Natural Killer cell receptors and decreased susceptibility to HIV infection

Salix Boulet

Faculty of Medicine, Department of Physiology

McGill University, Montreal

Submitted 2009

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

Table of Contents

Abstract

The human immunodeficiency virus (HIV) currently infects over 33 million individuals worldwide. The development of a protective HIV vaccine would provide the ideal tool to fight this pandemic. Recent clinical trials testing candidate T- and B-cell based vaccine strategies have failed to demonstrate that these were protective against infection. For many, these failures underline how little is known about what constitutes an efficient immune response against HIV and that increased knowledge about the role of innate immune cells may be required in order to design an efficient vaccine against HIV.

Natural Killer (NK) cells are part of the innate arm of the immune system and are involved in the control of several viral infections, including HIV. Epidemiological evidence has linked specific NK cell receptors, termed KIR3DS1 and KIR3DL1, to favourable clinical outcomes in HIV infected individuals. Whether these receptors would also be involved in protection from infection, and therefore could provide the basis for new vaccine strategies, is unknown.

To address this question, we have evaluated the genetic distribution of both KIR3DS1 and KIR3DL1 in a population of exposed uninfected individuals (EUs). EUs remain HIV seronegative despite repeated exposure to the virus through high-risk behavior. Understanding the immunological causes of their decreased susceptibility to infection may provide insights into vaccine design. In chapter II we demonstrate that KIR3DS1 homozygous individuals are overrepresented in the EU population. Additionnally, in chapter III, we provide evidence that the combined genotype of HLA-B*57 with a specific set of KIR3DL1 alleles is also overrepresented in the EU

population. Finally, in chapter IV, we demonstrate that NK cells from individuals carrying certain KIR3DL1/HLA genotypes linked to slower HIV disease progression and/or protection from infection have increased functional potential following stimulation.

The evidence presented in this thesis supports a role for NK cells, and particularly of KIR3DS1 and KIR3DL1, in the decreased susceptibility to HIV infection observed in EUs. Given that these cells are directly involved in viral suppression and are capable of modulating both the adaptive and innate arm of the immune response, understanding how NK cell mediate these activities may reveal new therapeutic strategies against HIV.

Résumé

Le virus de l'immunodéficience humaine (VIH) infecte présentement plus de 33 millions d'individus. Contre cette pandémie, la solution idéale serait le développement d'un vaccin. Toutefois, de récents essais cliniques évaluant l'efficacité des stratégies de vaccination visant à la stimulation des cellules T ou B contre le VIH n'ont pas réussi à démontrer que ces stratégies protégeaient contre l'infection. Pour plusieurs membres de la communauté scientifique, ces échecs démontrent que les caractéristiques d'une réponse immunitaire efficace contre le VIH sont encore méconnues et que le rôle des cellules du système immun inné devra sans doute être éclairci afin d'élaborer un vaccin efficace contre le VIH.

Les cellules NK (Natural Killer) font parties du système immunitaire inné et aident au contrôle de plusieurs infections virales, incluant les infections au VIH. Des études épidémiologiques ont lié certains récepteurs des cellules NK, appelés KIR3DS1 et KIR3DL1, à des indices cliniques favorables chez des individus infectés par le VIH. Que ces récepteurs puissent aussi pourvoir une forme de protection contre l'infection, et donc inspirer de nouvelles stratégies de vaccination, demeure encore incertain.

Afin de répondre à cette question, nous avons évalué la distribution génétique de KIR3DS1 et KIR3DL1 dans une population d'individus exposés séronégatifs (ESN). Les ESN demeurent séronégatifs aux anticorps du VIH malgré des comportements à risques. Comprendre les facteurs immunitaires permettant à cette population d'être moins susceptible à l'infection au VIH pourrait aider à l'élaboration d'un vaccin. Lors du chapitre II, nous démontrons que les individus KIR3DS1 homozygotes sont

surreprésentés dans la population d'ESN. De plus, dans le troisième chapitre, nos données soutiennent qu'une combinaison génétique du HLA-B*57 avec un sous-type particulier de KIR3DL1 et aussi surreprésentée dans la population d'ESN. Finalement, dans le chapitre IV, nous démontrons que les cellules NK provenant d'individus ayant des génotypes HLA/KIR3DL1 liés à une progression plus lente de la maladie VIH et/ou à une protection contre l'infection ont un potentiel de fonctionnalité accru suivant une stimulation.

Les données présentées dans cette thèse suggèrent que les cellules NK, plus particulièrement leurs récepteurs KIR3DS1 et KIR3DL1, sont impliquées dans une forme de résistance à l'infection observée chez les ESN. Puisque ces cellules sont directement impliquées dans le contrôle viral et peuvent moduler à la fois le système immun inné et adapté, éclaircir les mécanismes permettant aux cellules NK de diminuer la susceptibilité à l'infection pourrait révéler de nouvelle stratégie thérapeutique afin de contrer le VIH.

Preface

Chapter II: Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals

Salix Boulet, Saeid Sharafi, Nancy Simic, Julie Bruneau, Jean-Pierre Routy, Christos M Tsoukas, Nicole F. Bernard.

Published in : AIDS. 2008 Mar 12;22(5):595-9.

SB optimized and implemented the KIR genotyping assay, performed HLA and KIR genotyping, analyzed results and prepared the manuscript. SS and NS performed HLA and KIR genotyping. JB, JPR, and CMT are responsible for the cohorts from which clinical samples, behavioral and clinical information were obtained. NFB designed the research and prepared the manuscript.

Chapter III: A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection

Salix Boulet, Marianna Kleyman, Jenice Y Kim, Philomena Kamya, Saeid Sharafi, Nancy Simic, Julie Bruneau, Jean-Pierre Routy, Christos M Tsoukas, Nicole F. Bernard.

Published in: AIDS. 2008 Jul 31;22(12):1487-91

SB optimized and implemented the KIR allotyping assay, analyzed results and prepared the manuscript. MK optimized and implemented the KIR allotyping assay and performed KIR allotyping. JYK and PK performed KIR allotyping. SS and NS performed HLA genotyping. JB, JPR, and CMT are responsible for the cohorts from which clinical samples, behavioral and clinical information were obtained. NFB designed the research and prepared the manuscript.

<u>Chapter IV:</u> HIV protective KIR3DL1 and HLA-B genotypes influence natural killer cell function following stimulation with HLA-devoid cells

Salix Boulet, Julie Bruneau, Christos M. Tsoukas, Nicole F. Bernard

Revisions submitted to: Blood, 15 April 2009.

SB designed the study, performed the experiments and data analysis and prepared the manuscript; JB and CMT provided clinical samples; NFB designed the study and prepared the manuscript.

Acknowledgements

Completing a project as massive as a PhD thesis requires the help from family, friends, co-workers and collaborators. However, without the support from select individuals, it is likely that I wouldn't have made it through this Chinese torture.

To George, Kenneth and Yoav, thanks for helping me with the science, but most of all, thanks for making the years in the lab enjoyable. I know our friendship will last beyond the lab years. Hey, maybe one day we'll all be at Makflow.

To my mentor, Dr. Nicole Bernard, I would like to thank you for the opportunity you gave me when I first walked in your office. I had no idea what research was or even how a pipette worked and you still took me on board. Thanks for being easily available when I needed your input. Most of all, however, thank you for creating an environment which has made the journey more than just an academic experience, but also an enjoyable personal experience.

In addition to everything else, I owe my education entirely to you, my parents. You have transferred to me your thirst for knowledge. You have made sure I could choose my own path and you have supported my decisions blindly. You showed interest in my work even though I didn't volunteer much information.

To Geneviève, it's probably not easy to support someone who never discusses his work and who can never figure out what is next. Because you are willing to follow me, because you understand who I am and because you still stick with me despite my flaws, I love you.

List of abbreviations

7-TM : 7-transmembrane

ADCC: antibody dependent cellular cytotoxicity

AIDS: Acquired immunodeficiency syndrome

ANKD: Absolute natural killer cell deficiency

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

A-SAA: Acute-phase amyloid A protein

AZT: Azydothimidine

BAF: Barrier to autointegration factor

BID: BH3-interacting domain death agonist

CA: Capsid

CCL3L1: CC chemokine ligand 3 like-1

Cdk9: cyclin-dependent kinase 9

CI: Confidence interval

CMV: Cytomegalovirus

CNKD: Classical natural killer cell deficiency

cPPT: Central polypurine tract

CSW: Commercial sex worker

CTLD: C-type lectin-like domain

CTS: Central termination signal

DC: Dendritic cell

DC-SIGN: dendritic cell–specific intercellular adhesion molecule-3 grabbing nonintegrin

