involved in NK education as well, although these interactions should tune down NK responsiveness to altered self-cells (64, 65). HIV infected cells downmodulate cell surface levels of self-HLA-A, -B and -C ligands for iNKR, reducing or interrupting inhibitory signals through these receptors, shifting the balance of activating and inhibitory signals received by NK cells towards activation. This was the rationale for designing an Ab panel and a gating strategy that excluded KIR3DS1$^{+/-}$ cells co-expressing iNKR and aNKR. We found that a higher frequency of exclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells responded to siCD4^{-} cell stimulation. This ruled out the possibility for involvement of other i/aNKR in the responsiveness of KIR3DS1^{+} NK cells to siCD4^{-} stimuli. Blocking experiments provided further support for the conclusion that the higher responses of KIR3DS1^{+} than KIR3DS1^{-} NK cells to siCD4^{-} cell stimulation was due to the interaction between HLA-F and KIR3DS1. Pre-coating siCD4^{-} with KIR3DS1-Fc chimeric protein or 3D11 reduced the ability of these siCD4^{-} to stimulate exclusively gated KIR3DS1^{+} NK cells. Therefore, the ligation of KIR3DS1 on primary NK cells with HLA-F on siCD4^{-} cells is functional and responsible for activation of KIR3DS1^{+} NK cells. 3D11 was less effective than

KIR3DS1-Fc chimera protein at blocking the activation of KIR3DS1$^+$ NK cells. The reason for this is unclear as saturating concentration of both reagents was achieved. It is possible that mAb 3D11 has a higher off rate than the KIR3DS1-Fc chimeric protein, which may allow for more exposure of HLA-F to KIR3DS1. Alternatively, this result may suggest that KIR3DS1-Fc recognizes other ligands on siCD4$^-$ cells whose interaction with KIR3DS1 is not blocked by this mAb. Another possibility is that 3D11 does not entirely mask the binding site of KIR3DS1 on HLA-F. Reduction in the frequency of activated KIR3DS1$^+$ NK cells to levels at or below those seen for NK cell stimulation conditions where the interaction between KIR3DS1 and HLA-F was blocked by KIR3DS1-Fc chimeric protein suggests that the contribution of other NK receptors to activation is unlikely or minimal.

We found that a higher frequency of ILT2$^+$ than ILT2$^-$ NK cells responded to siCD4$^-$ cell stimulation characterized by several functional profiles. ILT2 is an iNKR that can also bind HLA-F with a high affinity when it is complexed with β_2-m and peptides (45). ILT2 does not bind HLA-F OCs (45, 66). The higher responses to autologous siCD4$^-$ by exclusively gated ILT2$^+$ than ILT2$^-$ NK cells suggest that educated ILT2$^+$ NK cells are recognizing autologous siCD4$^-$ cells as non-self. This would be the case if these cells predominantly expressed HLA-F as OCs, unable to interact with ILT2. Furthermore, the ability of HLA-F on siCD4$^-$ cells to interact with KIR3DS1 on NK cells and activate them is also consistent with HLA-F on these cells being expressed mostly as OCs as HLA-F- β_2-m-peptide complexes also do not bind KIR3DS1 (45).

In summary, the results reported in this manuscript provide a possible mechanistic explanation for the reduced risk of HIV infection observed in HESN KIR3DS1 hmzs, i.e. the rapid activation

of these NK cells by interactions with HLA-F expressing HIV-infected cells to elicit anti-HIV functions.

FIGURES AND LEGENDS

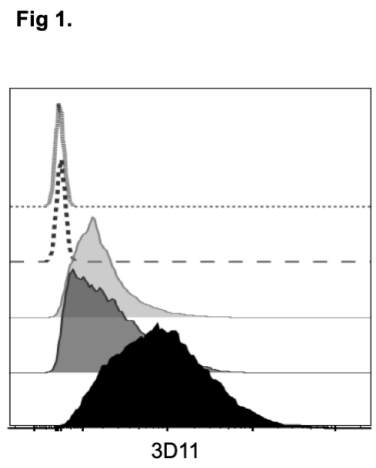

Fig 1. Effect of $CD4^+$ T cell activation and HIV infection on the mean fluorescence intensity of HLA-F expression. Flow cytometry plots of unstimulated HIV-uninfected $CD4^+$ T cells stained with a mouse IgG1 isotype control followed by PE-conjugated goat anti-mouse IgG (secondary antibody) (top panel), unstimulated $CD4^+$ T cell stained with anti-HLA-F specific monoclonal antibody (mAb) 3D11 and the same secondary antibody (2^{nd} panel), activated, HIV-infected sorted $CD4^-$ T cells stained with 3D11 and secondary antibody (3^{rd} panel), activated, HIV-infected sorted $CD4^{hi}$ T cells stained with 3D11 and secondary antibody (4^{th} panel) and activated, HIV-uninfected $CD4^+$ T cells stained with 3D11 and secondary antibody (5^{th} panel).

Fig 2.

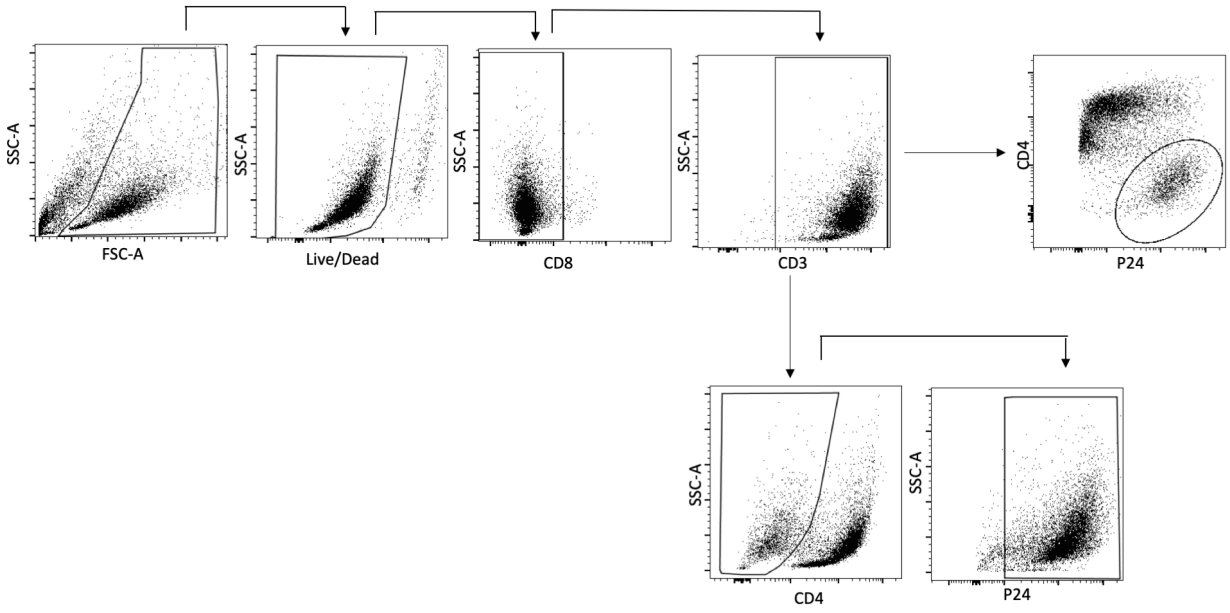

Fig 2. Gating strategy to detect CD4 expression on HIV-infected cells expressing p24 antigen. Magnetic bead purified $CD4^+$ T cells were isolated, activated for 4 days with recombinant, human IL-2 and anti-CD3/anti-28 tetrameric antibodies and spinoculated with HIV and cultured for 2 days. From these cells, live singlet lymphocytes were gated on that were $CD8^-$ and $CD3^+$. The far right-hand panel shows that most of the $p24^+$ cells in this population were $CD4^-$. The bottom panel shows that if $CD3^+CD8^-$ cells were sorted into $CD4^-$ and $CD4^+$ subsets, most of the $CD4^-$ cells were $p24^+$. This provided the rationale for using sorted HIV-infected $CD4^-$ cells ($siCD4^-$), which contain the majority of HIV-infected cells to stimulate NK cells. This strategy reduced the frequency of $siCD4^{hi}$ cells and activated $CD4^+$ T cells that both uninfected but that expressed HLA-F able to stimulate $KIR3DS1^+$ NK cells.

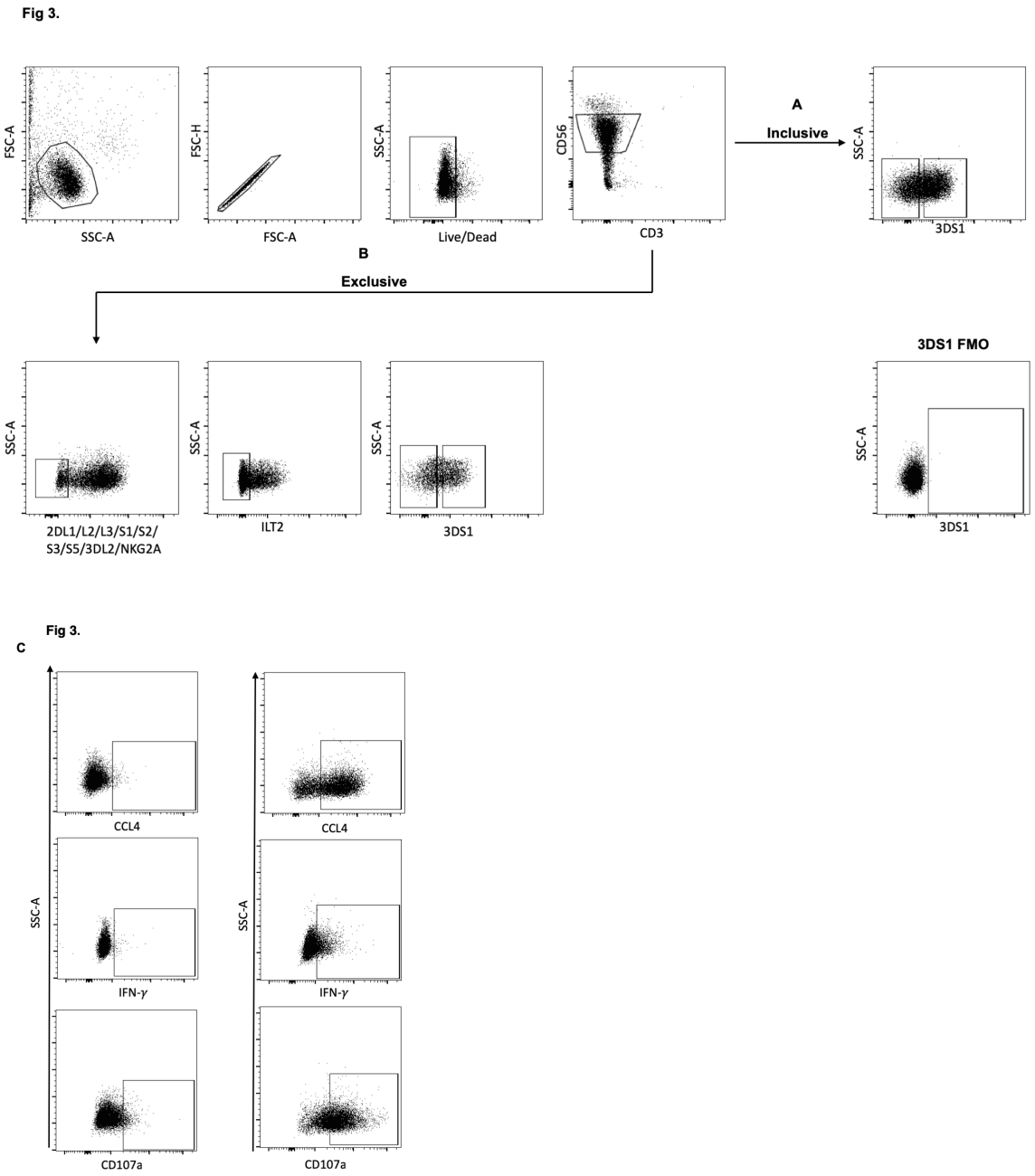

Fig 3. Gating strategy to detect inclusively and exclusively gated functional KIR3DS1^{+}CD56^{dim} NK cells. Live singlet lymphocytes were gated on, from which CD3^{-} CD56^{dim} cells were selected. (A) For inclusive gating, KIR3DS1^{+} and KIR3DS1^{-} NK cells were selected for examination of their functional profiles following stimulation with autologous sorted

HIV-infected $CD4^-$ ($siCD4^-$) cells. (B) For exclusive gating, $CD3^-CD56^{dim}$ NK cells that were negative for KIR2DL1/L2/L3/S1/S2/S3/S5, KIR3DL2 and NKG2A were gated on. From these cells, those negative for ILT2 were gated on to identify exclusively gated $KIR3DS1^+$ and $KIR3DS1^-$ cells. (C) Inclusively and exclusively gated $KIR3DS1^+$ and $KIR3DS1^-$ NK cells were examined for all possible combinations of CCL4, IFN-γ, and CD107a expression by Boolean gating. Functional gates were set on gated total unstimulated $CD3^-CD56^{dim}$ cells (NK cells). Unstimulated conditions were used to background subtract results obtained for HIV-stimulated conditions.

Fig 4.

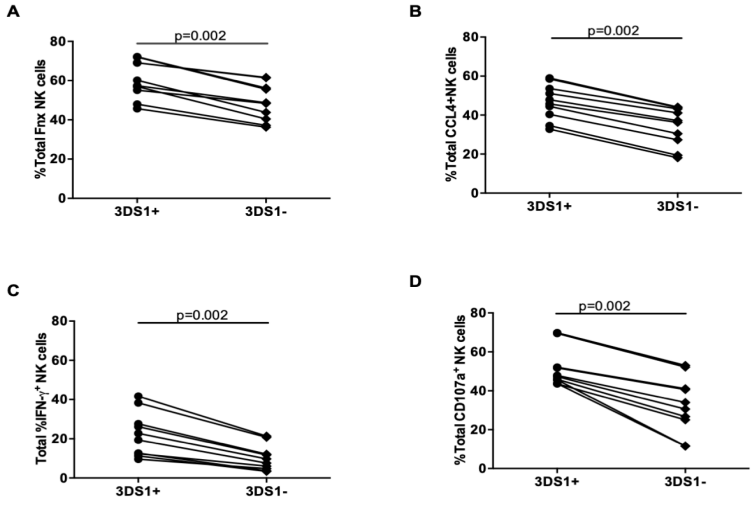

Fig 4. A higher frequency of inclusively gated KIR3DS1$^+$ than KIR3DS1$^-$NK cells responded to stimulation with sorted, HIV-infected CD4$^-$ cells. Shown on the y-axis is the frequency of inclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ NK cells exhibiting (A) the sum of all functions tested (% Total Fxn NK cells), (B) the sum of cells secreting CCL4 (% Total CCL4$^+$ NK cells), (C) the sum of cells secreting IFN-γ (% Total IFN-γ$^+$ NK cells) and (D) the sum of cells expressing CD107a (% Total CD107a$^+$ NK cells). The significance of between-group differences was assessed using Wilcoxon matched-pairs tests. P-values for comparisons between exclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ NK cells are shown over the lines linking the 2 populations being compared.