DIS: Dimer initiation signal

DMSO: Dimethyl sulfoxide

DNA: deoxyribonucleic acid

dsRNA: Double stranded ribonucleic acid

EBV: Epstein-Bar virus

EIA: Enzyme-linked immunoassay

eIF-2α: Elongation initiation factor-2α

Env: Envelope

EU: Exposed uninfected

FBS: Fetal bovine serum

FCS: Fetal calf serum

FDA: US Food and Drug Administration

FNKD: Functional natural killer cell deficiency

Gag: Group-specific antigen

GALT: Gut associated lymphoid tissue

Grb2: growth factor receptor bound protein 2

GRID: Gay-related immune deficiency

HAART: Highly active antiretroviral therapy

HIV: Human immunodeficiency virus

HLA: Human leukocyte antigen

HMGI(Y): high-mobility group DNA-binding protein

Hmz: Homozygote

HR: Helical region

Hsp60: Heat shock protein 60

HSV: Herpes simplex virus

HTLV: Human T-cell leukemia virus

Htz: Heterozygote

H-W: Hardy-Weinberg

iDC: Immature dendritic cell

IDU: Injection drug user

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

I or Ile: Isoleucine

ILT: Immunoglobulin-like transcripts

IN: Integrase

INR: Initiator

IRF-1: Interferon regulatory factor-1

ITAM: Immune tyrosine-based activation motif

ITIM: Immune tyrosine-based inhibitory motif

Kb: Kilobase

KIR: Killer immunoglobulin-like receptor

LAMP-1: Lysosomal-associated-membrane-protein 1

LGL: Large granular lymphocyte

LILR: Leukocyte Ig-like receptors

LPS: Lipopolysaccaride

LTNP: Long term non-progressor

LTR: Long terminal repeat

MA: Matrix

MCMV: Murine cytomegalovirus

MHC: Major histocompatibility complex

mRNA: messenger ribonucleic acid

MSM: Men having sex with men

MTCT: Mother to child transmission

MTOC: Microtubule-organizing center

NC: Nucleocapsid

NCAM: Neural cell adhesion molecule

NES: Nuclear exporting signal

NK: Natural Killer

NKD: Natural killer cell deficiency

NKR: Natural killer cell receptor

NLS: Nuclear localization signal

NNRTI: Non-nucleoside reverse transcriptase inhibitor

NOD: Non-obese diabetic

NRTI: Nucleoside reverse transcriptase inhibitor

OAS: 2',5'-oligoadenylate synthetase

OPV: Oral polio vaccine

OR: Odds ratio

PBMC: peripheral blood mononuclear cells

PBS: Primer binding site

PCR: Polymerase chain reaction

PFA: Paraformaldehyde

PI: Protease inhibitor

PIC: Pre-integration complex

PKR: Protein kinase dsRNA regulated

Pol: Polymerase

PPT: Polypurine tract

PR: Protease

rAd5: Recombinant Adenovirus 5

Rev: Regulator of expression of viral proteins

RNA: Ribonucleic acid

RNAPII: RNA polymerase II

RRE: Rev responsive element

RT: Reverse transcriptase

RTC: Reverse-transcription complex

SCID: Severe combined immunodeficiency

SDF-1: Stromal derived factor 1

SHIP: SH2-containing inositol phosphate 5-phosphatase

SHP: SH2-containing protein-tyrosine phosphatase

SIV: Simian immunodeficiency virus

SLAM: signaling lymphocytic activation molecules

SNP: Single nucleotide polymorphisms

STI: Sexually transmitted infection

TAP: Transporter associated with antigen processing

TAR: Transactivator responsive region

Tat: Transactivator

Thr or T: Threonine

TNF: Tumor necrosis factor

TRAIL: TNF related apoptosis-inducing ligand

TSG101: Tumor susceptibility gene 101

uNK: Uterine NK cells

VL: Viral load

WHO: World Health Organization

Chapter 1: Introduction

I. Human Immunodeficiency Virus

In 1981, reports of previously healthy American homosexual men affected by conditions such as *Pneumocystis carinii* pneumonia, Kaposi's sarcoma and disseminated cytomegalovirus were the first to describe what would, only a year later, affect over 500 individuals across the United States [1-4]. Initially found mostly in gay men, what was then called GRID (gay-related immunodeficiency) lead to T lymphocyte dysfunction and the reduced capacity of the immune system to control infections that were usually easily dealt with in healthy individuals. When GRID surfaced among heterosexual males and females, AIDS (acquired immunodeficiency syndrome) was used to replace the previous, stigmatizing term. Given the rapid spread of the disease, the scientific community was mobilized to understand what its cause was.

A. The cause of AIDS

It was in 1983 that Françoise Barré-Sinoussi et al. and Robert Gallo et al. reported the isolation and culture of a new T-lymphotropic retrovirus similar to HTLV (Human T-cell Leukemia Virus) from patients with symptoms of AIDS[5,6]. However, the group that would later receive a Nobel prize for their discovery remained cautious as to the relationship between the new virus, which would later be known as HIV (human immunodeficiency virus) and AIDS: "The role of this virus in the etiology of AIDS remains to be determined"[5]. In order to prove that a micro-organism is the cause of a particular disease, it must satisfy four postulates set forth by Robert Koch in the late 1800s:

1. the particular micro-organism must be found in every case of the disease
2. it can be isolated and grown in pure culture in the laboratory
3. it must reproduce the disease when a pure culture is inoculated in a susceptible host.
4. from the inoculated host, the micro-organism can be recovered.

There were several challenges associated with proving that HIV was the cause of AIDS[7]. Firstly, for ethical reasons, it was difficult to satisfy the latter two postulates by experimentally infecting human subjects with pure stocks of the virus. In addition, before disease is observed, there is a lengthy incubation period, which can last years. Finally, because of the nature of the syndrome, other infectious agents such as *Pneumocystis jiroveci* or human herpesvirus 8 (also known as Kaposi's sarcoma associated herpes virus) are often isolated from patients suffering from AIDS. Nevertheless, the first two postulates were met given that HIV was isolated and grown from virtually all patients with AIDS[5,6,8-10]. The third and fourth postulates were met when laboratory workers involved in the production of HIV developed AIDS after accidental work-related exposure to the virus[11]. These individuals reported no other risk factors and the same strain of virus produced in the laboratory was isolated from their blood samples. Although not included in Koch's postulates, one cannot ignore the evidence provided by drugs designed specifically to target HIV proteins, which produce a dramatic improvement in patients with the disease[12]. There is today no doubt that AIDS is caused by HIV, a virus responsible for one of the largest human epidemics.

B. CURRENT HIV EPIDEMIOLOGY

In 2001, leaders from 189 states joined together to respond to the HIV pandemic and produced the Declaration of Commitment on HIV/AIDS. While there have since been some measurable successes, such as increased funding of HIV programs, increased number of people with access to antiretroviral therapy and better prevention efforts in some countries, the epidemic continues to outpace the global response[13]. In 2007, an estimated 33 million individuals from every region of the globe were infected with HIV, with 2.7 million people becoming newly infected in that year alone (table 1)[14]. The severity of the epidemic is, however, regionally distinct, with biological and social factors strongly influencing the spread of the virus[15]. Biological factors affecting

World Health Organization

UNAIDS

Regional HIV and AIDS statistics and features, 2007

	Adults & children living with HIV	Adults & children newly infected with HIV	Adult prevalence (15–49) [%]	Adult & child deaths due to AIDS
Sub-Saharan Africa	22.5 million [20.9 – 24.3 million]	1.7 million [1.4 – 2.4 million]	5.0% [4.6% – 5.5%]	1.6 million [1.5 – 2.0 million]
Middle East & North Africa	380 000 [270 000 – 500 000]	35 000 [16 000 – 65 000]	0.3% [0.2% – 0.4%]	25 000 [20 000 – 34 000]
South and South-East Asia	4.0 million [3.3 – 5.1 million]	340 000 [180 000 – 740 000]	0.3% [0.2% – 0.4%]	270 000 [230 000 – 380 000]
East Asia	800 000 [620 000 – 960 000]	92 000 [21 000 – 220 000]	0.1% [<0.2%]	32 000 [28 000 – 49 000]
Latin America	1.6 million [1.4 – 1.9 million]	100 000 [47 000 – 220 000]	0.5% [0.4% – 0.6%]	58 000 [49 000 – 91 000]
Caribbean	230 000 [210 000 – 270 000]	17 000 [15 000 – 23 000]	1.0% [0.9% – 1.2%]	11 000 [9800 – 18 000]
Eastern Europe & Central Asia	1.6 million [1.2 – 2.1 million]	150 000 [70 000 – 290 000]	0.9% [0.7% – 1.2%]	55 000 [42 000 – 88 000]
Western & Central Europe	760 000 [600 000 – 1.1 million]	31 000 [19 000 – 86 000]	0.3% [0.2% – 0.4%]	12 000 [<15 000]
North America	1.3 million [480 000 – 1.9 million]	46 000 [38 00 – 68 000]	0.6% [0.5% – 0.9%]	21 000 [18 000 – 31 000]
Oceania	75 000 [53 000 – 120 000]	14 000 [11 000 – 26 000]	0.4% [0.3% – 0.7%]	1200 [<500 – 2700]
TOTAL	33.2 million [30.6 – 36.1 million]	2.5 million [1.8 – 4.1 million]	0.8% [0.7% - 0.9%]	2.1 million [1.9 – 2.4 million]

Table 1[14]**:** HIV/AIDS statistics worldwide, 2007. (Reproduced with kind permission from UNAIDS).

the spread of the epidemic include mode of transmission, viral strain, co-existence of endemic infections, gender, etc[16]. Sociologically, the virus underlines the inequalities found within each region, usually affecting the poor and/or disenfranchised of a society, such as the African Americans in the United States of America, injection drug users in Asian countries or women in some African countries[14,17]. In order to be able to combat HIV, all the factors affecting its regional spread must be considered to understand local epidemics.

Sub-Saharan epidemic

While some African countries report a stabilization of HIV prevalence (e.g. Uganda, Zambia, Tanzania, Kenya) due to prevention campaigns and increased accessibility to treatment, there are still over 20 million HIV carriers in sub-Saharan Africa[14,18]. In this region, the epidemic is unique in that more women than men are infected, possibly expressing the unequal social and economical status of women in some African countries, and the virus is transmitted mainly by heterosexual contact[19]. The epicenter of the pandemic is located in countries like Swaziland, Botswana, Zimbabwe, South Africa, Zambia, and Mozambique where 16-25% of the adult population/workforce and up to 32% of pregnant women are infected with HIV[20]. HIV has had devastating effects on the sub-Saharan population, killing an estimated 1.3 million sub-Saharans in 2007 alone[14]. Millions of children, many HIV positive, have lost both parents to the epidemic. Life expectancy, which had been increasing in some countries in the 1970-90s, has been decreasing since the beginning of the epidemic[19]. In addition, as the causative

agent of AIDS, an immunosuppressive disease, HIV has resulted in the recrudescence of endemic infections such as tuberculosis[19].