Fig 5.

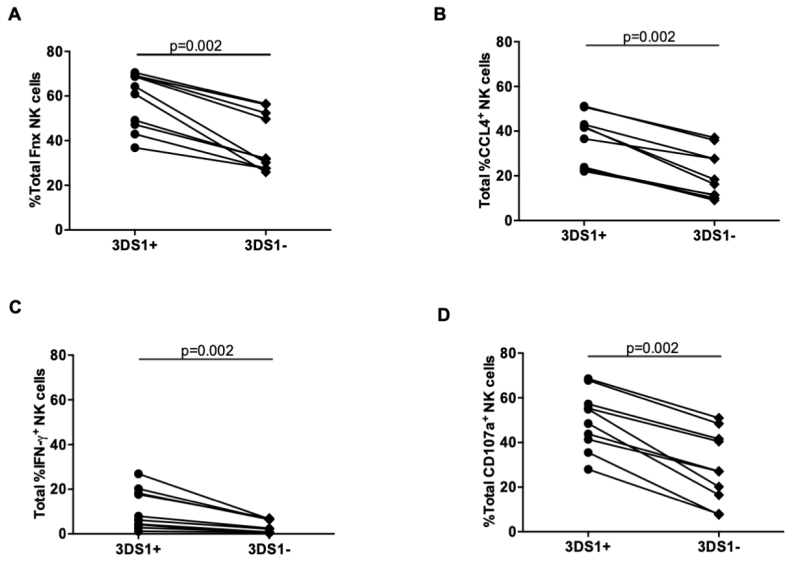

Fig 5. A higher frequency of exclusively gated KIR3DS1$^+$ than KIR3DS1$^-$ NK cells responded to stimulation with sorted, HIV-infected CD4$^-$ cells. Shown on the y-axis is the frequency of exclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ NK cells exhibiting (A) the sum of all functions tested (% Total Fxn NK cells), (B) the sum of cells secreting CCL4 (% Total CCL4$^+$ NK cells), (C) the sum of cells secreting IFN-γ (% Total IFN-γ$^+$ NK cells), and (D) the sum of cells expressing CD107a (% Total CD107a$^+$ NK cells). The significance of between-group differences was assessed using Wilcoxon matched-pairs tests. P-values for comparisons between exclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ NK cells are shown over the lines linking the 2 populations being compared.

Fig 6.

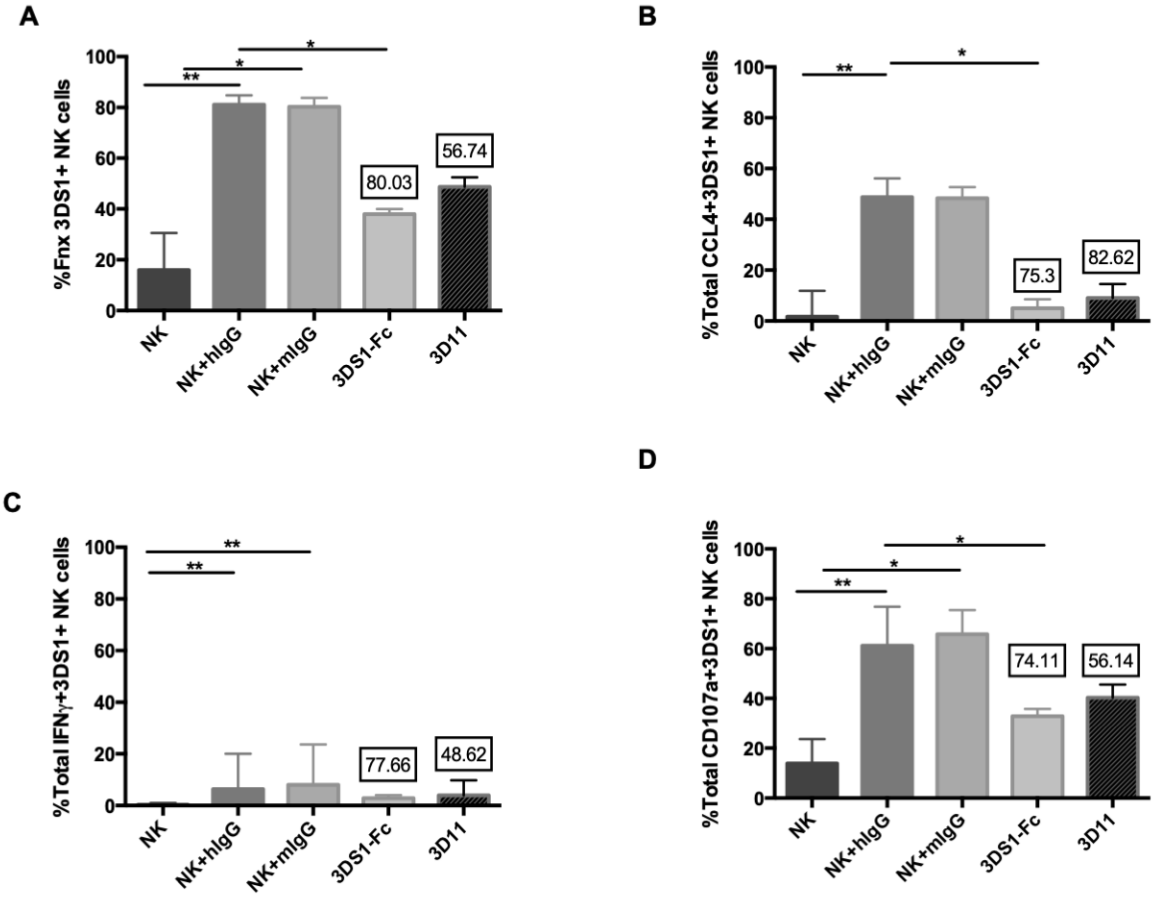

Fig 6. Blocking KIR3DS1 on NK cells suppresses the activation of KIR3DS1$^+$ NK cells stimulated by sorted, HIV-infected CD4$^-$ (siCD4$^-$) cells. Shown on the y-axis is the frequency of exclusively gated KIR3DS1$^+$ NK cells exhibiting (A) the sum of all functions tested (% Total Fxn NK cells), (B) the sum of cells secreting CCL4 (% Total CCL4$^+$ NK cells), (C) the sum of cells secreting IFN-γ (% Total IFN-γ$^+$ NK cells), and (D) the sum of cells expressing CD107a (% Total CD107a$^+$ NK cells) following no stimulation (NK), stimulation with siCD4$^-$ cells pre-coated with human IgG1 (hIgG1) or mouse IgG1 (mIgG1) isotype controls, KIR3DS1-Fc chimeric protein (3DS1-Fc) and monoclonal antibody 3D11 (3D11). Bar heights and error bars

represent the median and interquartile range. The significance of between-group results was assessed using Friedman tests with Dunn post-tests. The boxes over the 3DS1-Fc and 3D11 blocking conditions show the percentage of reduction in the frequency of functional cells from that seen for cells stimulated with siCD4^{-} cells under negative control blocking conditions. Statistically significant between-group comparisons are shown by a line linking groups being compared. Significant between-group differences are indicated as, * = $p \leq 0.05$, ** = $p \leq 0.01$.

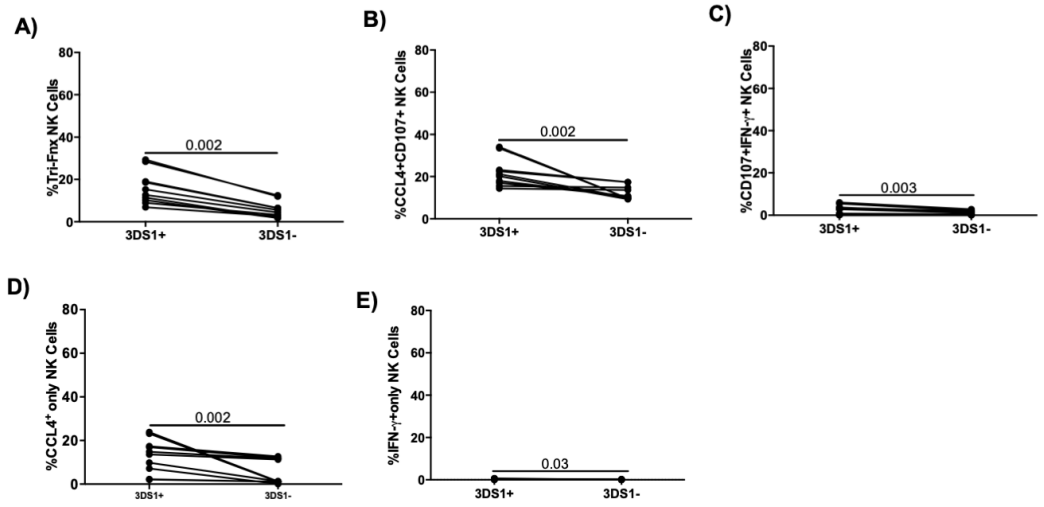

Fig. S1. A higher frequency of inclusively gated KIR3DS1$^+$ than KIR3DS1$^-$ NK cells responded to stimulation with sorted, HIV-infected CD4$^-$ cells with tri-functional, 2 bi-functional and 2 mono-functional profiles. Shown on the y-axis is the frequency of inclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ cells positive for (A) CCL4$^+$CD107a$^+$IFN-γ^+ (% Tri-Fxn NK cells), (B) CCL4$^+$CD107a$^+$, (C) CCL4$^+$IFN-γ^+, (D) CCL4$^+$ only and (E) IFN-γ^+ only NK cells. The significance of between-group differences was assessed using Wilcoxon matched pairs tests. P-values for comparisons between inclusively gated KIR3DS1$^+$ and KIR3DS1$^-$ NK cells are shown over the lines linking the 2 populations being compared.

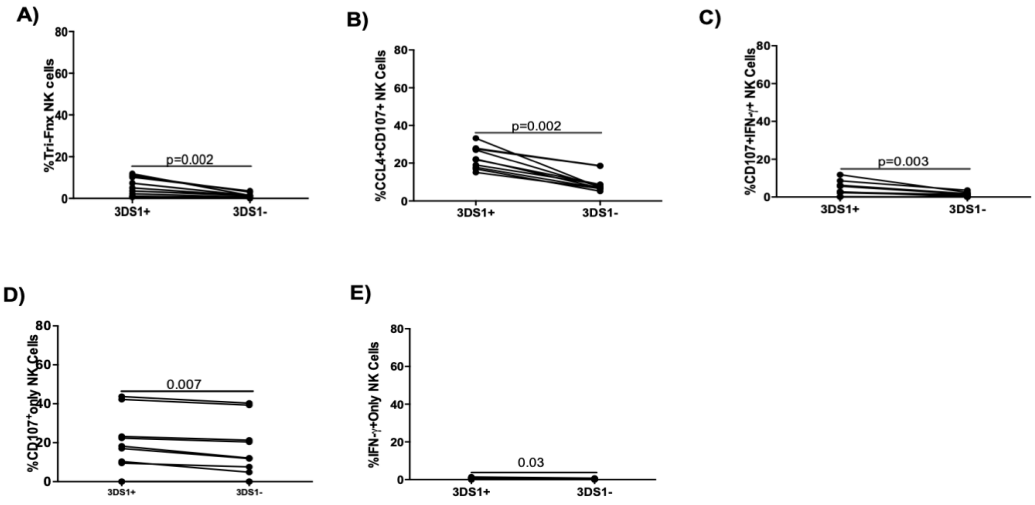

Fig S2. A higher frequency of exclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells responded to stimulation with sorted HIV-infected CD4^{-} cells with tri-functional, 2 bi-functional and 2 mono-functional profiles. Shown on the y-axis is the frequency of exclusively gated KIR3DS1^{+} and KIR3DS1^{-} cells positive for (A) CCL4^{+}CD107a^{+}IFN-γ^{+} (% Tri-Fxn NK cells), (B) CCL4^{+}CD107a^{+}, (C) CCL4^{+}IFN-γ^{+}, (D) CCL4^{+} only and (E) IFN-γ^{+} only NK cells. The significance of between-group differences was assessed using Wilcoxon matched pairs tests. P-values for comparisons between exclusively gated KIR3DS1^{+} and KIR3DS1^{-} NK cells are shown over the lines linking the 2 populations being compared.

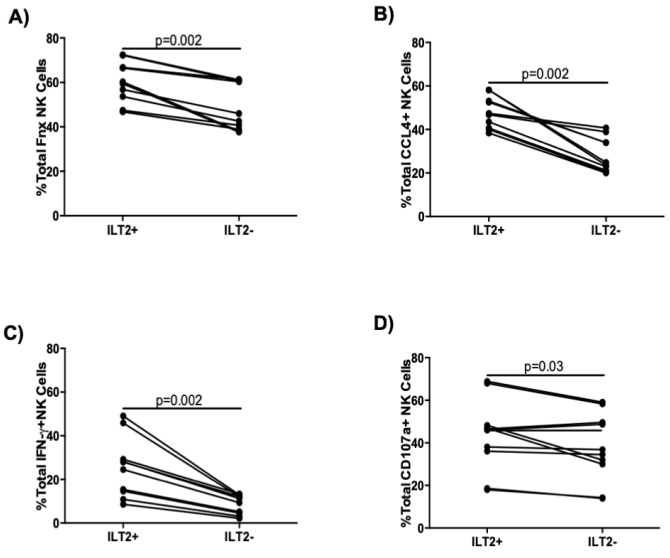

Fig S3. Comparisons of the frequency of functional ILT2^{+} versus ILT2^{-} cells following stimulation with sorted, HIV infected CD4^{-} cells. Shown on the y-axis is the frequency of exclusively gated ILT2^{+} and ILT2^{-} NK cells exhibiting (A) the sum of all functions tested (% Total Fxn NK cells), (B) the sum of cells secreting CCL4 (% Total CCL4^{+} NK cells), (C) the sum of cells secreting IFN-γ (% Total IFN-γ^{+} NK cells) and (D) the sum of cells expressing CD107a (% Total CD107a^{+} NK cells). The significance of between-group results was assessed using Wilcoxon matched pairs tests. P-values for comparisons between exclusively gated ILT2^{+} and ILT2^{-} NK cells are shown over the lines linking the 2 populations being compared.