Other epidemics

While the prevalence of HIV in Asian countries is relatively low, the fact that some of these countries such as India and China are very populous translates into elevated absolute numbers of HIV infected individuals. In this region, over 5 million individuals are infected with HIV[14]. Although, this epidemic was originally focused on injection drug users and commercial sex workers, it has started to spread outside these groups. This, combined with the fact that implementation of effective control programs has been delayed in some countries, will make the Asian epidemic a cause for concern in the near future[19].

With the fall of the Berlin wall and of the Iron Curtain, a flow of population and drugs combined with drastic political and economical changes stimulated an HIV epidemic that was quiescent in Eastern Europe[20]. Today, twice as many individuals (approximately 1.5 million) from Russia, Ukraine and the rest of Eastern Europe carry HIV compared to countries from Western and Central Europe.

With a prevalence of 1-2% and limited economic resources, countries of the Caribbean like Haiti were depicted as suffering an epidemic as severe as the one observed in Africa. However, there has been a decline or stabilization in HIV incidence in this region since the 1990s, perhaps due to better treatment coverage than in Africa[20].

Canada

The prevalence of HIV in Canada is relatively low with an estimated 58 000 Canadians living with HIV[21]. Men who have sex with men still account for the greater number of new infections (45%), followed by injection drug users (14%)[21]. Along with higher rates of poverty, lower education and increased suicide rates, Canadian aboriginals also differ from non-aboriginal Canadian citizens by an over-representation in the HIV epidemic at about 7.5% of all HIV infections in the country [21,22]. In this community, the virus mainly spreads due to injection drug use.

Specificity of regional epidemics

Based on genomic organization and evolutionary relationship, there are two distinct types of human immunodeficiency viruses, termed HIV-1 and HIV-2. HIV-1 is found globally and causes the vast majority of infections. On the other hand, HIV-2 is geographically restricted to West Africa, is somewhat less infectious and causes a less severe disease course[23]. The two HIV types can further be subclassified into distinct phylogenetic groups. HIV-1 is composed of three groups termed M (major), O (outlier) and N (non-M/O or new). Group M accounts for 90% of global infections and is divided into eleven clades (A through K) and recombinants with distinct geographic distributions (figure 1)[24]. HIV-2 strains can be phylogenitically classified into eight subtypes, termed A through H[25]. The explanation for the existence of different HIV types and groups is found in the origins of the virus.

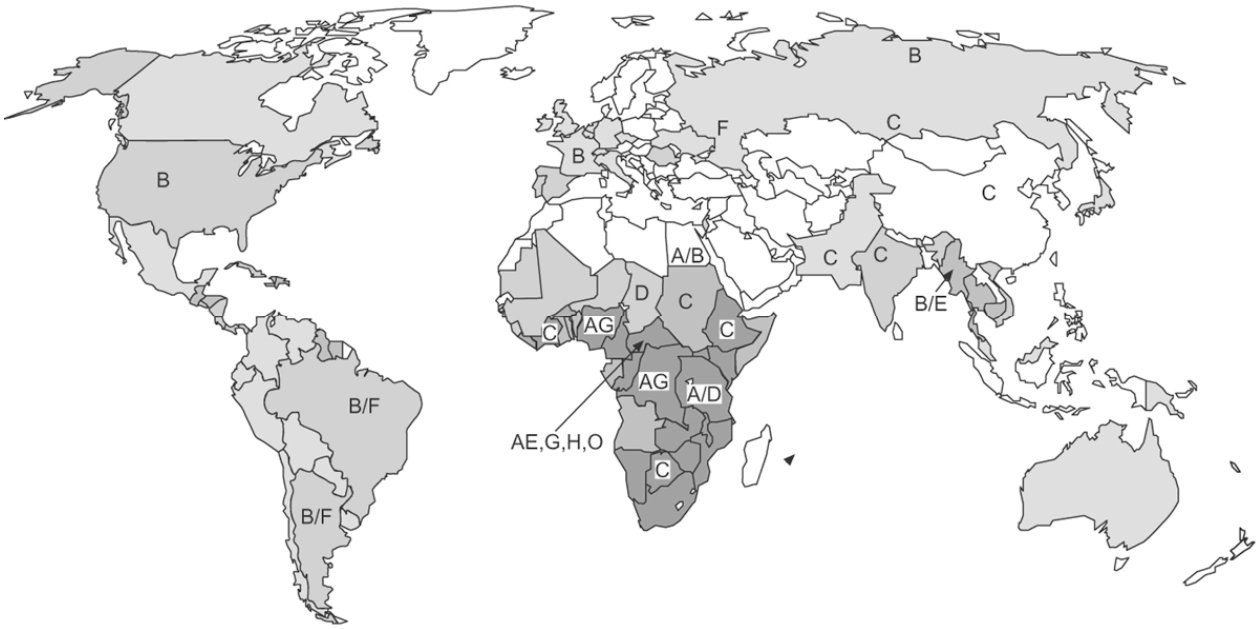


Figure 1[24]**:** Geographical distribution of HIV-1 group M clades. Letters indicate the predominant HIV-1 clade circulating in selected countries. (Reproduced with kind permission from Wolters Klumer Health, Wainberg MA. HIV-1 subtype distribution and the problem of drug resistance. AIDS. 2004;18 Suppl 3:S63-68).

C. ORIGINS OF HIV

When it became clear that HIV was similar biologically and molecularly to another virus found in several species of nonhuman primates, SIV (Simian Immunodeficiency Virus), there was speculation that the human form evolved from the simian virus[26-28]. This was supported by the fact that the latter's natural hosts (sooty mangabeys, African green monkeys, chimpanzees, etc.) were endemic to regions where HIV was rampant in the human population and that there existed plausible routes of transmission between nonhuman primates and humans[29].

Genus	Species/subspecies	Virus
Guenons (*Cercopithecus*)	Sykes' monkey (*C. albogularis*)	SIVsyk
	Blue monkey (*C. mitis*)	SIVblu
	L'Hoest monkey (*C. lhoesti*)	SIVlhoest
	Sun-tailed monkey (*C. solatus*)	SIVsun
	Hamlyn's monkey (*C. hamlyni*)	SIV?
	De Brazza monkey (*C. neglectus*)	SIVdeb
	Campbell's mona (*C. campbelli*)	SIVmon
	Wolf's mona (*C. wolfi*)	SIV?
African green monkeys (*Chlorocebus*)	Vervet monkey (*C. pygerythrus*)	SIVagmVer
	Grivet monkey (*C. aethiops*)	SIVagmGri
	Green monkey (*C. sabaeus*)	SIVagmSab
	Tantalus monkey (*C. tantalus*)	SIVagmTan
White-eyelid mangabeys (*Cercocebus*)	Sooty mangabey (*C. atys*)	SIVsm
	Red-capped mangabey (*C. torquatus*)	SIVrcm
Talapoins (*Miopithecus*)	Angolan talapoin (*M. talapoin*)	SIVtal
Black and white colobus (*Colobus*)	Mantled guereza (*C. guereza*)	SIVcol
Mandrills (*Mandrillus*)	Mandrill (*M. sphinx*)	SIVmnd/SIVmnd2
	Drill (*M. leucophaeus*)	SIVdrl
Chimpanzee (*Pan*)	Western chimpanzee (*P. troglodytes troglodytes*)	SIVcpz(*P.t.t.*)
	Eastern chimpanzee (*P. troglodytes schweinfurthii*)	SIVcpz(*P.t.s.*)
Patas monkeys (*Erythrocebus*)	Patas monkey (*E. patas*)	SIVagmSab
Baboons (*Papio*)	Yellow baboon (*P. cynocephalus*)	SIVagmVer
	Chacma baboon (*P. ursinus*)	SIVagmVer

Table 2[29]**:** African nonhuman primates infected with SIV. (From Hahn BH, et al. Science. 2000;287:607-614. Reprinted with permission from AAAS).

Sequencing analyses of HIV-1, HIV-2 and all the available African SIV (see table 2) revealed that HIV-1 is more closely related to SIVcpz which infects a specific chimpanzee subspecies (*Pan troglodytes troglodytes*) than to HIV-2, which is closely related to SIVsm of sooty mangabeys (*Cercocebus atys*) (see figure 2)[30,31]. While the amino acid sequence similarity of HIV-2 and SIVsm proteins ranged from 62-87%, the most striking similarity was the presence of Vpx, a protein only found in these two

viruses[30]. Similarly, there is a high level of homology between HIV-1 and SIVcpz is strong and the protein Vpu is not found in other lentiviruses[29]. Phylogenetic studies have shown that each of the HIV-1 (M, N, O) and HIV-2 (A-K) subtypes has arisen from a distinct SIVcpz or SIVsm strain[25]. The different HIV-1 group M clades are closely related and have evolved from a single cross-species transmission event and diverged from their common SIVcpz ancestor through the mutational capacity of the virus (see Chapter I, section IE)[31]. Interestingly, SIVcpz itself has been shown to have also evolved from a cross-species transmission. SIVcpz phylogeny indicates that it is a hybrid virus created via in-host recombination of SIVrcm and SIVgsn, viruses found in red-capped mangabeys (*Cercocebus torquatus*) and greater spot-nosed monkeys (*Cercopithecus nictitans*), respectively. These are smaller primate species hunted by chimpanzees[32].

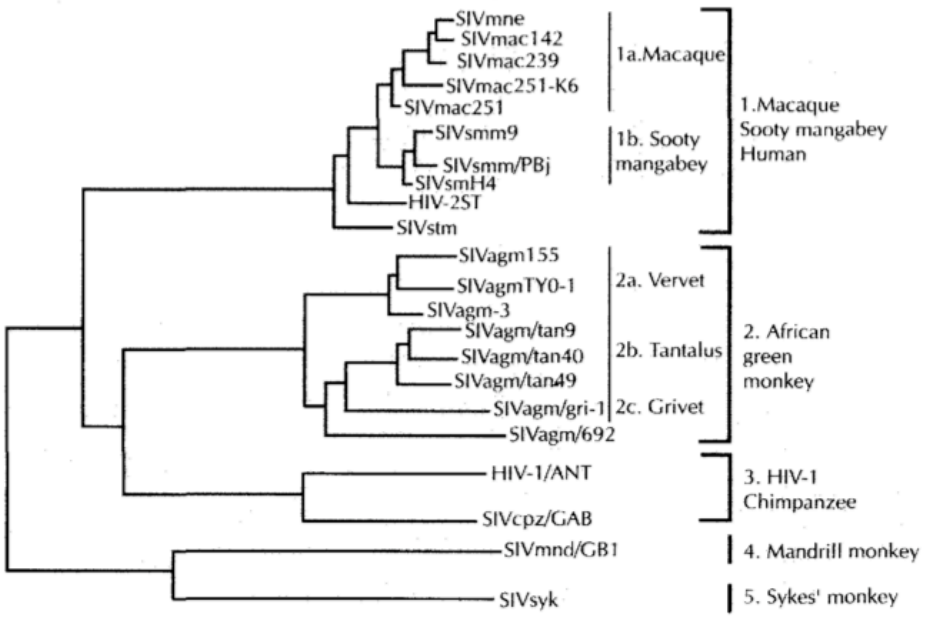


Figure 2[33]: Phylogenetic tree of HIV-1 and SIV. (Reprinted from Current Opinion in Genetics & Development, Vol. 6, Hirsch et al, Phylogeny and natural history of the primate lentiviruses, SIV and HIV, 798-806, 1995, with permission from Elsevier).

The different origins of HIV-1, HIV-2 and their subtypes suggests that cross-species transmission from nonhuman primates to humans has occurred more than once, and could theoretically happen again.

From primates to humans

Two main theories have been put forward to explain how SIVcpz made the transition to humans[29]. The first suggests that exposure to infected animal blood during hunting or butchering of bush meat favored this event. The second theory, based on the presumption that SIV-infected nonhuman primate kidneys were used in the preparation of an attenuated oral polio vaccine (OPV), pins the emergence of HIV on OPV trials carried out in the Kisangani region of the Democratic Republic of Congo in the 1950s. The OPV theory has since been refuted by demonstrating that the SIVcpz strains endemic to the areas where these clinical trials were implemented is related to SIVcpz found in *Pan troglodyte schweinfurthii*, not to the one found in *Pan troglodyte troglodyte*[34]. It is thus more likely that hunting of nonhuman primates resulted in the transmission of SIV to the human population. If this is the case, why did HIV infection emerge as an epidemic only in the 20th century, given that hunting of these animals is an ancient tradition? It is thought that sociobehavioral changes associated with this century could have favored the emergence of a new disease. Although not fully understood, these changes could include urbanization, social disruption, prostitution, the use of nonsterilized needles, etc[29].

While the oldest accessible human sample infected with HIV-1 group M is from 1959, the most recent estimate of the first transmission from nonhuman primates to

humans is 1908 (with a confidence interval ranging from 1884 to 1924), further disproving the OPV theory of transmission[35]. This date was obtained by reconstructing the evolutionary history of HIV-1 through an estimation of the rate of change of the virus by comparing the envelope protein sequences of several specimens collected over more than a decade.

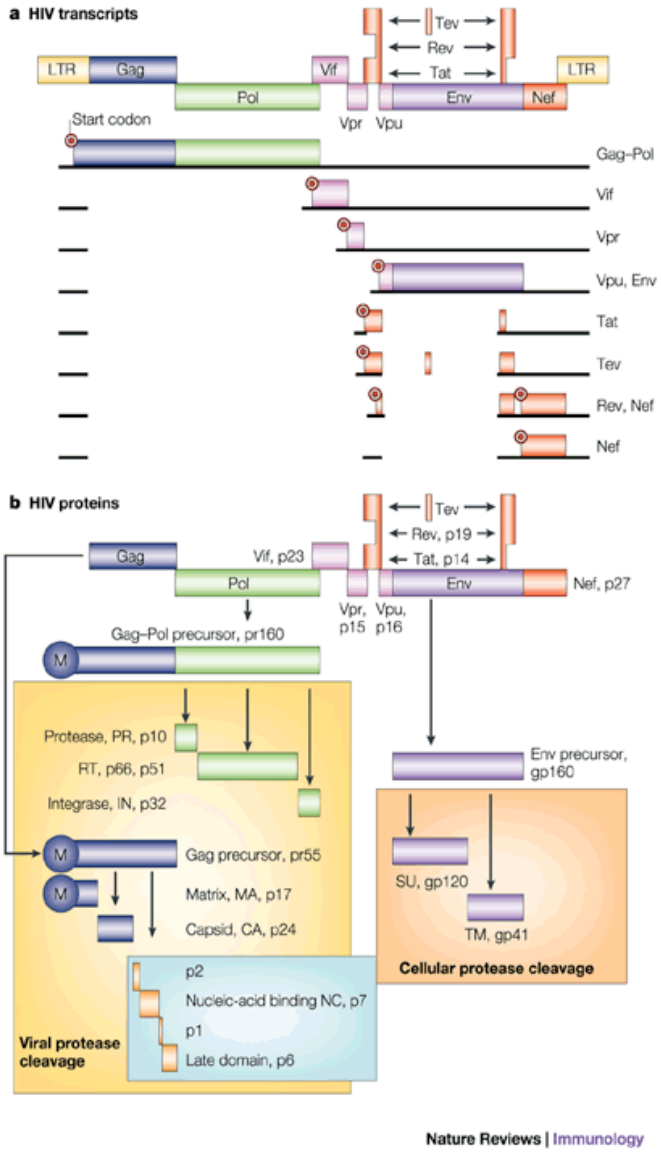


Figure 3[36]**:** The HIV genome and its proteins. A. Black lines denote different splicing patterns above which start codons and coding sequences are shown. B. Shows the origin of each HIV protein. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, 3:97-107, 2003).

D. HIV-1 GENOME

HIV belongs to the genus *Lentivirus* of the *Retroviridae* (retrovirus) family of viruses. Retroviruses are unique among viruses because their diploid genome is composed of an inverted dimer of plus sense single stranded RNA (ribonucleic acid) that is reverse transcribed (thus the term retro) to DNA (deoxyribonucleic acid) before integrating into the human genome[37]. *Lentiviruses* are characterized by a slow development of the clinical illness[38]. The 10 kilobase (kb) long HIV genome (see figure 3) encodes for genes common to all retroviruses; *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope glycoproteins)[39]. In addition, HIV-1 also encodes for regulatory (Tat and Rev) and accessory (Vpu, Vif, Vpr and Nef) proteins. The latter are termed accessory to reflect the fact that they are not strictly required for viral replication. In total the viral genome contains open reading frames for 16 proteins synthesized from 10 transcripts[36]. This rich protein content in a relatively small genome is made possible by translational frameshifts and alternative splicing of the HIV (figure 3). The *gag* gene encodes a polyprotein precursor, Pr55, which is cleaved by the viral protease into the matrix (MA), capsid (CA) and nucleocapsid (NC) and p6 proteins (figure 4)[39]. If a frameshifting event occurs during *gag* translation, the resulting polyprotein, Pr160, can be cleaved by the viral protease to form *pol*-encoded proteins, reverse transcriptase (RT), integrase (IN) and protease (PR)[39]. While the *gag* and *pol* polyprotein precursors are cleaved by the viral protease, gp160, the *env* precursor, is cleaved by cellular protease, resulting in gp120 and gp41[39]. The function of each viral protein is summarized in figure 4.