Table 1. Antibodies

Antibodies	Supplier	Clone	Color
Anti-CD3	Biolegend	OKT3	BV785
Anti-CD56	Biolegend	HCD56	BV711
Anti-CD158e1/e2	Beckman Coulter	Z27.3.7	PE
Anti-2DL1/S1/S3/S5	Biolegend	HP-MA4	FITC
Anti-NKG2A	Miltenyi Biotech	REA110	FITC
Anti-2DL1/L2/L3	BD Biosciences	CH-L	FITC
Anti-3DL2	R&D systems	539304	FITC
Anti-ILT2	Biolegend	GHI/75	PC7
Viability dye	Invitrogen	N/A	Blue
Anti-CCL4	BD Biosciences	D21-1351	AF700
Anti-IFN-γ	Biolegend	B27	BV510
Anti-CD107a	Biolegend	H4A3	BV421
Purified anti-Human HLA-F	Kind gift from Dr.Geraghty	3D11	N/A
F(ab')2 goat anti-mouseIgG	eBioscience	polyclonal	PE
KIR3DS1-Fc Chimeric protein	R&D Systems	N/A	N/A
Anti-CD3	Miltenyi Biotech	REA613	APC
Anti-CD4	Miltenyi Biotech	REA623	VioBlue
Anti-CD8	Miltenyi Biotech	REA734	PE-Vio770

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CHAPTER 4

Education of NK cells single positive for inhibitory killer immunoglobulin receptors determines responsiveness to autologous HIV infected $CD4^+$ T cells.

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Author contributions:

Z.K. designed the study, performed the experiments and data analysis, and prepared the chapter; F.P.D. performed some of the optimization experiments, J.B. and B.L. provided clinical samples; C.R provided reagents; D.E.G. performed KIR region gene typing and allotyping; N.F.B designed the study, participated in data analysis and edited the chapter.

RATIONALE AND RESEARCH OBJECTIVES

Our results from previous chapters demonstrated that KIR3DS1^{+} NK cells respond to autologous HIV infected cells through their interaction with HLA-F present on the surface of HLA-null and HIV-infected target cells. Epidemiological and functional studies showed that the carriage of other *KIR/HLA* combinations encoding NKR ligand pairs was protective against HIV infection and slowed the rate of HIV progression. In chapter 4 of this thesis I studied the anti-HIV responsiveness of educated versus uneducated single positive KIR2DL1^{+}, KIR2DL2^{+} KIR2DL3^{+} and KIR3DL1^{+} NK cells.

Publication status

This work is under revision for publication

ABSTRACT

There are several studies showing the importance of specific KIR/HLA combinations in protection from HIV infection and slower time to AIDS. NK cells acquire their effector functions through a process called "education" requiring the interaction of inhibitory NK cell receptors (iNKRs) with their HLA class I cognate ligands. HLA-C allotypes are ligands for inhibitory Killer Immunoglobulin-like Receptors (iKIRs) KIR2DL1, KIR2DL2 and KIR2DL3 whereas KIR3DL1 interacts with HLA-Bw4. HIV reduces the expression of cell surface HLA-A, B and C on HIV infected CD4 T cells (iCD4). Here we investigated whether education through iKIR/HLA influences NK responses against autologous iCD4. Enriched NK cells were stimulated with autologous iCD4 for 6 hours. We then assessed the frequency of NK cells that were single positive (sp) for KIR2DL1, KIR2DL2, KIR2DL3 or KIR3DL1 for all combinations of CCL4, CD107a and IFN-γ responses. A higher frequency of educated, than uneducated, spKIR2DL1$^+$ NK cells responded to iCD4 by secreting CCL4 and IFN-γ. A higher frequency of educated, than uneducated, spKIR2DL2$^+$ and spKIR2DL3$^+$ NK cells responded to autologous iCD4 by producing CCL4. We also found that the density of both KIR3DL1 and Bw4 ligands and their affinity for each other influenced the frequency of spKIR3DL1$^+$ NK cells responding to iCD4. Both NK cell education and HIV mediated changes in HLA expression influence NK responses to HIV.

INTRODUCTION

Natural Killer (NK) cells are components of the innate immune system with important roles in anti-viral and anti-tumor responses (1). They can respond to viruses at early stages of infection by secreting cytokines and chemokines and by degranulating, which can result in target cell killing (1-4). NK cell functional status depends on the integration of signals received from inhibitory and activating NK receptors (NKRs) (5). Among the NKRs, Killer-Immunoglobulin (Ig)-like receptors (KIRs) are a large family of receptors expressed on NK cells and other lymphocyte subsets (6). The ligands for KIRs are HLA class I, which are expressed on all nucleated cells. Binding of inhibitory KIRs (iKIRs) to their HLA ligands generate inhibitory signals that prevent NK cells from responding to healthy HLA expressing self-cells. NK cells acquire their effector functions through a process called "education". NK cell education occurs when subsets of NK cells bearing iKIRs bind to self HLA molecules during their development. These educated NK cells are then primed for activation when they encounter target cells with reduced levels of HLA ligands due to virus infection or tumor transformation. The $iKIR^+$ NK subsets unable to interact with self HLA during development remain uneducated and are hyporesponsive to target cell stimulation (7-12).

KIR and HLA genes are highly polymorphic and map to different chromosomes. Consequently, possibly due to allelic variation in both KIRs and HLA molecules, a person can have both educated, uneducated NK cells and also can have NK cells with varied levels of education and responses to stimulation (13-16).

KIR2DL1, KIR2DL2 and KIR2DL3 recognize HLA-C allotypes. HLA-C molecules are divided into two groups, C1 and C2. C1 allotypes have an asparagine at position 80 of their heavy chain and are ligands for KIR2DL3. C2 group allotypes have a lysine at this position and are ligands for KIR2DL1 and KIR2DS1 receptors (14, 17, 18). KIR2DL2 is an intermediate receptor that also binds C1 allotypes. However, based on cell-free assays, KIR2DL2 was also shown to recognize some C2 allotypes (14, 19).

KIR haplotypes can be divided into haplotypes A and B, based on KIR gene content. KIR haplotype A contains mostly iKIRs while KIR B haplotypes include varying numbers of activating KIRs (aKIRs) in addition to the genes present in KIR haplotype A. The alleles encoding KIR2DL2 and KIR2DL3 receptors are alleles at the same locus; KIR2DL2 is present in KIR haplotype Bs while KIR2DL3 maps to KIR haplotype A. KIR2DL1 receptors are encoded by a separate KIR locus present in both haplotypes A and B. In general, KIR2DL1^{+} NK cells are educated through binding of KIR2DL1 to C2 whereas KIR2DL2^{+} and KIR2DL3^{+} NK cells are educated through binding of these receptors to C1 ligands. In KIR haplotype A, KIR2DL1 allotypes have a high affinity for C2 subtypes while KIR2DL3 allotypes bind weakly to C1 variants (13). In KIR B haplotypes, KIR2DL1 allotypes bind C2 isoforms weakly while KIR2DL2 allotypes have a high affinity for C1 ligands (13).

KIR3DL1 is another iKIR recognizing a subset of HLA-A and –B antigens containing Bw4 motifs (20-22). Allotypes belonging to the Bw4 group differ from the remaining HLA-Bw6 variants (Bw6) at amino acids 77-83 of the HLA heavy chain (23). There is a dimorphism at position 80 in Bw4 allotypes. Presence of an isoleucine (80I) or a threonine (80T) at this position

influences KIR3DL1 binding affinity for its ligands. In general, Bw4*80I allotypes have a stronger affinity than Bw4*80T subtypes for KIR3DL1 receptors leading to more potent education and responsiveness (24). Bw6 allotypes do not interact with KIR3DL1. Thus, KIR3DL1^{+} NK cells from donors lacking Bw4 allotypes remain uneducated through this receptor. KIR genes are highly polymorphic. These polymorphisms influence their cell surface expression, avidity and specificity for HLA ligands. *Boudreau et al.* showed that the expression levels of both the KIR3DL1 receptor and Bw4 ligand and the strength of binding of the receptor to its ligand predict NK education and responses to HLA null cells and autologous HIV infected cells (24). So far, 77 allotypes have been identified for KIR3DL1, which are categorized into four groups based on their surface expression. These include KIR3DS1, KIR3DL1-null allotypes (null) with no detectable cell surface expression, KIR3DL1-low (low) and KIR3DL1-high (high) allotype groups (25-27). KIR3DL1-low and –high allotypes bind to Bw4 subtypes with various affinities. KIR3DS1 does not interact with Bw4 molecules, with one reported exception (24, 28). The KIR3DL1*004 null allotype may contribute to NK cell education despite it not being expressed on the cell surface (29-31). *Martin et al.* reported that the presence of the homozygous *KIR3DL1* genotype encoding at least 1 KIR3DL1-high allotype (KIR3DL1*h/*y) co-carried with *HLA-B*57*, which encodes a Bw4*80I isoform (**h/*y+B*57*) was associated with slower time to AIDS and lower HIV viral load in infected individuals compared to carriers of the receptor or ligand alone or neither (32). NK cells expressing combinations of KIR3DL1-high with Bw4*80I allotypes, such as HLA-B*51, –B*52 and B*57 have higher functional capacity to produce IFN-γ when stimulated with HLA null cells (33-37).

HIV infection downmodulates the expression of HLA-A, B and C (38, 39). Therefore, it is expected that iKIR$^+$ NK cells educated through these HLA molecules will have higher responses to autologous HIV infected CD4 T (iCD4) cells than their uneducated counterparts. We tested this hypothesis by stimulating NK cells with autologous iCD4 and examining the induction of CCL4, CD107a and IFN-γ functions in educated versus uneducated single positive (sp) KIR2DL1$^+$, KIR2DL2$^+$, KIR2DL3$^+$ and KIR3DL1$^+$ NK cells. We showed that educated spKIR2DL1$^+$, spKIR2DL2$^+$ and spKIR2DL3$^+$ NK cells exhibited higher responses to iCD4 cells than their uneducated counterparts, likely due to reduced levels of HLA-C on iCD4 cells. Expression levels of both KIR3DL1 and its Bw4 ligand and receptor/ligand binding affinity both influenced spKIR3DL1$^+$ NK cell education potency and responsiveness.

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 Researche du Centre Hospitalier de I'Université de Montreal and the Research Ethics Board of the McGill University Health Center. All subjects were healthy adults who provided written informed consent for the specimen collection and subsequent analyses.

Study population

Table 1 a, b, c and d shows the HLA and KIR genotypes and allotypes of all subjects included in this study. All were healthy HIV-negative individuals recruited from the St. Luc cohort in Montreal QC, Canada.

Genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) or from Epstein-Barr virus (EBV)-transformed cells using QIAamp DNA Blood Mini Kits (Qiagen, Mississauga, ON, Canada) as per manufacturer's instructions. HLA-I alleles were typed by sequencing using commercial reagents (Atria Genetic Inc., South San Francisco, CA). KIR gene content and allele typing was carried using the ScisGo KIR v3 typing kit (Scisco Genetics Inc., Seattle, WA) according to the manufacturers protocol. Briefly, the method, modified from the approach described in *Nelson et al*. (40), employs an amplicon-based 2-stage PCR, followed by sample pooling and sequencing using a MiSeq v2 PE500 (Illumina, San Diego, CA). Results were assembled using custom software supplied as an adjunct to the ScisGo KIR v3 typing kit.

Cells

PBMCs were isolated from leukaphoresis samples by density gradient centrifugation (Lymphocytes Separation Medium, Wisent Bioproducts, St-Bruno, QC, Canada) and cryopreserved in 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO); 90% fetal bovine serum (FBS, Wisent). CD4 and NK cells were isolated from frozen PBMCs by negative selection using EasySep^{TM} human CD4^{+} T cell isolation kits and EasySep^{TM} human NK cell enrichment kits for CD4 and NK isolation, respectively (Stemcell Technologies, Inc., Vancouver, BC,

Canada). Purity was verified by flow cytometry and was an average of 99.1% and 96.3% for CD4 and NK cells, respectively.

Preparation of HIV infected CD4 T cell stimulators

Isolated autologous CD4 cells were stimulated with ImmunoCult Human CD3/CD28 T cells activator and 100 international units (IU)/ml of recombinant human Interleukin-2 (hrIL-2) in RPMI 1640 medium; 10% FBS; 2 mM L-glutamine; 100IU/ml penicillin and 100mg/ml Streptomycin (R10-100) for 4 days at 37ºC in a humidified 5% CO_2 incubator. On day 4, stimulated $CD4^+$ T cells were infected with HIV-$1_{JR\text{-}CSF\text{-}VSVG}$ in R10-100 for 4 hrs. These HIV infected CD4 cells will henceforth be called iCD4. Next, cells were washed and cultured for 3 days in R10-100. Uninfected CD4 (CD4) were cultured in parallel with iCD4 for 3 days in R10-100. On day 3, both CD4 and iCD4 cells were used to stimulate NK cells.

Detection of HLA-A, -B and C on the surface of CD4 cells

Expression levels of HLA-A, B and C were measured on CD4 and iCD4 by using the monoclonal antibody (mAb), W6/32, a pan-HLA-specific Ab, which also recognizes HLA-E and DT-9, and Ab specific for HLA-C. Both CD4 and iCD4 were stained with UV Live/Dead fixable dead cell stain kit as per manufacturer's directions, followed by surface staining using fluorochrome conjugated Abs: CD3-BV785 (OKT3-Biolegend), CD4-BV421 (OKT4-Biolegend), CD8-PC7 (SK1-Biolegend), HLA-A/B/C-FITC (W6/32-Biolegend), HLA-Bw4-APC-Vio770 (REA274, Miltenyi) and unconjugated HLA-C Ab (DT-9-EMD Millipore) in separate wells. After surface staining, cells were washed, fixed with 2% paraformaldehyde (PFA) and permeabilized for intracellular staining (ICS) for HIV Gag p24, using anti-p24-PE

(KC57-Beckman Coulter). DT-9 binding was detected by adding BV605 goat anti-mouse secondary Ab (Biolegend) to washed CD4 and iCD4 cells. Cells were then fixed and permeabilized before ICS with anti- p24 specific PE. For both W6/32 and DT-9 staining, cells were washed and resuspended in 2% PFA after ICS till acquisition within 1 hour (hr). $1x10^6$ to $1.4x10^6$ cells were acquired using an LSRFortessa X20 flowcytometer (BD Biosciences). We measured the mean fluorescent intensity (MFI) of HLA-A/B/C and HLA-C as well as the frequency of cells positive for p24 in both CD4 and iCD4 cells.

NK cell stimulation and staining for flow cytometry

$1x10^6$ overnight rested NK effector (E) cells were co-cultured with either CD4 or iCD4 cells as target cells (T) at ratio of 1:1 (E:T) in R10 for 6 hrs at 37ºC in a humidified 5% CO_2 incubator. Brefeldin A (Sigma-Aldrich, ON, Canada) (24 ug/ml) and monensin (BD Biosciences, Mississauga, ON, Canada) (3 ug/ml, GolgiStop) were added 1 hr after the initiation of the co-culture. NK cells cultured in R10 alone served as an unstimulated negative control. An aliquot of NK cells from each study subject was tested for their ability to respond to 5.8 ug/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, ON, Canada) and 1.2 ug/ml Ionomycin (Sigma-Aldrich, ON, Canada) stimulation. All cells were responsive to this positive control stimulus. After the 6 hr stimulation, cells were sequentially stained for 1) viability using UV Live/Dead staining (Thermofisher, Massachusetts, US) and surface-stained with Abs to CD3-BV785 (OKT3-Biolegend), CD14-BV785 (M5E2-Biolegend), CD19-BV785 (HIB19-Biolegend), CD56-BV605 (HDC56-Biolegend), KIR2DL1-APC-vio770 (REA284-Miltenyi Biotech), KIR2DL3-FITC (180701-R&D systems), KIR2DS2/L3-Alexa Fluor 647 (1F12, a kind gift from Christelle Retière, Établissement Français du Sang, Nantes, France), KIR3DL2-Alexa

Fluor 594 (DX31), KIR3DL1/S1-PE (REA168-Miltenyi Biotech), NKG2C-PE-vio770 (REA205-Miltenyi Biotech), CD107a-BV711 (H4A3-Biolegend) and NKG2A-Biotin (REA110-Miltenyi Biotech) for 30 min at 4º C. 2) Abs to KIR2DL2/L3/S2-Percp-Cy5.5 (DX27-Biolegend) and KIR2DL1/S1-Vio-Blue (REA1010-Miltenyi Biotech) for 15 min at 4º C. Next, cells were washed and incubated with 3) Qdot 655 streptavidin conjugate for 20 min at 4º C. After surface staining, cells were washed and fixed with 2% PFA followed by permeabilization and ICS with anti-CCL4-AF700 (D21-1351-BD Biosciences) and anti-IFN-γ-BV510 (B27-BD Biosciences). Samples were then washed twice and fixed with 2% PFA and acquired within 1 hour.