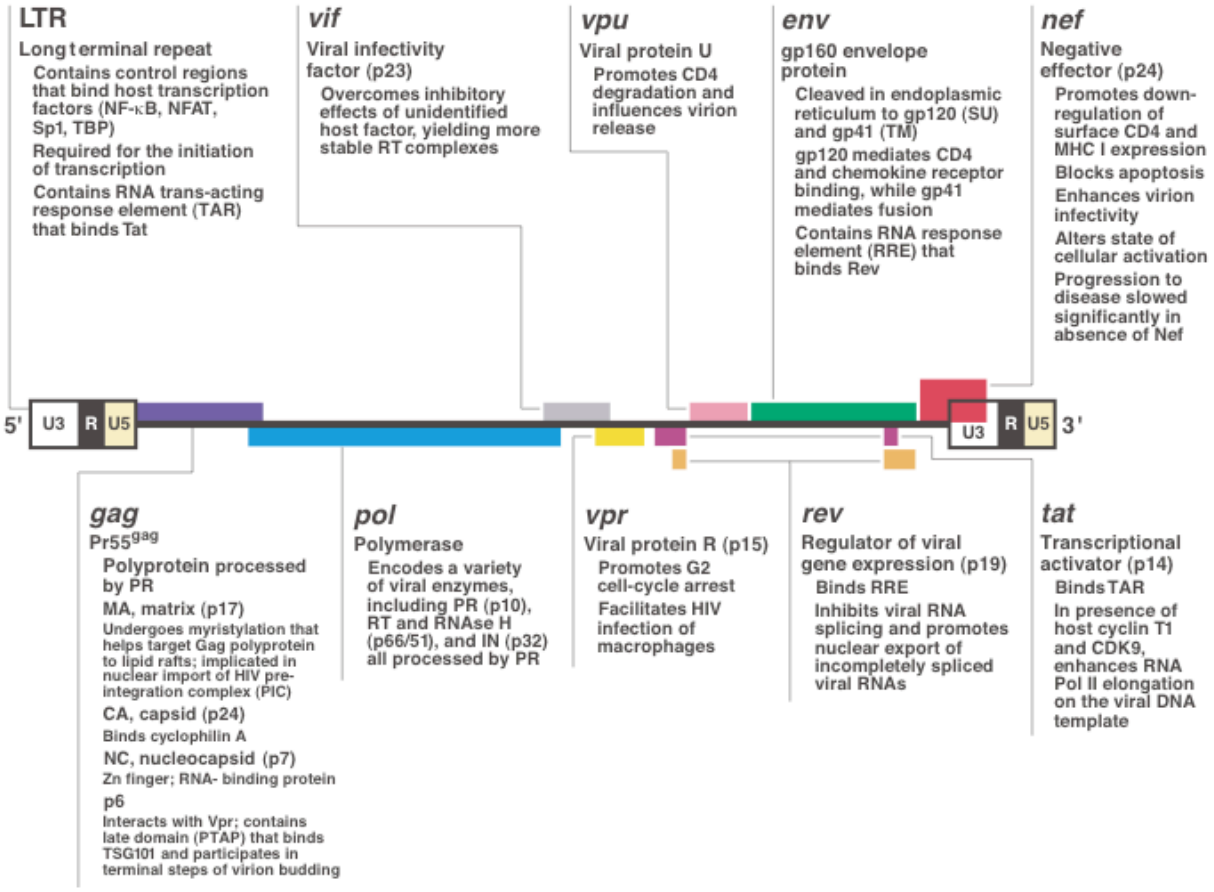


Figure 4[40]**:** Function of HIV-encoded proteins. (Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine, 8:673-680, 2002).

E. HIV-1 LIFE CYCLE

Like the life cycle of other viruses, HIV-1 replication is divided into early (figure 5) and late stages (figure 8). While the early stages are principally directed at creating an environment for the virus to enter the host cell and replicate its genetic material, the late stages drive the assembly of new virions.

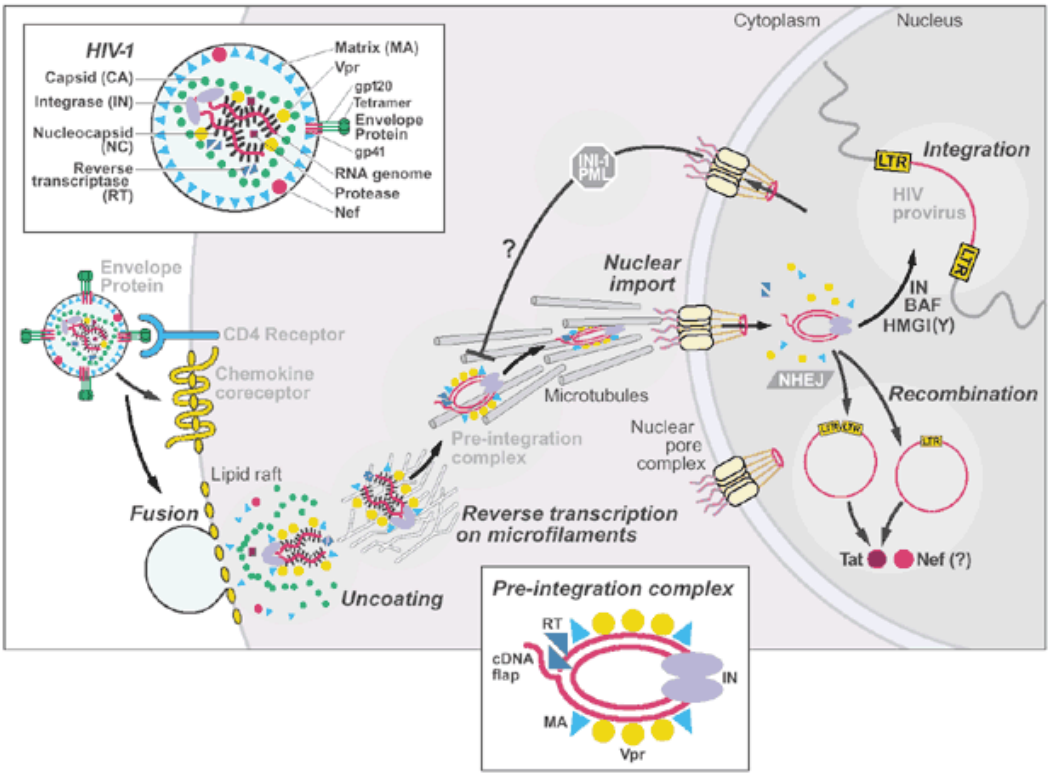


Figure 5[40]: Early stages of HIV replication. (Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine, 8:673-680, 2002).

Entry

Although HIV can enter cells via an endocytic pathway, which usually results in its inactivation in lysosomes, its main point of entry is mediated by the interaction of a trimeric complex of Env gp41 and gp120 proteins with cellular receptors and co-receptors (figure 6)[41]. The transmembrane protein gp41 contains two helical regions, termed HR1 and HR2, and a fusion peptide, which are involved in cell entry. Gp120 is a surface protein that mediates the initial interaction of the virus with the host cell by binding CD4. The native function of CD4, which is found on T-helper cells (CD4+ T cells), macrophages, microglia and dendritic cells, is to act as the co-receptor for MHC

(major histocompatibility complex) class II molecules during the immune stimulation of T-helper cells[42].

Upon binding of CD4, gp120 undergoes a conformational change, which exposes the co-receptor binding sites. While the main co-receptors involved in HIV entry are the seven-transmembrane (7-TM) chemokine receptors CCR5 and CXCR4, several other members of this receptor family can be used as co-receptors: CCR2, CCR3, CCR8, etc[38]. The discovery that CD4 was not sufficient for viral entry was based on the observation that HIV isolates from infected individuals display variable replication efficiency in different CD4+ cells (i.e. different tropism). Some isolates, termed M-tropic, replicate efficiently in macrophage cultures while other isolates are T-tropic and have an elevated replicative capacity in T-cell lines[39]. The tropism of a virus isolate is determined by a variable region (the V3-loop) in gp120 and determines, after the conformational change induced by the binding of gp120 to CD4, the interaction of gp120 with either CCR5 for M-tropic viruses (or R5 viruses) or CXCR4 for T-tropic viruses (or X4 viruses)[39,43]. Following this event, another conformational change, this time in gp41, allows the interaction between HR1 and HR2 and the formation of a six-bundle structure (see figure 6) that results in the insertion of the highly hydrophobic gp41 N-terminal fusion peptide into the target cell membrane. At that point, the viral and cellular lipid bilayers can fuse together, allowing entry of the viral core into the cell.

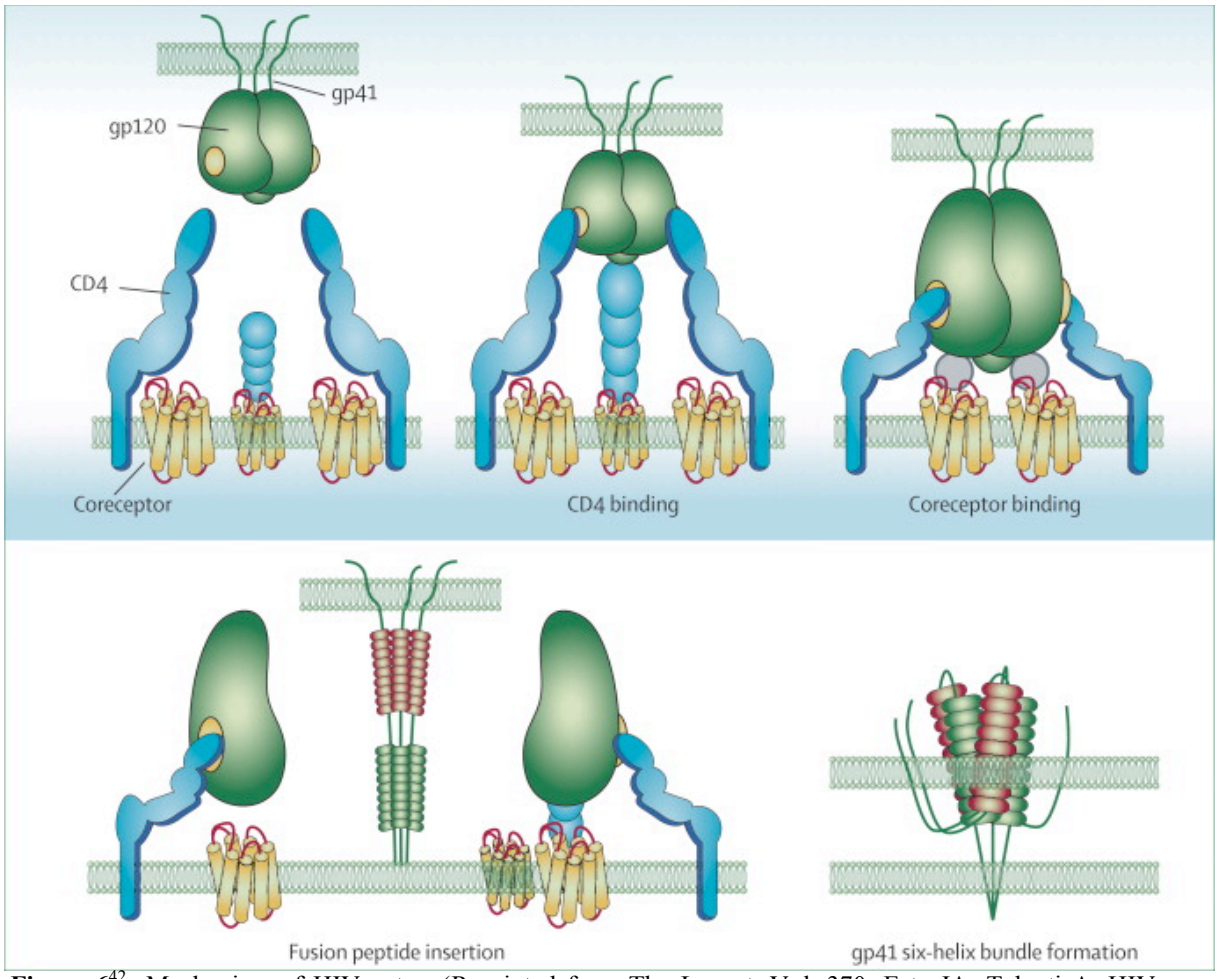


Figure 6[42]**:** Mechanism of HIV entry. (Reprinted from The Lancet, Vol. 370, Este JA, Telenti A, HIV entry inhibitors, 81-88, 2007, with permission from Elsevier).