Flow cytometry analysis

Between 1.5 x 10^6 and 1.8 x 10^6 total events were acquired for each sample using an LSR Fortessa X-20 flow cytometer (BD Biosciences). Results were analyzed using FlowJo software version 10.3 (Treestar, Ashland, OR). Single stained beads (Comp Beads; BD) were used to apply compensation. We used the gating strategy shown in Figure 1 to gate on spiKIRs$^+$ NK cells. We measured the frequency of NK cells exhibiting the sum of the frequencies of all functions tested (total) and the sum of the frequencies of each functional subset secreting CCL4 (total CCL4), secreting IFN-γ (total IFN-γ) and expressing CD107a (total CD107a). These functional subsets were assessed for spKIR2DL1, spKIR2DL2, spKIR2DL3 and spKIR3DL1 NK cell populations. The data presented were background subtracted using results for the matched CD4 control condition.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used for data analysis and graphical presentation. We used Mann-Whitney test to compare difference between two unmatched groups and Kruskal-Wallis tests for comparisons of more than 2 unmatched groups.

RESULTS

HIV infection is associated with downmodulation of HLA-C on iCD4 cells

HLA-A, B and C expression is downmodulated on HIV infected CD4 cells (20, 38, 41). However, HIV dependent downmodulation of HLA-C is virus strain dependent (41-43). To investigate whether the $HIV_{JRCSF\text{-}VSVG}$ virus strain, downmodulated HLA-C expression on iCD4, we stained both CD4 and iCD4 with HLA-C-specific mAb DT-9. As shown in Figure 2b, c, iCD4 cells expressed HLA-C with a significantly lower MFI that did CD4 cells. Staining iCD4 and CD4 cells with the pan-HLA-specific mAb W6/32 confirmed that the MFI of HLA-A, B and C expression was significantly lower on iCD4 than on CD4 cells (Figure 2 b, d).

A higher frequency of educated, than uneducated, spKIR2DL1$^+$ NK cells responded to iCD4 cells

We next tested whether reduced HLA-C expression on iCD4 was enough to abrogate inhibitory signals through KIR2DL1/HLA-C2 interactions. Responses of spKIR2DL1$^+$ NK cells from subjects carrying at least one *C2* allele (educated) versus no *C2* alleles (uneducated) to iCD4 were compared. Figure 1a shows the strategy used to gate on spKIR2DL1$^+$ NK cells. Figure 3 shows that a significantly higher frequency of educated, than uneducated, spKIR2DL1$^+$ NK cells

responded to iCD4 stimulation for the sum of all functions tested, total CCL4 and total IFN-γ production (Figure 3a-c, p=0.002 for both sum of all functions and total CCL4, p=0.01 for total IFN-γ secretion, Mann-Whitney tests). There was a trend towards higher CD107a expression for educated versus uneducated spKIR2DL1$^+$ NK cells that did not achieve statistical significance (Figure 3d, p=0.13, Mann-Whitney test). These results confirmed that HLA-C is downmodulated on iCD4 cells enough to interrupt inhibitory signals mediated by KIR2DL1/C2 interactions and reverse inhibition of educated spKIR2DL1$^+$ NK cells.

A higher frequency of educated, than uneducated, spKIR2DL2$^+$ and spKIR2DL3$^+$ NK cells responded to iCD4 cells

We next investigated whether HLA-C downmodulation abrogated the inhibitory signals received by KIR2DL2 and KIR2DL3 interacting with HLA-C1 molecules on iCD4 cells. To address this, we classified subjects into those who were educated and uneducated through there receptors based whether they carried at least one *C1* allele (C1/C1 and C1/C2) versus no *C1* alleles (C2/C2 homozygotes), respectively. There are no KIR2DL2 specific Abs available so we used the gating strategy describe in Figure 1b to gate on spKIR2DL2 and spKIR2DL3 NK cells. Figure 4 shows that a significantly higher frequency of educated, than uneducated, spKIR2DL2$^+$ NK cells responded to iCD4 stimulation for the sum of all functions tested and for total CCL4 (p=0.0008 for both, Mann-Whitney test) (Figure 4a, b). We observed a trend towards a higher frequency of IFN-γ secreting and CD107a expressing educated versus uneducated spKIR2DL2$^+$ NK cells that did not achieve statistical significance (p= 0.14 and p=0.18 for IFN-γ and CD107, respectively. Mann-Whitney tests, Figure 4c, d).

Fig. 5 shows that a significantly higher frequency of educated, than uneducated, spKIR2DL3$^+$ NK cells responded to iCD4 stimulation for the sum of all functions tested and total CCL4 secretion (p=0.005 and p=0.0004, respectively, Mann-Whitney tests, Figure 5a, b). We observed no significant differences in the frequency of IFN-γ secreting and CD107a expressing educated versus uneducated spKIR2DL3$^+$ NK cells (p=0.37 and p=0.91 for IFN-γ and CD107a, respectively. Mann-Whitney tests) (Figure 5c, d).

We also questioned whether stronger binding between KIR2DL2 and C1 than between KIR2DL3 and C1 allotypes led to a higher level of education and iCD4 stimulated function of NK cells from *KIR2DL2/C1* than *KIR2DL3/C1* carriers. Figure 6 shows that the responsiveness of spKIR2DL2$^+$ NK cells to iCD4 stimulation was significantly higher than that of spKIR2DL3$^+$ NK cells for the sum of all functions and total CCL4 secretion (p=0.03 and p=0.004, respectively, Mann-Whitney tests) (Figure 6a, b). Differences for the responsiveness of KIR2DL2$^+$ and KIR2DL3$^+$ NK cells to iCD4 for total IFN-γ secretion and total CD107a expression did not differ significantly (p=0.19 and p=0.16 for total IFN-γ and CD107a, respectively. Mann-Whitney tests, Figure 6c, d).

Expression of KIR3DL1 allotypes on the surface of NK cells

The *KIR3DL1/S1* locus is the most polymorphic KIR region gene. The study subject KIR3DL1 allotypes are shown in Table 1d. REA168, used to stain KIR3DL1/S1 revealed that KIR3DL1 allotypes are expressed at various mean fluorescence intensities (MFI) ranging from 2897 to 34858 (Figure 7a, b).

Expression of HLA-Bw4 allotypes and their downmodulation on iCD4

Bw4 subtypes have variable expression densities on the surface of PBMCs (24). We confirmed this by staining donor PBMCs with an antibody specific for HLA-Bw4 antigens. Figure 8a shows the MFI of HLA-Bw4 staining of $CD4^+$ T cells from carriers of different *HLA-B* alleles. $CD4^+$ T cells from *Bw4* homozygous donors expressed Bw4 antigens at a higher MFI than *Bw4*/*Bw6* heterozygotes expressing only 1 Bw4 copy. Background levels of staining for Bw4 allotypes was illustrated by staining CD4+ T cells from *Bw6* homozygotes (hmzs) with this Ab (Figure 8b). HIV infection leads to Nef mediated downmodulation of HLA-A and -B. We examined the level of Bw4*80I and Bw4*80T subtype downmodulation on iCD4. For this analysis, we included study subjects that expressed either only Bw4*80I or Bw4*80T antigens. Our results showed that HIV infection led to a higher percent reduction in Bw4*80I than Bw4*80T expression on iCD4 cells (Figure 9a-c).

Variation in KIR3DL1/Bw4 NK cell education potency determines the frequency of KIR3DL1$^+$ NK cell functional responses to iCD4

Bw4*80I molecules have a higher affinity for KIR3DL1 than Bw4*80T subtypes. *Boudreau et al*. demonstrated that KIR3DL1 and HLA-Bw4 expression levels, in addition to their affinity for each other, predict the frequency of NK cells that degranulate (i.e express CD107a) in response to HLA null and HIV infected cells (24). Here, we extended these observations by examining the frequency of $KIR3DL1^+$ NK cells that responded to autologous iCD4 by secreting CCL4 and IFN-γ, in addition to expressing CD107a. We assumed that a greater magnitude of downmodulation of Bw4*80I than Bw4*80T ligands by HIV on iCD4 cells would interrupt inhibitory signals through KIR3DL1/Bw4*80I interactions to a greater extent than

KIR3DL1/Bw4*80T interactions. To address this, study subjects were stratified based on their KIR3DL1 and Bw4 allotypes into 4 different groups: 1) Bw6 hmzs (regardless of KIR3DL1 allotype) as a negative control for KIR3DL1$^+$ cell education, 2) KIR3DL1-low/Bw4*80I, 3) KIR3DL1-high/Bw4*80I and 4) KIR3DL1-high/Bw4*80T. Of all these groups, NK cells from group 4 responded to autologous iCD4 with the highest frequency of cells characterized by the sum of all functions and by CCL4 secretion but not for total IFN-γ secretion or total CD107a expression (Figure 10). We observed a trend towards a higher frequency of NK cells from groups 2 and 3, than from group 4, responding to iCD4 cells for sum of all functions and for total CCL4, though the differences did not achieve statistical significance, probably due to the small sample size.

DISCUSSION

In this work, we studied the functional profiles of sp KIR2DL1$^+$, KIR2DL2$^+$, KIR2DL3$^+$ and KIR3DL1$^+$ NK cells stimulated by autologous iCD4 cells. Educated sp KIR2DL1 responded more potent than their uneducated counterparts by secreting CCL4 and IFN-γ. A higher frequency of educated than uneducated sp KIR2DL2$^+$ and KIR2DL3$^+$ NK cells responded to iCD4 cells by producing CCL4. We demonstrated that the expression level of both KIR3DL1 and their Bw4 ligands and their affinity for each other influenced the frequency of spKIR3DL1$^+$ NK cells responding to iCD4. Altogether, both NK cell education and HIV mediated changes in HLA expression affect NK cell responses to HIV.

Educated NK cells have been reported to play an important role in anti-viral activity, including that to HIV. NK cells acquire their functional potential during a process called education in which iNKRs interact with their HLA-I ligands during NK cell development. NK education is influenced by the expression levels of iNKRs on the surface of NK cells and HLA-I on stimulatory/target cells, the strength of binding between receptor/ligand an also the number of iNKRs to self HLA-I ligands (14, 24, 34, 37, 44). NK cells activation occurs when of inhibitory signals mediated through iNKRs/HLA-I interactions are interrupted as a result of the downmodulation of self HLA-I ligands on HIV infected target cells. Here, we investigated the consequences of education through iKIRs and their ligands on NK cell responsiveness to autologous iCD4 cells.

HIV infection reduces the expression levels of HLA-A, B and C on iCD4 cells. *Apps et al*. showed that HLA-C expression is decreased on iCD4 cells by Vpu, an accessory HIV protein (41). *Korner et al*. demonstrated that HIV strains differ in their ability to reduce the expression of HLA-C on iCD4 cells. (42). $HIV_{JRCSF\text{-}VSVG}$, the isolate used here to infect CD4 cells, decreased the cell surface expression of both HLA-C1 and C2 subtypes on iCD4 cells to an average MFI of 19,547 from 27,114 on uninfected CD4 cells.

The frequency of functional educated spKIR2DL1$^+$ NK cells from donors carrying at least one copy of a *C2* allele responding to autologous iCD4 was higher than that of their uneducated counterparts from C1/C1 hmzs. The expression of the KIR2DL1 ligand, C2, was reduced on iCD4 cells, compared to that uninfected CD4$^+$ T cells. This C2 downmodulation relieved inhibitory signals mediated by KIR2DL1/C2 interactions enough to shift the balance of NK cells

inhibitory and activating signals towards spKIR2DL1$^+$ NK cells activation in response to iCD4 stimulation. The gating strategy we used enabled us to gate on KIR2DL1$^+$ NK cells exclusively, thus reducing the effect of signals received through other i/aNKRs. Together, these results demonstrate that NK cells responses to HIV not only depend on education of NK cells via iKIR/HLA interactions but also on changes in HLA expression on iCD4 cells governed by HIV.

The frequency of spKIR2DL2$^+$ NK cells from donors carrying at least one allele encoding C1 group antigens responding to iCD4 stimulation were higher than that of C2 hmzs. C1 allotypes have a relatively high affinity for KIR2DL2 and their interaction educate KIR2DL2$^+$ NK cells. HIV infection downmodulates C1 ligands on autologous iCD4 cells, reducing inhibitory signals generated through KIR2DL2/C1 interactions. This leads to educated spKIR2DL2$^+$ NK cell activation. SpKIR2DL2$^+$ NK cells from *C2* hmzs remain uneducated and hyporesponsive to autologous iCD4. *Moesta et al*. reported that KIR2DL2 and KIR2DL3 are less specific for C1 than KIR2DL1 for C2 subtypes (14). They showed that KIR2DL2-Fc and KIR2DL3-Fc fusion proteins also bound to some single HLA-C2 allotype coated beads. The highest level of binding was to HLA-C*05:01 and HLA-02:02 allotypes. However, we did not find that KIR2DL2/L3$^+$ NK cells from carriers of these promiscuous *C2* subtypes had higher responses to autologous iCD4 than when they originated from carriers of non-promiscuous *C2* subtypes. Our results may differ from those predicted by the findings of *Moesta et al*. for several reasons First, *Moesta et al*. measured cytotoxicity using a functional assay in which NK clones, transduced with KIR2DL2 and KIR2DL3, were co-cultured and the 721.221 cell line transfected with a panel of C2 allotypes that did not include HLA-C*05:01 and HLA-C*02:02 (14). At this time, we do not know whether KIR2DL2$^+$ and KIR3DL3$^+$ NK clones would kill 721.221 cells transfected with

these higher affinity C2 ligands. The read out for the functional assays we performed was measurement of the frequency of NK cells positive for CCL4, CD107a and IFN-γ. Our experiments were performed using primary NK cells and autologous iCD4 rather than NK clones and 721.221 cells as effector and target cells, respectively. *Alter et al.* showed that the presence of HIV peptide could influence KIR/HLA binding and shift responses from inhibition to activation or *vice versa* (45). With respect to this finding, we used autologous iCD4 as stimulatory cells whereas *Moesta et al.* showed binding between KIR2DL2/L3 and C2 allotypes in the absence of HIV (14). We used an Ab panel and a gating strategy that allowed us to assess the functions of exclusively gated KIR2DL2$^+$ primary NK cells stimulated with autologous iCD4. Work by others using either KIR2DL2 transduced NK clones or inclusively gated KIR2DL2 NK cells co-expressing other NKR demonstrated that NKR other than KIR2DL2 can affect the education of these KIR2DL2$^+$ NK cells. KIR2DS2 is particularly important to consider in this regard. KIR2DS2 is in a close linkage disequilibrium (LD) with KIR2DL2 such that all carriers of *KIR2DL2* also carry *KIR2DS2* genes. The gating strategy we used in this report to isolate and study, spKIR2DL2$^+$ NK cell responses to iCD4 allowed us to eliminate the possibility that these responses were due to co-expression of other NKR co-expressed on this subset of NK cells.