The requirement for CD4 and CCR5 or CXCR4 co-receptors restricts the capacity of HIV to infect only certain types of human cells, mainly $CD4^+$ T cells and macrophages, but replication has been reported in dendritic cells, NK cells and $CD8^+$ T cells[44-46]. Because DC-SIGN, a C-type lectin found on immature dendritic cells, is capable of binding gp120, dendritic cells are capable of trans-infection, in addition to becoming infected themselves[44,47]. Trans-infection involves the internalization of HIV particles in a non-lysosomal compartment, where it is kept infectious until the carrying dendritic cell encounters a target T cell.

The events that follow fusion of the viral and cellular membrane are poorly understood. In a process termed uncoating, cellular MAP kinase and cyclophilin A and viral proteins Nef and Vif participate in the disassembly of the viral capsid, exposing the viral genome. In association with host and viral molecules required for stable reverse transcription and subsequent stages of the HIV life cycle (reverse transcriptase, integrase, matrix, nucleocapsid, Vpr, Vif, $tRNA^{lys}$, etc.), the diploid viral RNA genome now forms the RTC (reverse-transcription complex) that can dock with actin microfilaments, leading to reverse transcription[40].

Reverse transcription

Conversion of the viral RNA to DNA is carried out in the RTC by the viral reverse transcriptase (RT), a two-subunit enzyme that has both RNase H and DNA polymerase activity. The steps leading to production of HIV DNA are summarized in figure 7. Briefly[39], initiation of reverse transcription occurs when $tRNA^{lys}$ binds the viral primer binding site (PBS) which allows RT to synthesize a short minus-strand DNA fragment towards the 5' end of the plus-sense RNA genome, forming a DNA/RNA hybrid. The RNase H activity of RT then degrades the RNA portion of the hybrid,

generating a DNA fragment that can 'jump' from the 5' to the 3' end of the RNA genome

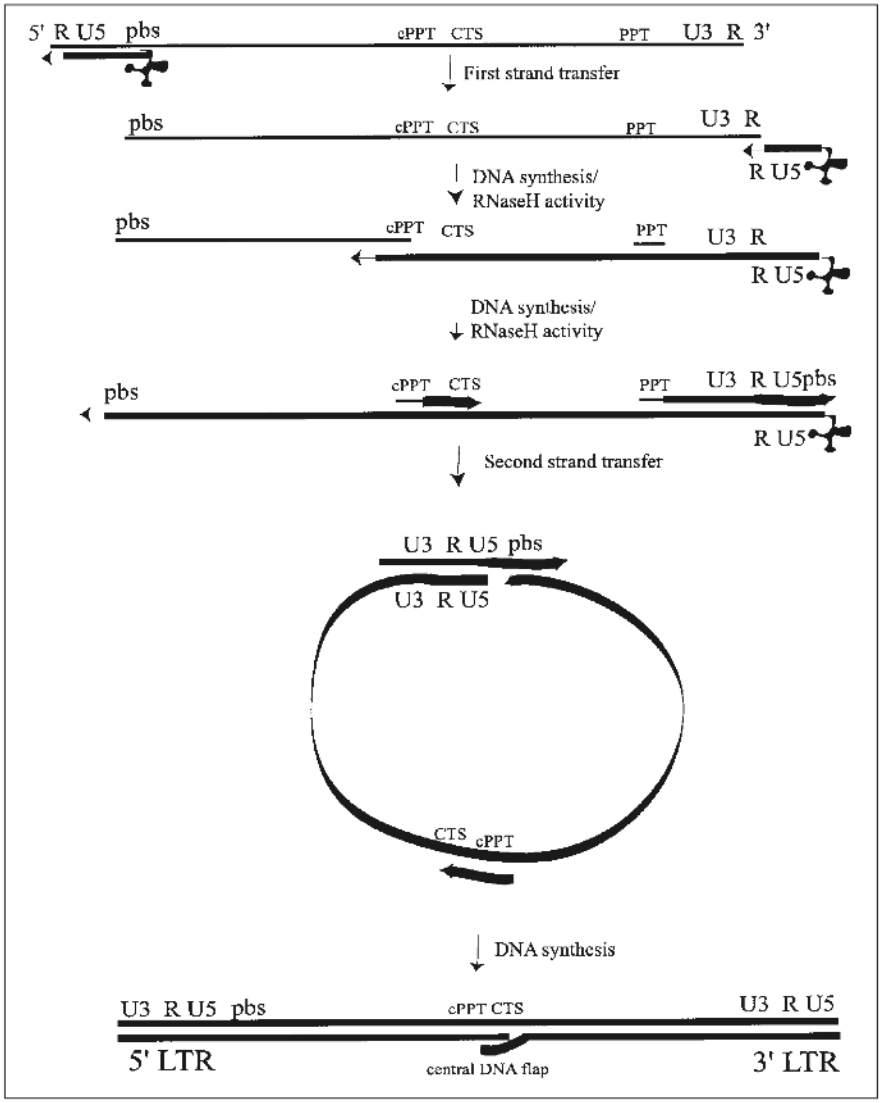


Figure 7[39]**:** HIV reverse transcription steps. Thin lines denote RNA fragments and thick lines DNA fragments. PBS: primer binding site. PPT: polypurine tract. cPPT: central PPT. CTS: central termination signal. (With kind permission from Springer Science+Business Media: Somat Cell Mol Genet, HIV-1 replication, 26, 2001, 13-33, Freed EO, Figure 5).

by interacting with short homologous regions (R-regions). This is called first-strand transfer. Synthesis of the minus DNA strand can then occur by using the 3' end of the transferred DNA fragment as a primer. Synthesis of the plus-sense DNA strand can be initiated at both the polypurine tract (PPT) and the central PPT (cPPT), purine-rich sequences that have resisted RNase H degradation. Once the tRNA bound to the PBS is removed by RNase H, a second strand transfer can occur, allowing plus-strand synthesis to proceed, stopping at the end of the minus strand and at the central termination signal (CTS). Given that the CTS is downstream of the cPPT, an estimated 100 plus-strand DNA nucleotides are displaced resulting in a central DNA flap which has been implicated in the import of the viral genome to the host-cell nucleus.

The process of reverse transcription induces variability among the different viral particles through two mechanisms. Firstly, RT does not have proof-reading capacity resulting in a high mutation rate ($3x10^{-5}$ per cycle of replication)[48]. Secondly, in each viral particle there are two RNA strands that are most likely not genetically identical (see first point). Because there are two strand transfers that occur during reverse transcrition, each of these can result in the 'jump' from one RNA strand to the other, creating a novel recombinant DNA genome containing sequences from both parental RNAs[49]. These inherent 'flaws' of the replication cycle confer HIV with a mutational capacity that has been problematic for both the immune response and drug development.

Nuclear import and integration

Following reverse transcription, the double-stranded viral DNA, in association with proteins such as IN, MA, Vpr and RT, forms the pre-integration complex (PIC)[40].

The PIC must translocate from the cytoplasm to the nucleus such that the viral genetic material can integrate into the host genome. However, the PIC is too large to diffuse into the nucleus through the nuclear pore and must be actively transported[50]. Several mechanisms have been proposed to mediate nuclear import of the PIC[40]. The central DNA flap could bind host proteins containing nuclear-targeting signals[51]. Conversely, MA, Vpr and IN all contain nuclear localization signals (NLS)[52-54]. While the NLS in the MA protein is canonical (a short stretch of basic amino acids) and is recognized by host importins, the NLS of Vpr and IN is non-canonical. Additionally, it is thought that Vpr, by binding proteins of the nuclear-pore complex, could bypass the importin system altogether. Redundancy of mechanisms allowing the import of the PIC into the nucleus could allow infectiousness of HIV in different cell targets, from the activated T-cell to the resting macrophages[40].

Once in the nucleus, IN and host proteins HMGI(Y) (high-mobility group DNA-binding protein) and BAF (barrier to autointegration) are required for integration of viral DNA. While the role of HMGI(Y) and BAF is not clear, it is known that IN clips off nucleotides from the 3' termini of both strands of viral DNA, resulting in recessed 3' ends, and makes a staggered cleavage in the cellular DNA[39,55]. After, the recessed 3' ends of viral DNA join the ends of the cleaved cellular DNA, host repair enzymes fill in the gaps between viral DNA and host DNA. While integration is not specifically limited to an area in the human genome, HIV favors integration in active genes and some intergenic hotspots[56]. Once integrated, the virus, known at this stage as the provirus, behaves like a gene and remains latent.