The frequency of educated KIR2DL3$^+$ NK cells from donors carrying at least one *C1* group allele responding to iCD4 was higher than those from uneducated KIR2DL3$^+$ NK cells from individuals lacking C1 allotypes. HLA-C1 downmodulation on iCD4 abrogates the inhibitory signals through KIR2DL3/C1 pairs shifting the balance of signals received towards activation of educated NK cells by autologous iCD4 cell stimulation. In contrast, KIR2DL3$^+$ NK cells from

C2/C2 hmzs were hyporesponsive to iCD4 stimulation. The frequency of iCD4 induced functional KIR2DL3^{+} NK cells from donors positive for promiscuous C2 allotypes able to bind KIR2DL3 was not higher than when from individuals with non-promiscuous C2 isotypes (14). The reasons for this likely include those discussed above but also likely include the lower affinity of KIR2DL3 than KIR2DL2 receptors for their ligands due to substitutions/mutations in D1 and D2 domains of these two receptors.

There are several studies that show the importance of specific KIR3DL1/HLA-B allotype combinations in protection from HIV infection and slower time to AIDS (32, 46-49). *Martin et al*. demonstrated the role of highly educated KIR3DL1-high/Bw4*80I subtype combinations in delayed progression to AIDS (32). *Kim et al*. showed that Bw4 copy number influenced the responsiveness of KIR3DL1^{+} NK cells (50). *Boudreau et al*. demonstrated that various KIR3DL1/HLA-B subtype pairs degranulated differentially in response to HLA null and autologous iCD4 stimulation (24). Both receptor/ligand pair affinity and cell surface density of receptors and ligands contributed in NK responsiveness.

Our results are in line with those reported by *Boudreau et al*. with some differences. We observed that NK cells from carriers of the KIR3DL1-high/Bw4*80I combination had the highest frequency of NK cells with anti-HIV functions of the four groups tested due to this combination having the highest receptor ligand binding affinity and expression density. As expected, the functionality of NK cells from *Bw6* hmz donors was the lowest, given that Bw6 is not a ligand for KIR3DL1. Of note, when either KIR3DL1 or HLA-Bw4 are expressed at lower

densities, lower NK cell anti-HIV responsiveness ensues. In our hands, stronger binding between KIR3DL1-low and Bw4*80I did not compensate for the lower density of receptor expression.

In conclusion, we demonstrated the impact of education through iKIR/HLA pairs on NK cell responsiveness to autologous iCD4. The higher frequency of educated NK cells responding to iCD4 cells by secreting CCL4 in all cases and for CCL4 and IFN-γ when inhibition through the KIR2DL1 receptor is relieved, highlights the potential role of educated NK cells in viral control by inducing the production of CC-chemokines, which compete with HIV to bind to the CCR5, co-receptor for HIV entry and by eliciting IFN-γ, an important antiviral cytokine (36, 51).

FIGURES AND LEGENDS

Figure 1a

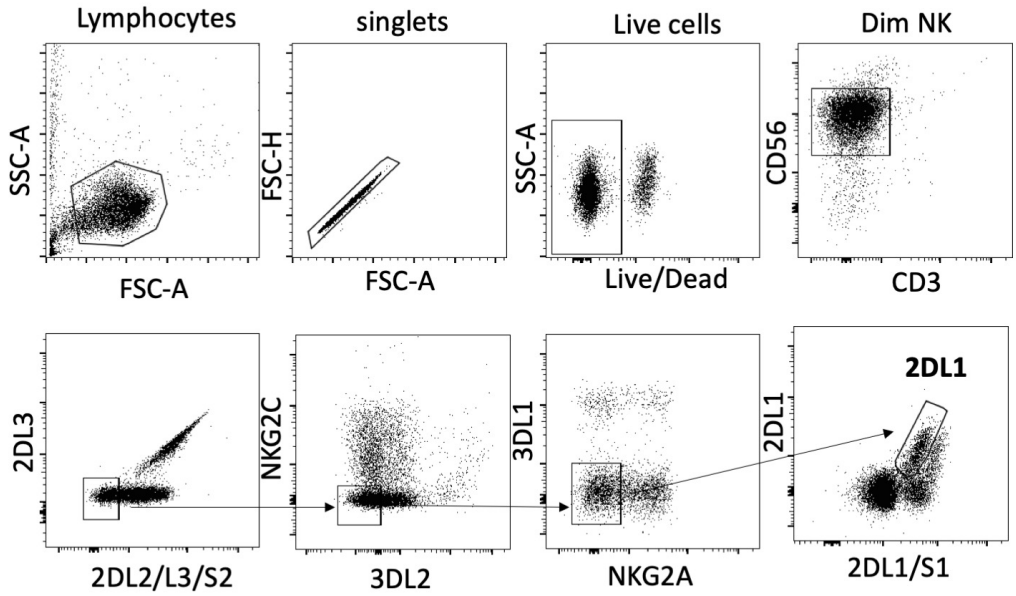

Figure 1a. Gating strategy used to gate on single positive (sp) KIR2DL1^{+} NK cells. Live, singlet lymphocytes were gated on from which CD3^{-}CD56^{dim} cells were selected. SpKIR2DL1 NK cells gated from KIR2DL2^{-}KIR2DL3^{-}KIR2DS2^{-}KIR3DL2^{-}NKG2C^{-}NKG2A^{-}KIR3DL1^{-}KIR2DS1^{-} NK cells. SSC-A = side scatter area; FSA-A = forward scatter-area; FSC-H = forward scatter height; 2DL3 = KIR2DL3; 2DL2/L2/S2 = KIR2DL2/KIR2DL3/KIR2DS2; 3DL2 = KIR3DL2; 3DL1 = KIR3DL1; 2DL1 = KIR2DL1; 2DL1/S1 = KIR2DL1/KIR2DS1.

Figure 1b

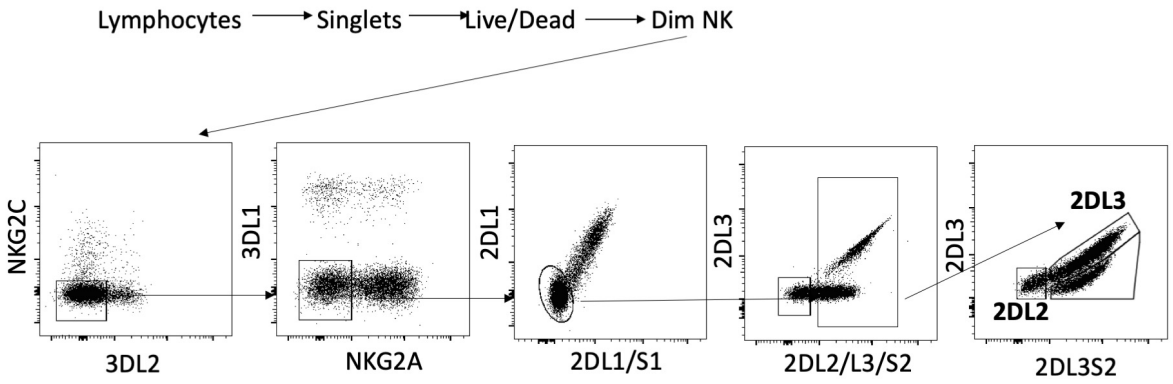

Figure 1b. Gating strategy used to gate on spKIR2DL2 and spKIR2DL3 NK cells. Live, singlet lymphocytes were gated on from which $CD3^{-}CD56^{dim}$ cells were selected. SpKIR2DL2 and spKIR2DL3 NK cells were gated from $NKG2C^{-}KIR3DL2^{-}KIR3DL1^{-}NKG2A^{-}KIR2DL1^{-}KIR2DS1^{-}KIR2DS2^{-}$ NK cells. Abbreviated are the same as for Figure 1a

Figure 1c

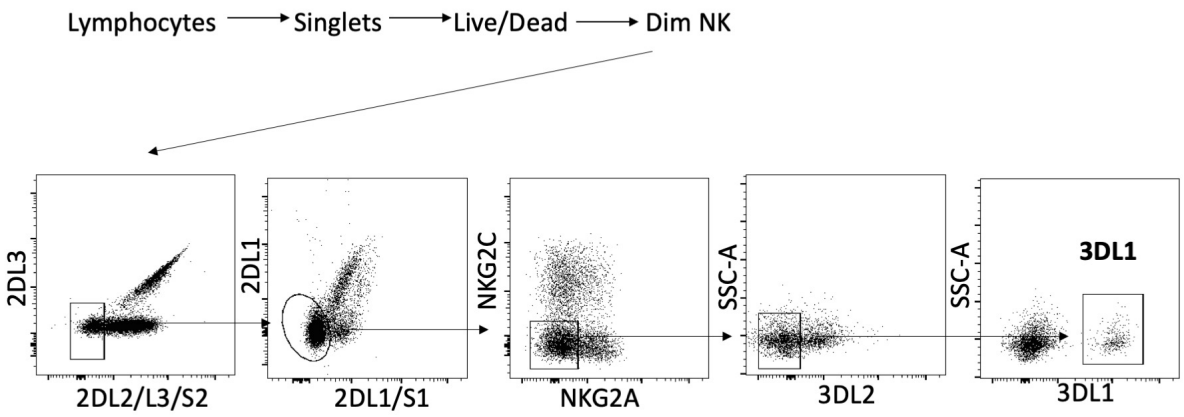

Figure 1c. Gating strategy used to gate on spKIR3DL1 NK cells. Live, singlet lymphocytes were gated on from which $CD3^{-}CD56^{dim}$ cells were selected. SpKIR3DL1 NK cells gated from $KIR2DL2^{-}KIR2DL3^{-}KIR2DS2^{-}KIR2DL1^{-}KIR2DS1^{-}NKG2A^{-}NKG2C^{-}KIR3DL2^{-}$ NK cells. Abbreviated are the same as for Figure 1a

Figure 2

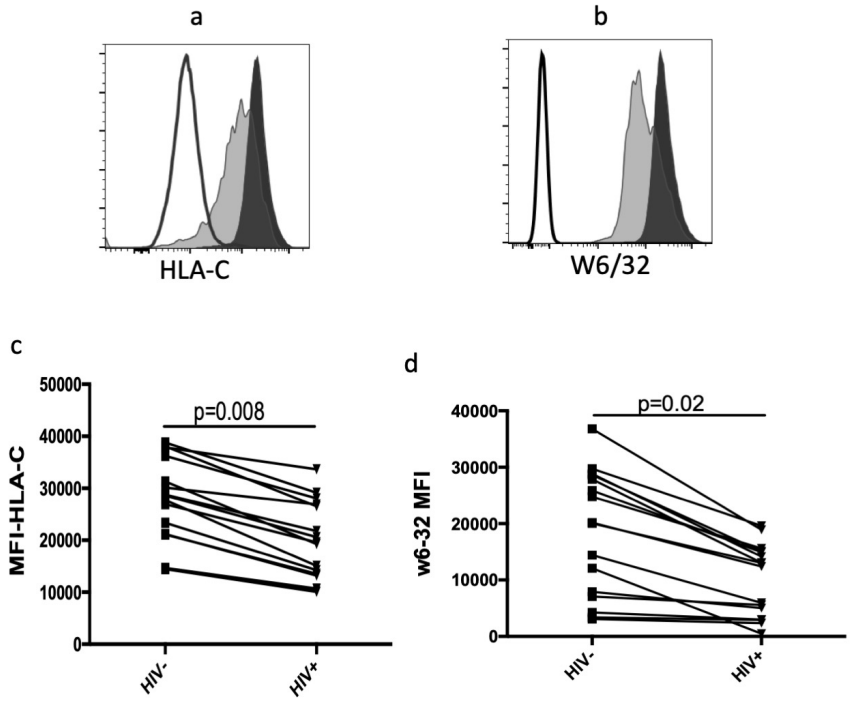

Figure 2. HIV infection downmodulates HLA-A, B and C expression on HIV infected CD4 (iCD4) cells. (a) Staining for HLA-C on unCD4 (within the same culture) (dark gray histogram) iCD4 (light gray histogram). The open histogram depicts negative control staining. (b) Staining for HLA-A, B and C expression on CD4 (dark gray histogram) and iCD4 (light gray histogram). The open histogram depicts negative control staining. (c) Mean fluorescence intensity of HLA-C staining and (d) HLA-A, B and C staining on uninfected CD4 (HIV-) and iCD4 (HIV^+) cells.