Expression phase

The mechanisms underlying the switch from a latent to an active phase of replication are still unclear but they depend on the type of cell infected and its activation state. For example, an activated T cell enhances production of cellular factors that stimulate transcription initiation and decreases production of factors that inhibit this process. The viral LTR (long terminal repeat) contains promoter and enhancer sequences that regulate transcription of viral DNA into RNA. Upstream from the transcription start site, Inr (initiator), TATA box and three SP1-binding site are found[57]. These direct the binding of cellular RNA polymerase II (RNAPII). Basal transcription of the HIV LTR is very low, usually ending in the synthesis of short non-adenylated transcripts, stabilized by an RNA stem loop called the TAR (transactivation response) element[58]. Further upstream of the LTR transcription start site are enhancer sequences that bind transcription factors such as NFAT and NF-κB. These are translocated to the nucleus after cellular activation and enhance transcription slightly, making them likely candidates for the switch between HIV latency and activation[40,58]. The increased transcriptional stability induced by NFAT and NF-κB leads to an increased likelihood of viral protein production, including Tat (transactivator). Tat binds the TAR (both in the DNA and transcribed forms) element found in the LTR and recruits cyclin T1 and Cdk9 (cyclin-dependent kinase 9), thus phosphorylating the C-terminal domain of RNAPII and increasing its efficiency to elongate[59,60]. Thus Tat is essential for replication in the host and its increased production marks the initiation of the late stages of viral replication.

Transcription in the nucleus of the HIV coding sequence results in a large number of viral RNAs which can be divided into three classes: 1) fully spliced RNA which are

translated into HIV proteins Rev, Tat and Nef, 2) partially spliced RNA that encode the Env, Vif and Vpu proteins and 3) unspliced RNAs, which function as the messenger RNA for Gag and Pol polyprotein precursors or genomic RNA packaged into virions[39]. The latter two classes of RNA must therefore be found in the cytoplasm, which is complicated by the fact that the majority of cellular messenger RNAs are fully spliced before transport out of the nucleus. The HIV Rev (regulator of expression of viral proteins) protein is essential in the transport of unspliced or partially spliced RNA[61]. These all contain an RRE (Rev responding element), a highly structured RNA sequence located in the *env* coding region which mediates the binding of approximately eight Rev molecules per RNA strand. A NES (nuclear exporting signal) and a NLS (nuclear localization signal) contribute to the shuttling of Rev between the nucleus and cytoplasm, allowing the movement of HIV unspliced or partially spliced RNA towards the cellular translational machinery.

Viral assembly and budding

HIV particles are generated in two majors steps, assembly (figure 8) and budding. The Gag uncleaved polyprotein precursor Pr55 contains three major domains involved in the viral assembly and budding processes[41]. The M domain (membrane targeting) is myristoylated thereby targeting Gag to the lipid bilayer of the plasma membrane by interacting with the negatively charged acidic phospholipids. The I domain (interaction) mediates interaction of Gag monomers whereas the L domain (late) is involved in budding of the virus via its ubiquitylation and interaction of its PTAP (proline-tyrosine-alanine-proline) motif with TSG101 (tumor susceptibility gene 101), a cellular protein

involved in vacuole formation. Mutation in the PTAP tetrapeptide, which is located in the p6 region of Gag, results in viruses unable to pinch off from the plasma membrane[62]. The Gag NC (nucleocapsid) region interacts with the HIV RNA's packaging signal located at the 5' end of the *gag* initiation codon via a zinc finger motif, allowing the viral genome to be recruited[63]. Retroviral RNAs are dimerized by the DIS (dimer initiation signal), a sequence at the 5' end of the genome[39]. Finally, the MA region interacts with the cytoplasmic tail of env gp41, which interacts non-covalently with gp120 on the cell surface, to recruit Env to the virion[39].

Following PTAP-mediated release from the plasma membrane, HIV protease (PR) cleaves the Gag and GagPol polyprotein precursors, which causes a series of structural changes highlighted by the formation of an electron-dense conical core. This process, termed maturation is required for viral infectivity[64].

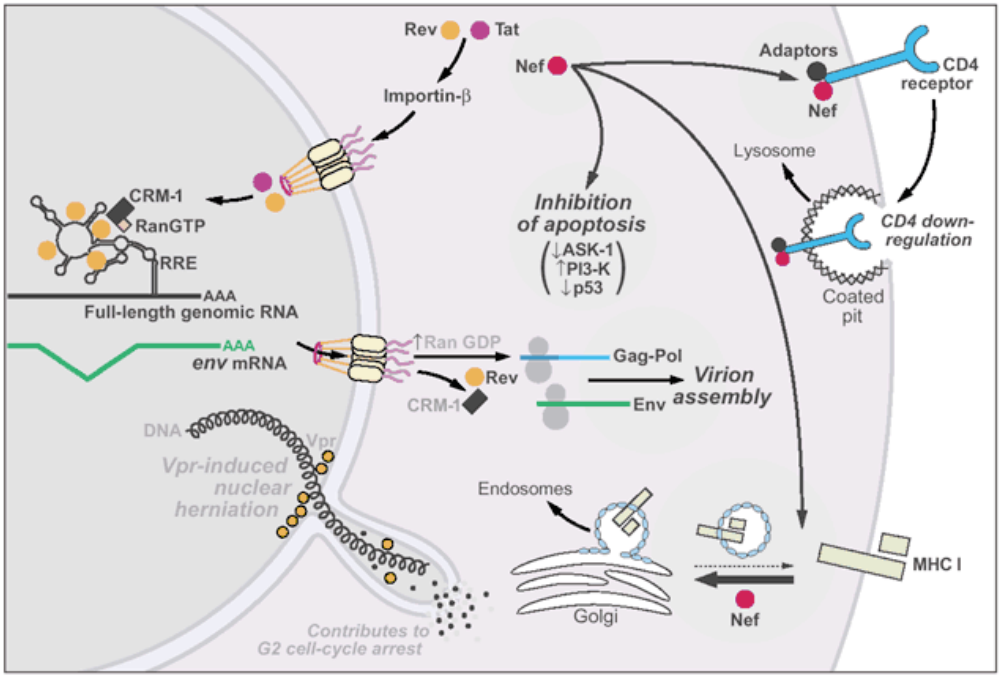


Figure 8[40]: Late stages of HIV replication. (Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine, 8:673-680, 2002).

F. TRANSMISSION

Because it is found in bodily fluids, such as blood and genital secretions, HIV is transmitted via sexual contacts, MTCT (mother to child transmission) or exposure to infected blood (injection drug use, transfusion, occupational exposure). The efficiency of all these modes of transmission is, however, strongly dependent on the fluid viral loads. Increased virus concentrations in blood, semen or breast milk correlate directly with increased risk of transmission[65-68]. Since viral load varies during HIV disease course, the stage of infection of the transmitting individual is an important factor of efficiency of transmission (figure 9). In the acute phase of the infection, HIV replication is largely unchecked by host defenses, leading to a peak in viral titer[69]. This peak, accompanied by the fact that individuals in the acute phase of infection are usually unaware of the fact that they have become infected, and thus less likely to take precautionary measures,

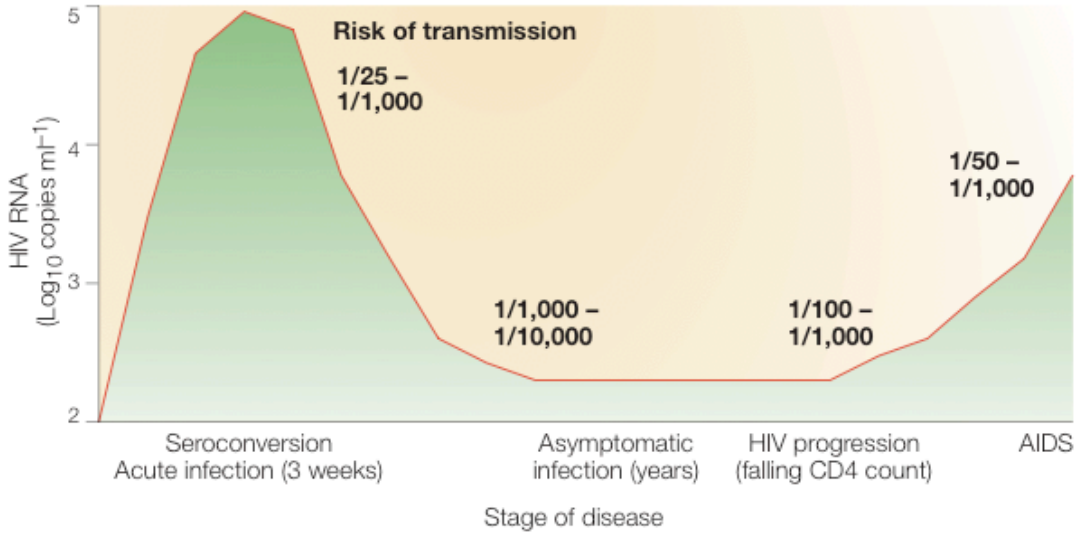


Figure 9[70]**:** Effect of viral load on risk of sexual HIV transmission during different stages of disease. (Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Microbiol, 2:33-42, 2004).

makes the acute phase very dangerous for HIV transmission[71].