Figure 3

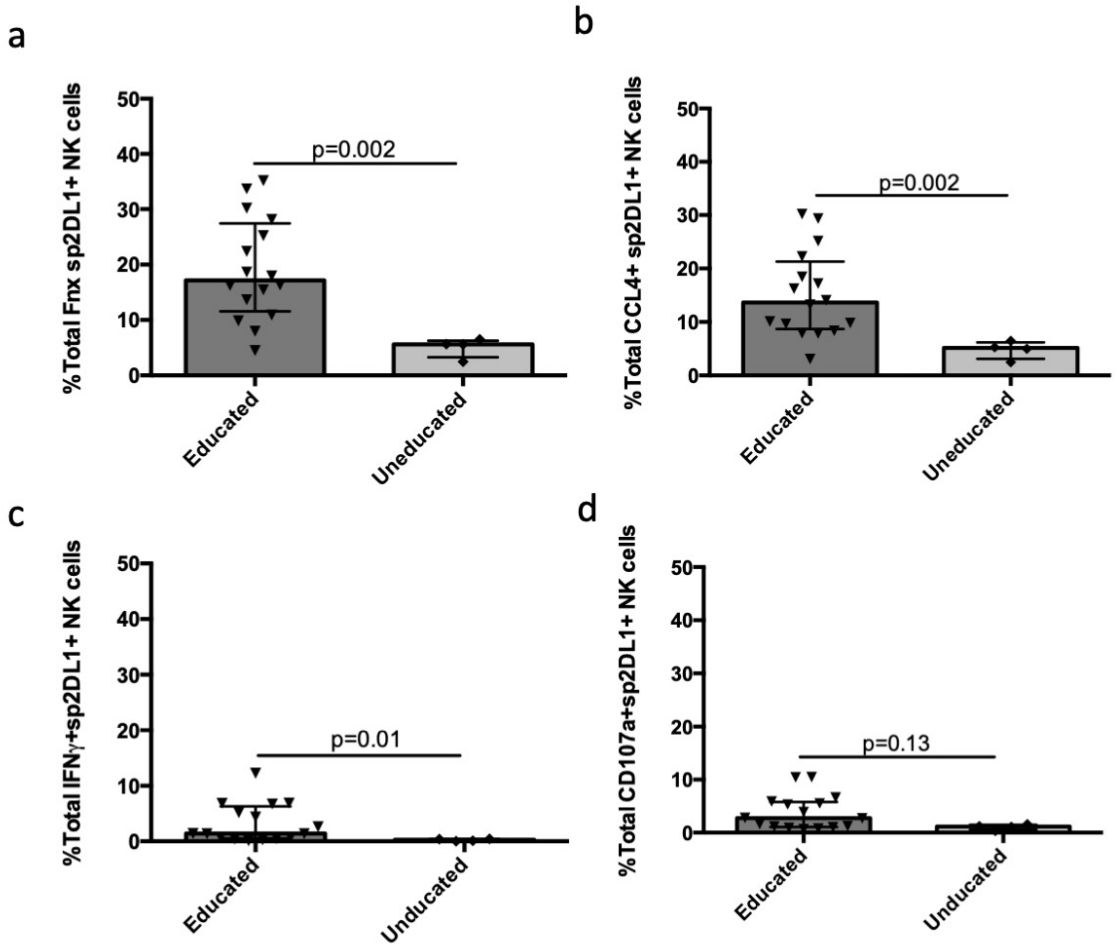

Figure 3. A higher frequency of educated than uneducated spKIR2DL1⁺ NK cells responded to iCD4 stimulation. The y-axis shows the frequency of functional cells characterized by (a) the sum of all functions tested, (b) the sum of cells secreting CCL4, (c) the sum of cells secreting IFN-γ and (d) the sum of cells expressing CD107a. Each point indicates results for a single individual. The bars heights and error bars show the median and interquartile range for the data set. P-values are indicated over the bar linking the data sets being compared.

Figure 4

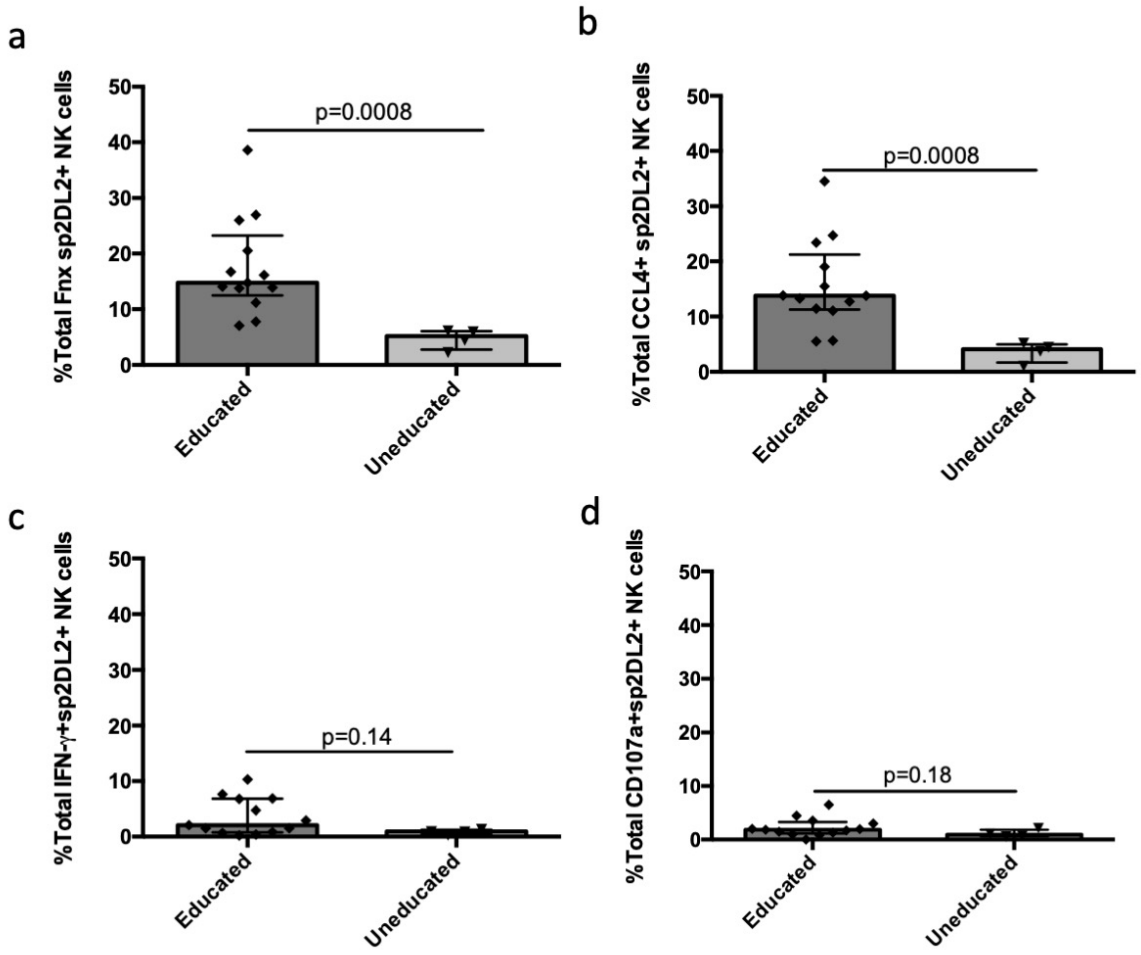

Figure 4. A higher frequency of educated than uneducated spKIR2DL2^{+} NK cells responded to iCD4 stimulation. The y-axis shows the frequency of functional cells characterized by (a) the sum of all tested functions, (b) the sum of cells secreting CCL4, (c) the sum of cells secreting IFN-γ and (d) the sum of cells expressing CD107a. Each point indicates results for a single individual. The bars heights and error bars show the median and interquartile range for the data set. P-values are indicated over the bar linking the data sets being compared.

Figure 5

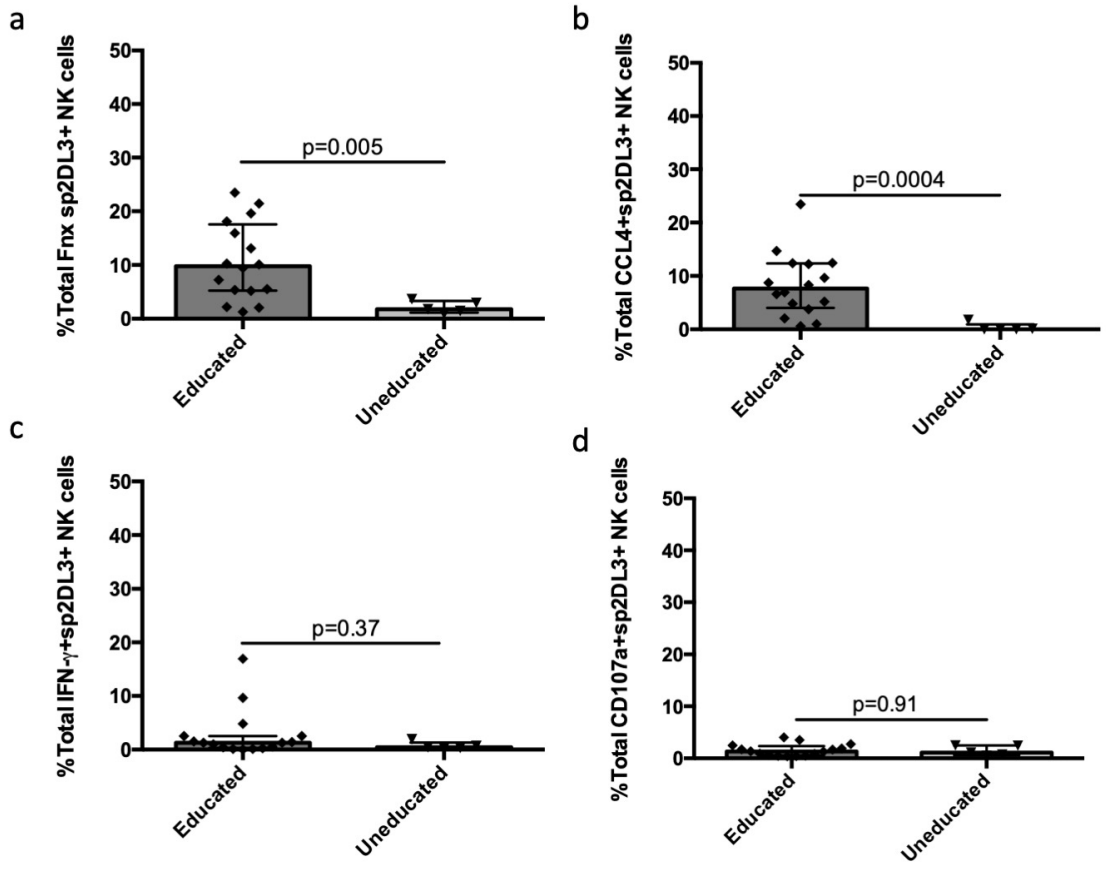

Fig 5. A higher frequency of educated, than uneducated, spKIR2DL3^{+} NK cells responded to iCD4 stimulation. The y-axis shows the frequency of functional cells characterized by (a) the sum of all tested functions, (b) the sum of cells secreting CCL4, (c) the sum of cells secreting IFN-γ and (d) the sum of cells expressing CD107a. Each point indicates results for a single individual. The bars heights and error bars show the median and interquartile range for the data set. P-values are indicated over the bar linking the data sets being compared.

Figure 6

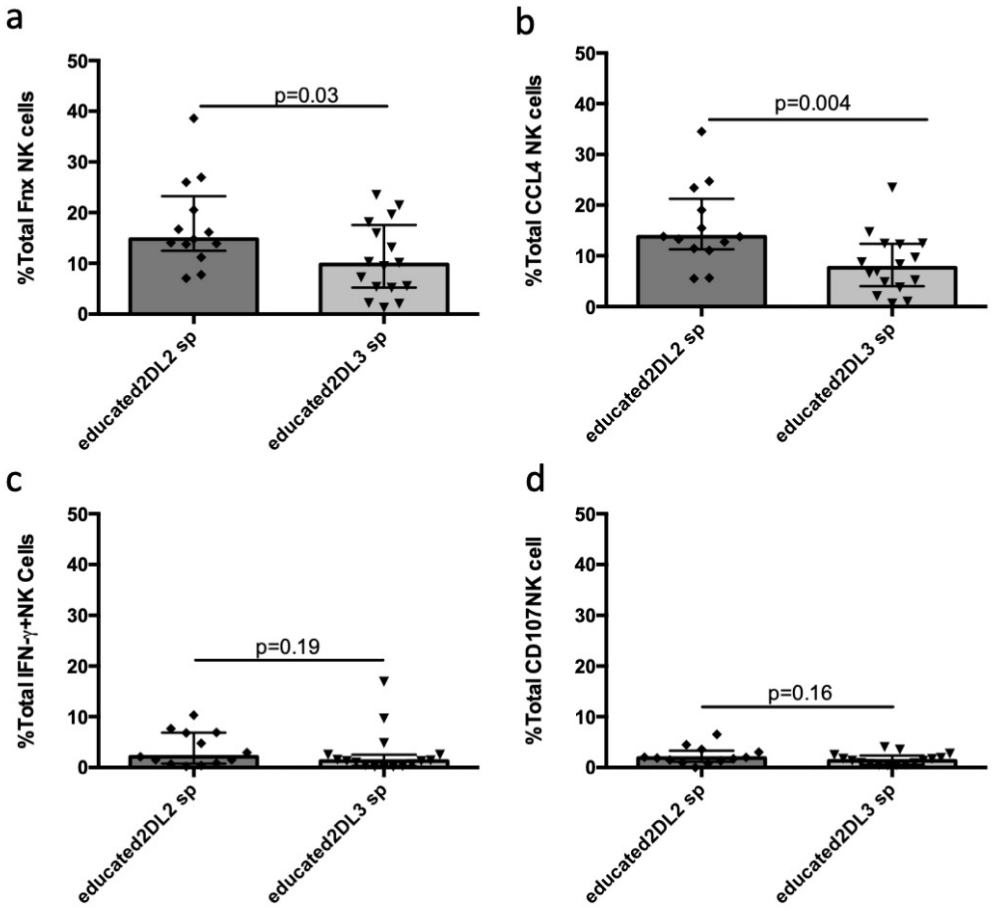

Fig 6. Stronger binding of educated spKIR2DL2^{+} than spKIR2DL3^{+} NK cells with their cognate ligands results in higher responses to iCD4 stimulation. The y-axis shows the frequency of functional cells characterized by (a) the sum of all functions tested, (b) the sum of cells secreting CCL4, (c) the sum of cells secreting IFN-γ and (d) the sum of cells expressing CD107a. Each point indicates results for a single individual. The bars heights and error bars show the median and interquartile range for the data set. P-values are indicated over the bar linking the data sets being compared.

Figure 7

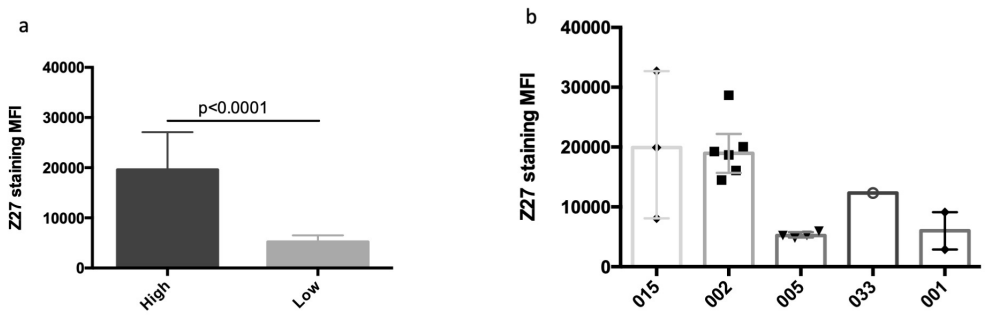

Figure 7. KIR3DL1 allotypes are expressed at different intensities on NK cells. The y-axis shows the mean fluorescence intensity (MFI) of expression of (a) KIR3DL1-high versus KIR3DL1-low allotypes, (b) the MFI of individual KIR3DL1 allotype staining**.** Each point indicates results for a single individual. The bars heights and error bars show the median and interquartile range for the data set.

Figure 8

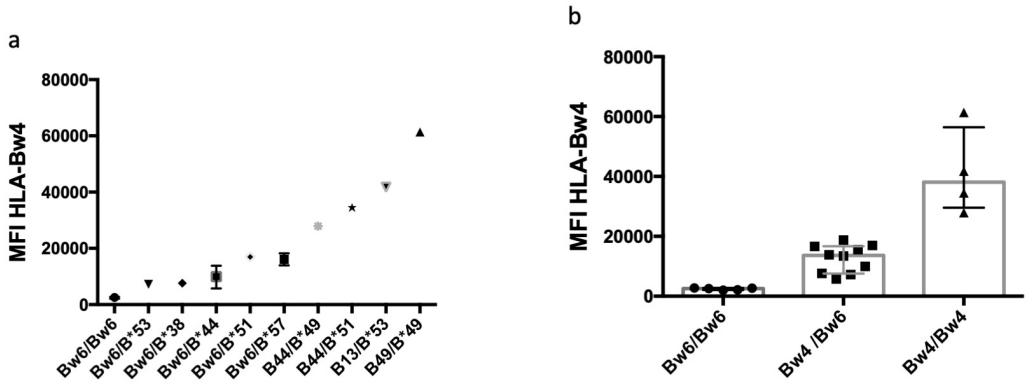

Figure 8. HLA allotypes are expressed at distinct intensities on CD4 T cells. The y-axis shows the MFI of expression of (a) HLA-Bw4 antigens from individuals carrying distinct HLA genotypes using an Ab specific for Bw4 only. (b) The y-axis shows the MFI generated by staining CD4 T cells from *Bw6* homozygotes (hmzs), *Bw4*/*Bw6* heterozygotes and Bw4 hmz subjects with an Ab specific for HLA-Bw4. Each point indicates results for a single individual. Bars heights and error bars represent the median and interquartile range for the data set.

Figure 9

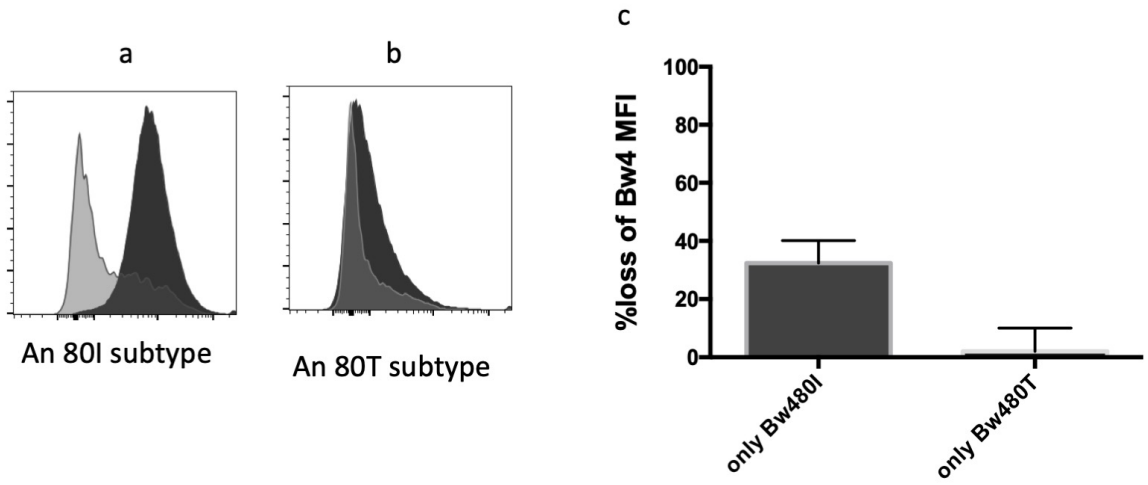

Figure 9. HIV infection reduces the expression of HLA-Bw4*80I to higher extend than HLA-Bw4*80T on iCD4. (a) A Bw4 specific antibody was used to stain iCD4 (light gray histogram) and uninfected CD4 T cells (dark gray histogram) from a subjects who expressed HLA-Bw4*80I and (b) HLA-Bw4*80T. (c) The y-axis shows the percent loss in MFI of the expression of Bw4 antigens in iCD4 from a subjects expressing only HLA-Bw4*80I antigens or only HLA-Bw4*80T antigens. Bars heights and error bars represent the median and interquartile range for the data set.

Figure 10

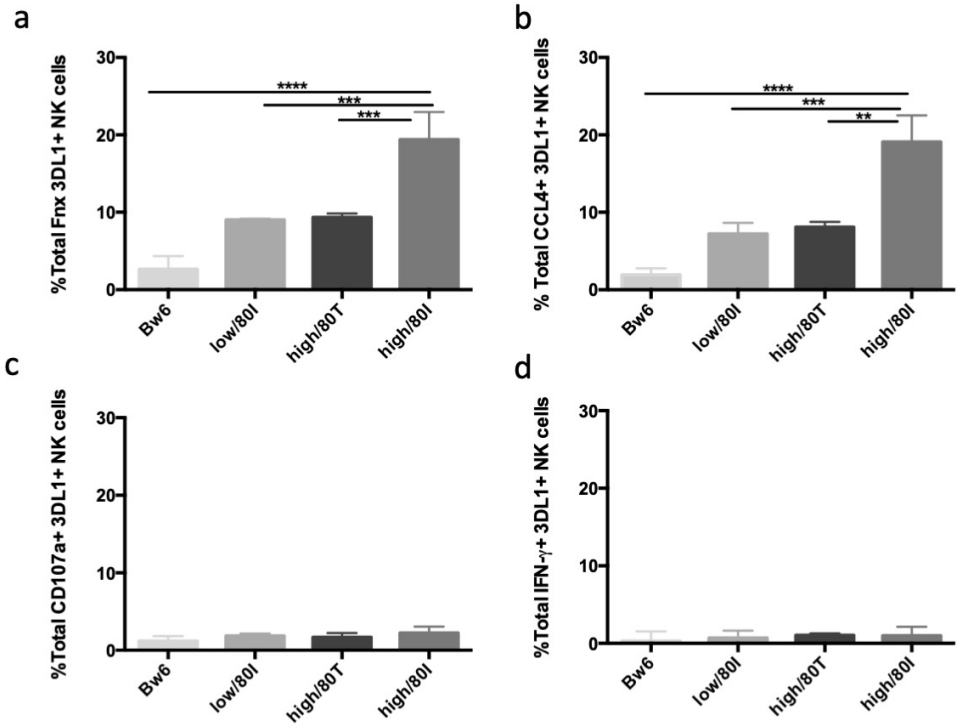

Figure 10. KIR3DL1/HLA-B subtype combinations predict the frequency of KIR3DL1$^+$ activated by autologous iCD4 cells. The y-axis shows the percent of KIR3DL1$^+$ NK cells responding to autologous iCD4 by expressing (a) the sum of all functions tested, (b) the sum of cells secreting CCL4, (c) the sum of cells secreting IFN-γ and (d) the sum of cells expressing CD107a. Bars heights and error bars represent the median and interquartile range for the data set. P-values are indicated over the lines linking the data sets being compared. "**" = $p<0.01$; "***" = $p<0.001$; "****" = $p<0.0001$.

Tables

Table 1a. Study population HLA-C and 2DL1 genotypes and allotypes.

DONOR	HLA-C1C2	HLA-C	HLA-C	2DL1 (ALLELE 1)	2DL1 (ALLELE 2)
1	C1/C1	03:03	12:02	003	003
2	C1/C1	07:02	07:02	003	003
3	C1/C1	07:02	16:01	004	004
4	C1/C1	03:03	12:03	001/002	004
5	HTZ	05:01	14:02	004	004
6	HTZ	06:02	07:01	004	004
7	HTZ	06:02	07:01	003	003
8	HTZ	07:02	16:02	001/002	003
9	HTZ	05:01	08:02	001/002	004
10	HTZ	05:01	07:02	003	003
11	HTZ	06:02	08:02	001/002	004
12	C2/C2	04:01	06:02	004	004
13	C2/C2	02:02	04:01	001/002	004
14	C2/C2	02:02	04:01	004	004
15	C2/C2	04:01	04:01	001/002	003
16	C2/C2	05:01	06:02	001/002	004
17	C2/C2	05:01	06:02	001/002	001/002
18	C2/C2	05:01	06:02	003	003
19	HTZ	04:01	07:02	001	002
20	HTZ	03:03	05:01	003	007

Table 1b. Study population HLA-C and 2DL2 genotypes and allotypes.

DONOR	HLA-C1/C2	HLA-C	HLA-C	2DL2 (ALLELE 1)	2DL2 (ALLELE 2)
1	C2/C2	04:01	06:02	001	003
2	C2/C2	02:02	04:01	001	2DL3
3	C2/C2	02:02	04:01	001	003
4	C2/C2	05:01	06:02	001	003
5	C1/C1	07:01	16:01	003	2DL3
6	C1/C1	07:01	07:02	003	2DL3
7	HTZ	02:02	08:02	003	2DL3
8	HTZ	05:01	14:02	001	001
9	HTZ	05:01	08:02	001	2DL3
10	HTZ	05:01	07:02	003	2DL3
11	HTZ	06:02	08:02	001	2DL3
12	C1/C1	03:03	03:04	001	2DL3
13	C1/C1	07:02	16:01	001	003
14	C1/C1	03:01	12:03	001	2DL3
15	HTZ	06:02	07:01	001	001
16	HTZ	04:01	07:02	003	2DL3
17	C1/C1	07:01	07:01	?	2DL3

Table 1c. Study population HLA-C and 2DL3 genotypes and allotypes.\

DONOR	HLA-C1/C2	HLA-C	HLA-C	2DL3 (ALLELE 1)	2DL3 (ALLELE 2)
1	C2/C2	02:02	04:01	001	2DL2
2	C2/C2	04:01	04:01	001	002
3	C2/C2	05:01	06:02	002	2DL2
4	C2/C2	05:01	06:02	002	002
5	C2/C2	05:01	06:02	001	001
6	C1/C1	07:01	07:01	001	001
7	C1/C1	07:01	16:01	002	2DL2
8	C1/C1	08:02	12:03	001	001
9	HTZ	02:02	08:02	001	2DL2
10	HTZ	06:02	07:01	001	001
11	HTZ	07:02	16:02	002	002
12	HTZ	03:04	06:02	001	001
13	HTZ	05:01	08:02	002	2DL2
14	HTZ	05:01	07:02	001	2DL2
15	HTZ	03:03	04:01	002	2DL2
16	HTZ	06:02	08:02	002	2DL2
17	HTZ	03:03	05:01	001	001
18	C1/C1	03:03	03:04	001	2DL2
19	C1/C1	03:03	12:03	002	2DL2
20	HTZ	05:01	07:01	?	2DL2
21	C1/C1	07:01	07:01	?	2DL2

Table 1d. Study population HLA-A, B and 3DL1 genotypes and allotypes.

DONOR	BW4/BW6	HLA-A	HLA-A	HLA-B	HLA-B	3DL1/S1	3DL1/S1
1	BW6 HMZ	02:01	02:01	15:01	03:01	015	3DS1
2	BW6 HMZ	02:01	11:01	?	40:02	002	3DS1
3	BW6 HMZ	02:01	02:01	05:01	01:01	001	002
4	BW6 HMZ	02:01	33:03	15:01	35:08	002	005
5	HTZ	03:01	11:01	40:02	57:01	015	3DS1
6	BW4 HMZ	03:01	32:01	13:02	53:01	001	3DS1
7	HTZ	29:01	36:01	07:02	53:01	001	002
8	BW4 HMZ	02:01	24:02	44:02	51:01	002	005
9	BW4 HMZ	01:01	03:01	44:03	49:01	001	002
10	HTZ	01:01	23:01	14:01	38:05	001	008
11	HTZ	01:01	03:01	14:02	57:01	004	015
12	BW4 HMZ	01:01	31:01	49:01	49:01	001	004
13	HTZ	02:01	02:01	15:01	44:02	033	3DS1
14	HTZ	02:01	29:02	07:02	44:03	002	3DS1
15	HTZ	01:01	24:02	07:02	18:01	001	015
16	HTZ	02:01	03:01	07:02	51:01	004	002
17	HTZ	02:01	03:01	07:02	51:01	005	054
18	BW4 HMZ	02:01	24:02	44:02	51:01	002	005
19	HTZ	02:01	02:01	07:02	57:01	004	005
20	HTZ	24:02	26:01	15:01	57:01	005	005

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CHAPTER 5

DISCUSSION

In chapter 2 of this thesis, we showed that HLA-F was expressed on the HLA-null cell line, 221. Co-culture of 221 cells with PBMC activated a higher frequency of KIR3DS1^{+} than KIR3DS1^{-} NK cells to express CD69, to secrete CCL4 and IFN-γ and to express CD107a. The functionality of KIR3DS1^{+} NK cells was not due to co-expression of other i/aNKR that may influence the education of KIR3DS1^{+} NK cells, although NKG2D and NCRs were not included in exclusive channels. We demonstrated that interaction of KIR3DS1 by HLA-F on 221 cells was responsible for the higher frequency of stimulated exclusively gated KIR3DS1^{+} than KIR3DS1^{-} NK cells (although KIR3DS1^{-} NK cells were responsive but to significantly less extent than KIR3DS1^{+} NK cells) as blocking of interaction between KIR3DS1 with HLA-F decreased the frequency of KIR3DS1^{+} NK cells responsive to 221 cells. As HLA-F is only expressed on the surface of activated lymphocytes therefore is not expressed constitutively, it does not tune down the responsiveness of KIR3DS1^{+} NK cells. It seems the ligation of KIR3DS1 to HLA-F overrides the inhibitory signals through iNKR conjugated with their ligands. *Garcia-Beltran et al.* showed that HLA-F plate bound are able to stimulate KIR3DS1^{+} NK clones thus I could conclude that KIR3DS1 is able to mediate function on its own; however, it is possible that the presence of other activating receptors results in higher responsiveness of KIR3DS1^{+} NK cells. In chapter 3 of this thesis, we showed that HLA-F was present on the surface of activated unCD4 as well as on p24^{lo}CD4^{hi} and siCD4^{-} cells. HIV infection reduced the expression of HLA-F on siCD4^{-} cells compared to that on activated unCD4 and p24^{lo}CD4^{hi} cells. However, siCD4^{-} cells expressed enough HLA-F to activate KIR3DS1^{+} NK cells. Co-culture of siCD4^{-} cells with isolated primary

NK cells stimulated a higher frequency of KIR3DS1^{+} than KIR3DS1^{-} NK cells to secrete CCL4 and IFN-γ and express CD107a. The higher functionality of KIR3DS1^{+} NK cells responding to siCD4^{-} cells was not due to co-expression of other a/iNKR on the surface of KIR3DS1^{+} NK cells. Interruption of the interaction of KIR3DS1 with HLA-F resulted in a decreased frequency of exclusively gated functional KIR3DS1^{+} NK cells responding to autologous siCD4^{-} stimulation to levels lower than that seen in the absence of blocking or in the presence of isotype control reagents. KIR3DS1-Fc fusion proteins and HLA-F specific mAbs reduced the frequency of functional KIR3DS1^{+} NK cells stimulated with autologous siCD4^{-} cells to levels that did not differ significantly from those of unstimulated KIR3DS1^{+} NK cells. In chapter 4, we confirmed that education of NK cells expressing iKIRs predicts their responsiveness to autologous iCD4. We demonstrated that the infecting HIV strain used was able to down-modulate expression of HLA-C in addition to HLA-A and -B on the surface of iCD4. Our results confirmed that a higher frequency of educated, than uneducated, spKIR2DL1^{+} NK cells responded to iCD4 by secreting CCL4 and IFN-γ (although NKG2D and NCRs were not included into our exclusive gating strategy). A higher frequency of educated, spKIR2DL2^{+} and spKIR2DL3^{+} NK cells responded to autologous iCD4 than their uneducated counterparts by producing CCL4. The density of both KIR3DL1 and Bw4 ligands and their affinity for each other predicted the frequency of spKIR3DL1^{+} NK cells responding to iCD4. Thus, both potency of NK cell education and the downmodulation of HLA by HIV infection contributed to NK cell responsiveness to autologous iCD4 cells.

HLA-F is a high avidity ligand for KIR3DS1

We demonstrated that KIR3DS1, expressed on primary NK cells, binds to HLA-F present on the surface of autologous iCD4 cells. By blocking the interaction between KIR3DS1 and HLA-F using specific mAbs recognizing HLA-F or using KIR3DS1-Fc fusion protein, we confirmed that binding of KIR3DS1 with HLA-F is functional and responsible for activating KIR3DS1$^+$ NK cells. Using a mAb panel able to detect a/iNKR and a gating strategy that excluded NK cells co-expressing these a/iNKR we showed that the potent functions of KIR3DS1$^+$ NK cells against autologous iCD4 was not due to co-expression of these a/iNKR expressed on KIR3DS1$^+$ NK cells.

HLA-F together with HLA-E and -G are non-classical HLA-1b antigens. Although the role of HLA-E and HLA-G in immune functions has been well studied, that of HLA-F has not been well described until recently. HLA-F has been reported to play a protective role in pregnancy and in amyotrophic lateral sclerosis (ALS) (392, 393).

HLA-F presents an open-ended peptide binding groove (394). There are ten amino acid lining the peptide binding site of HLA-I antigen that govern the interaction of HLA-I molecules with their bound peptides. Five of these amino acids are conserved in HLA-F. The other 5 residues in the peptide binding site of HLA-F differ from those conserved residues in the peptide binding sites of other HLA-I antigens. This may be a reason for HLA-F often being expressed as an OC independently of bound peptide on the surface of activated cells and cell lines (394, 395). One hypothesis proposes that HLA-F is an empty HLA-I molecule. An alternate hypothesis proposes

that HLA-F contributes to immune responses in a form where it is heterodimerized with other HLA-I molecule H chains. HLA-F, in complex with β2m, was reported to be present on placental cells. Therefore HLA-F may be expressed in complex with β2m and/or peptide as well (396).

HLA-F was reported to be associated with the TAP-peptide loading complex. Together these findings suggest that HLA-F can be expressed in different isoforms either as OCs or in complex with β2m, and/or peptide and/or HLA heavy chains depending on cells type and the status of the cell it is expressed on (397). HLA-F binds NKRs, such as ILT2, KIR2DS4, KIR3DL2, KIR3DL1 and KIR3DS1 and consequently can regulate immune responses through these receptor/ligand interactions (312, 374, 397, 398).

Garcia-Beltran *et al.* demonstrated that HLA-F OCs are high avidity ligands for KIR3DS1 and the interaction between KIR3DS1 and HLA-F is functional in terms of NK cell degranulation and antiviral cytokine/chemokine production (312). Although the results described in chapters 2 and 3 of this thesis are in the line with those described by *Garcia-Beltran et al.*, there are discrepancies between their results and mine as well. For example, *Garcia-Beltran et al.* used NK cell clones rather than primary NK cells for their experiments. They investigated the stimulation of NK cells by plating KIR3DS1^{+} NK cell clones into the wells of 96-well plates coated with HLA-F monomers (312). By contrast, we co-cultured primary NK cells with autologous sorted siCD4^{-} cells. Producing NK cell clones requires the presence of IL-2 in order to drive NK cell proliferation in wells containing NK cells present at the limiting dilutions needed to obtain clonal NK cell populations. This IL-2 stimulation may change NK cell responsiveness in ways that differ from *ex vivo* NK cells. The primary NK cells we used for the experiments described in chapters 2 and 3

are naïve to IL-2 and thus better reflect the responsiveness of *ex vivo* NK cells. In *Garcia-Beltran et al.,* the assessment of NK cell stimulation was performed in the absence of HIV infected cells (312). However, NK cell receptor/ligand binding has been shown to be altered by the presence of HIV peptides (399). In our experiments siCD4^{-} cells provided the stimulus for KIR3DS1^{+} NK cells. This stimulus would provide relevant HIV peptides, should they have been required to stimulate KIR3DS1^{+} NK cells, again reflecting a potentially more biologically relevant experimental system in which to study the interaction of HIV infected cells stimulating KIR3DS1^{+} NK cells.

Future directions for this research would be to sort for enriched NK cells from KIR3DS1 hmzs that are spKIR3DS1^{+} and KIR3DS1^{-}. These NK cells would be co-cultured with sorted autologous siCD4^{-} cells. This would eliminate any possible interaction of NK cells co-expressing other a/iNKRs. This may be an improvement on the strategy used in chapters 2 and 3 where an exclusive gating strategy was applied to total NK cells to detect KIR3DS1$^{+/-}$ NK cells. Although the exclusive gating strategy we used allowed us to gate on KIR3DS1$^{+/-}$ NK cells exclusively after the stimulation step, during stimulation, total NK cells were present and may have contributed to the responsiveness of the KIR3DS1$^{+/-}$ NK cells to the stimuli. Impediments to this approach would be the large numbers of cells required for these experiments, and the costs associated with the large amounts of Abs, reagents to isolate NK cells and sorting time that would be required.

HIV infection interrupts interactions between ILT2 and HLA-F to elicit the activation of educated ILT2$^+$ NK cell function

We found that a higher frequency of ILT2$^+$ than ILT2$^-$ NK cells responded to siCD4$^-$ cell stimulation. In line with our results, *Dulberger et al*. demonstrated that ILT2 does not interact with HLA-F OCs whereas HLA-F/β2m/peptide complexes bind the iNKR, ILT2 (279). This finding suggests that the HLA-F present on siCD4$^-$ is in the form of an OC. If this is the case, ILT2$^+$ NK cells would be activated by siCD4$^-$ cells through missing-self recognition leading to the higher frequency of activated ILT2$^+$ than ILT2$^-$ NK cells.

Based on previous studies, the conformation of the HLA-I molecules and their ability to bind their NKRs changes in the presence of HIV peptides. An interesting avenue to explore would be the structure of ILT2/HLA-F complexes on unCD4 versus iCD4 cells using crystallography techniques to determine whether HLA-F is present predominantly as an OC on iCD4. Such an investigation has the potential to confirm whether ILT2 fails to bind HLA-F because of it being present as an OC on activated CD4 cells or because of the presence of HIV peptides in complex with the HLA-F heavy chain and β2m on iCD4 that results in a conformational change no longer able to interact with ILT2 receptors.

Educated spKIR2DL1$^+$ NK cells contribute in anti-HIV responses

We demonstrated that educated spKIR2DL1$^+$ NK cells responded more potently than their uneducated counterparts to autologous iCD4 cells. NK cells acquire their functional potential during the NK cell education process in which iNKRs interact with their HLA-I ligands. NK cells become activated due to the interruption of inhibitory signals through iNKR/HLA-I interactions because of the downmodulation of self HLA-I ligands on infected target cells, and also owing to activating signals through the interaction of activating receptors with their ligands.

We demonstrated that HIV reduces the expression of HLA-C on iCD4. The consequence of this downmodulation of HLA-C is that a higher frequency of activated educated than uneducated spKIR2DL1$^+$ NK cells are induced by stimulation with autologous iCD4 as a result of dowmodulated HLA-C2 group ligand expression on iCD4 cells.

Our gating strategy allowed us to gate on KIR2DL1$^+$ NK cells exclusively, eliminating the effect of signals received through other i/aNKRs. All studies done to assess the functionality of KIR2DL1 to iCD4 were done using either on NK cell clones or cell lines transduced with KIR2DL1. Work done by others using primary NK cells did not use an Ab panel that I designed for this work nor a gating strategy permitting gating on spKIR2DL1 cells.

Together, these results demonstrate that NK cells responses to HIV not only depend on education of NK cells via iKIR/HLA interactions but also on changes in HLA expression on iCD4 cells governed by HIV.

Interaction between KIR2DL2 and KIR2DL3 with HLA-C1 results in more potent NK cell activation by autologous iCD4.

We found that a higher frequency of educated, than uneducated, spKIR2DL2$^+$ and spKIR2DL3$^+$ NK cells responded to autologous iCD4. Considering the downmodulation of HLA-C on iCD4, we concluded that the inhibitory signals mediated through KIR2DL2/C1 and KIR2DL3/C1 interactions were diminished, resulting in more potent anti-HIV responses from educated spKIR2DL2 and spKIR2DL3 than their uneducated counterparts.

There is no specific Ab available to recognize KIR2DL2. Therefore, we used specific combinations of mAbs recognizing different combinations of KIR2D receptors. The 180701 Ab recognized KIR2DL3 only, the DX27 Ab recognized KIR2DL2, KIR2DL3 and KIR2DS2 and the 1F12 Ab recognized KIR2DL3 and KIR2DS2. spKIR2DL2 was distinguished as double negative by staining with 180701 and 1F12. spKIR2DL3 was distinguished by staining with 180701 and 1F12. spKIR2DS2 was distinguished by staining with 180701 and 1F12. This is the strategy that we used to identify spKIR2DL2$^+$ NK cells.

It has been shown that co-expression of other NKR on KIR2DL2$^+$ NK cells influenced education and response of these NK cells. In this regard, KIR2DS2 is important to take into consideration as KIR2DS2 is in a close LD with KIR2DL2. Virtually all carriers of *KIR2DL2* also carry

KIR2DS2 genes. The gating strategy we used allowed us to eliminate the possibility that these responses were due to co-expression of other NKR co-expressed on this subset of NK cells.

Béziat et al. designed an Ab panel recognizing human a/iNKRs for which no specific Abs exist (400). In this thesis, we modified *Béziat et al.'s* mAb panel and gating strategy in order to be able to detect spKIR NK cells so that their responses to autologous iCD4 could be examined.

Dr. Peter Parham's laboratory demonstrated that KIR2DL2 and KIR2DL3 are less specific for C1 than is KIR2DL1 for C2 subtypes (308). However, the experimental approach they used to arrive at this conclusion differs from the one we used to examine the anti-HIV function of the NK cell subsets in several ways. First, the binding specificity of the KIR2DL2 and KIR2DL3 iKIRs was examined by using KIR2DL2-Fc and KIR2DL3-Fc fusion proteins and a panel of beads coated with single HLA-C2 allotypes. By contrast, we investigated the interaction between receptor/ligand pairs expressed on primary NK cells and autologous iCD4. Second, the Parham group evaluated cytotoxicity as measured by ^{51}Cr release assay as a functional read out using NK cell clones and 221 cells transduced with specific C2 allotypes that have a weak affinity for KIR2DL2 and KIR2DL3. The work done by the Parham group did not address anti-HIV responsiveness, which may affect iKIRs binding to HLA-I bearing HIV peptide (399). The read out for the functional assays we performed was the measurement of the frequency of NK cells positive for CCL4, CD107a and IFN-γ. Our experiments were performed using primary NK cells and autologous iCD4 rather than NK cell clones and 221 cells as effector and target cells, respectively. In line with the finding that KIR2DL2 allotypes bind more strongly than KIR2DL3

subtypes to HLA-C1 group antigens, we found a higher frequency of educated KIR2DL2^{+} NK cells responded to HIV stimulation than educated KIR2DL3^{+} NK cells Fig 6, chapter 4).

KIR/HLA combinations could be informative in HIV prognosis

In line with the effect of education on the responsiveness of KIR2DL2^{+} and KIR2DL3^{+} NK cells we also found that education through KIR3DL1/Bw4 influenced the responsiveness of NK cells to HIV. *Boudreau et al*. showed that various KIR3DL1/HLA-B subtype pairs degranulate differentially in response to HLA null and autologous iCD4 stimulation (318). Both receptor/ligand pair affinity and cell surface density of receptors and ligands contributed in NK responsiveness.

Boudreau et al.'s findings are in line with our data with some differences. They found more potent NK cell degranulation when NK cells were from carriers of the *KIR3DL1-high/Bw4*80I* than other genotype combinations. We also showed that the KIR3DL1-high/Bw4*80I KIR/HLA combination responded to HIV with the highest frequency of functional NK cells. *Boudreau et al*. found that NK cell degranulation was the predominant function of NK cells from *KIR3DL1-high/Bw4*80I* carriers compared to NK cells from carriers of other *KIR/HLA* combinations. We showed that a higher frequency of NK cells from carriers of *KIR3DL1-high/Bw4*80I* secreted CCL4 than NK cells from carriers of the other tested genotypes. *Boudreau et al*. did not test for NK stimulation by cytokine/chemokine secretion (318). By contrast, we found no significant between-genotype combination differences for CD107a expression. One reason for these

discrepant results for CD107a expression may be due to the smaller size of the population included in our study or the HIV isolates that were used to infect NK cells. *Boudreau et al*. used envelope-deleted DHIV3 virus whereas we used $HIV_{JR\text{-}CSF}$.

Increasing the size of the study population would be an important future direction for this project. Given that we have preliminary results, it is possible to use this information to perform sample size calculations in order to select a sample size that would provide 80% power to observe between groups differences with an $\alpha<0.05$ for each function tested. In the results described in chapter 4, most of the total functionality is attributable to CCL4 secretion. CCL4 and the other CC-chemokines are induced as early as 1 hr after NK cell stimulation (239). IFN-γ and TNF-α secretion take longer to develop after stimulation (239). It may be that a longer stimulation interval would permit IFN-γ and CD107a expression to be visualized more effectively. A time course experiment would determine whether this is the case and what the duration of the stimulation period should be to obtain optimal results for these 2 functions.

In a setting where I would have access to unlimited resources, I would sort KIR/HLA typed NK cells into sp cells for each i/aKIRs. These would then be stimulated/co-cultured with autologous $siCD4^-$ cells. In this way, only the NK subset of interest would be co-cultured with $siCD4^-$ cells making our conclusion more concrete. Another avenue of interest would be to design an experiment that examines the cytolysis of target cells (iCD4) such as the ^{51}Cr release assay or Annexin V (annexin V) target cell staining.

FINAL CONCLUSION AND SUMMARY

In conclusion, data presented in this thesis characterize the anti-HIV function of NK cells subsets. They describe anti-HIV responses of previously unknown NK cell subsets. Results presented here provide new insights into the molecular mechanisms mediating the protective effect of NK cell subjects such as KIR3DS1^{+} and KIR3DL1-high NK cells. What needs to be done in future is more research to characterize other protective NK subsets to complement what has been described here. Such information could serve as a springboard toward further defining protective mechanisms for NK cells to HIV, other infectious diseases and cancers.

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