Sexual transmission

Between 75-85% of individuals infected with HIV worldwide have acquired the virus via sexual contact, the majority through unprotected heterosexual intercourse[72]. Different sexual practices have varying risks of transmission. The probability of contracting HIV via vaginal intercourse with no other co-factors is approximately 0.0011 per coital act, which is almost eight times less than by receptive anal intercourse (0.0082)[73,74]. Although less probable, cases of infection between female partners and via oral sex have been reported[75-77]. The presence of other sexually transmitted infections (STI), such as syphilis or gonorrhea, can increase the likelihood of transmission of HIV via sexual contact. STIs can increase viral titers in the index individual, damage mucosal surfaces, change the genital microenvironment or stimulate immunological changes favoring infection[70]. While condom use has been shown to dramatically decrease HIV transmission, the need for a female-controlled method of protection has lead to the development of microbicides, topically applied anti-microbial chemical agents[78]. However, these have yet successfully passed phase III clinical trials[79]. Finally, epidemiological associations and randomized clinical trials have shown that circumcision decreases likelihood of HIV transmission during sexual contact[80]. This phenomenon has been explained by the susceptibility of the foreskin in uncircumcised men to tears, abrasions, and STIs. In addition, Langerhans cells and other target cells in the foreskin increase HIV absorption. Mathematical models have shown that implementing circumcision programs in countries with widespread HIV prevalence would lead to

marked reductions in HIV infection in men (and subsequently in women) in a cost-effective manner[81]. However, several challenges, including evaluating social acceptability and health concerns, need to be addressed before such a program is put in place[82].

MTCT

While MTCT (or vertical transmission) has been decreased to less than 1% in the western world, in some developing regions transmission rates are still over 10%[68]. This disparity is related to the challenges faced in these countries in terms of HIV testing, care and prevention. The issue is complicated by the fact that vertical transmission can occur before birth (*in utero*), during delivery (intrapartum) or after birth via breastfeeding.

In utero transmission, which usually occurs during the third trimester, results when HIV crosses the placenta. However, the majority of vertical transmissions occur during the delivery process, when the neonate passes through the birth canal and is exposed to infected maternal blood or genital secretions[68]. The rate of transmission that occurs because of breast milk feeding is hard to distinguish from late *in utero* or intrapartum transmission because of the proximity of these events, but it was evaluated to be 16% in a randomized trial in Kenya comparing HIV seropositive mothers that were breastfeeding to those that were formula feeding[83]. In some countries, infected women continue to breastfeed their newborns because of the stigma often associated with not breastfeeding, because of the cost associated with alternative feeding methods or because of the lack of access to clean water[68]. Interestingly, recent evidence suggests that avoidance of breastfeeding, a policy that has worked well in the western world, may not

be advantageous in HIV endemic countries because of the beneficial properties of maternal milk on the immune system of the infant. This was supported by an increase in overall mortality rates in formula-fed infants caused by diarrhea and pneumonia when compared to a group of breastfed infants that were also given zidovudine (to decrease the risk of HIV transmission)[84]. While strict formula feeding has the advantage of zero-risk transmission, mothers may still occasionally breastfeed even when formula is supplied to them to avoid stigma and complexities associated with formula feeding[85]. Often, this means that formula-based prevention programs have similar HIV transmission rates as breastfeeding plus zidovudine programs[84].

The evolution of HIV infection in children is more rapid with a median survival ranging from 75 to 90 months and only 70% of individuals infected via MTCT reaching the age of 6 years[16]. Fortunately, obstetric interventions, such as cesareans, alternatives to breastfeeding and the advent of HAART (highly active antiretroviral therapy) have all lead to reduction of MTCT in regions where these are available. HAART, a prophylactic measure for the infant, also decreases maternal viral loads. In resource poor countries, where HAART is currently not available, short-course zidovudine (or AZT) or single-dose nevirapine can decrease the incidence of vertical transmission incidence[86].

Injection drug use transmission

Globally, an estimated 3 million injection drug users (IDU) are infected with HIV[87]. The most affected regions are eastern Europe, southeast Asia and Latin America[87]. Transmission of HIV in this case occurs when IDU inject themselves with contaminated syringes. The probability of infection per injection with a contaminated

syringe was estimated to be 0.0067[88]. Usually, programs based on increasing the availability of sterile injection equipment, either through pharmacy sale or needle-exchange programs, lead in decreased HIV-risk behavior[89].

Blood transmission

There are still 5-10% of global new infections that occur through transfusion of infected blood[90]. Highly sensitive screening tests used in developed countries have minimized the risk of HIV transfusion. However, according to the WHO (World Health Organization), only 75% of the blood supply in Africa is screened for the presence of HIV[91]. Improving this statistic by training health-care workers and expanding blood management systems will be essential for preventing new infections. Not surprisingly, blood transfusion with infected blood carries the highest risk of transmission, with approximately 90% of recipients seroconverting[92].

Other factors in transmission efficiency

Other than route of exposure, factors that affect the likelihood that an infection will occur after exposure include viral strain and host genetics[70]. Different HIV clades may vary in their viral replication capacity (therefore influencing viral load) or may differ in their target cell tropism. The topic of the influence of human genetics on HIV infection will be covered in section IV.

G. CLINICAL COURSE OF INFECTION

Following exposure, in the case of a productive HIV infection, T cells, macrophages and dendritic cells surrounding the inoculation site can become infected. In the first few days, only a small number of cells located in the draining lymph node, mostly resting CD4+ T cells, sustain a modest primary amplification[93]. This first wave of amplification is thought to be largely supported by dendritic cells (DC), which either become infected themselves or bind the virus via DC-SIGN (see section IE) and then migrate to T cell-rich regions of lymph nodes[93]. This would bring the virus in

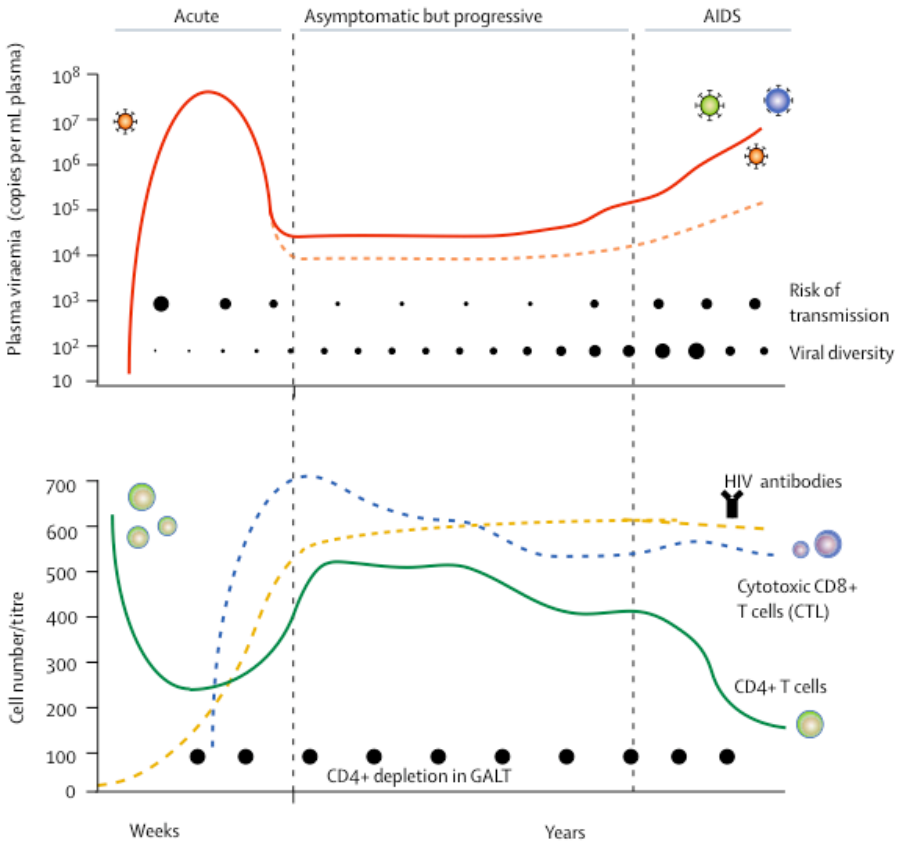


Figure 10[18]**:** Natural history of untreated HIV infection. (Reprinted from The Lancet, Vol. 368, Simon V, Ho DD, Abdool Karim Q, HIV/AIDS epidemiology, pathogenesis, prevention and treatment, 489-504, 2006, with permission from Elsevier).

close contact with its favored target cell, activated T cells. DC-mediated primary dissemination partly explains the fact that R5- and not X4-tropic viruses are the usual strain transmitted to a new host[94]. The CCR5 co-receptor is expressed on Langerhans cells (subcutaneous DCs) as well as macrophages and CD4+ T cells. Following primary amplification, infected T lymphocytes and virions can migrate into the bloodstream and drive secondary amplification by infecting susceptible cells in the gastrointestinal tract, spleen and bone marrow[18]. Because the receptors and co-receptors for HIV are found on several different cell types (CD4+ T cells, macrophages, dendritic cells), the virus is now found in various replicative states and tissue compartments[95]. This spread creates a diverse viral reservoir, which is difficult for the host immune response to eradicate and which complicates treatment. The established infection can be clinically divided into three stages: acute infection, asymptomatic phase and AIDS (figure 10).

Acute infection

Symptoms of HIV infection usually appear 2-6 weeks following exposure and resemble those of EBV (Epstein-Bar Virus) or influenza virus infections[96]. While it is thought that the infection is symptomatic in up to 90% of individuals, the non-specificity of these symptoms (see table 3) leads to its underdiagnosis[94]. Laboratory tests, often on blood samples, can be used to confirm infection. Diagnostic tests are usually based either on the detection of host responses to HIV (serological tests) or on the detection of the virus itself. Highly sensitive, highly specific, cost-effective and time-efficient, EIA (enzyme immunoassays) and western blots are commonly used in HIV testing. In both

cases, the patient's plasma is incubated with viral proteins (usually p24, gp41 and gp120). If the individual is infected and has produced anti-HIV antibodies, these will bind the viral protein. This complex can then be detected by electrophoretic separation (western blot) or enzyme-linked anti-human antibodies (EIA, can also be based on enzyme-linked antigens)[97]. Because they depend on the production of HIV specific antibodies, these tests

Table 3[94]**:** Common symptoms of acute HIV infection

Clinical symptom	Patients, %
Fever	80
Fatigue	70
Weight loss	70
Pharyngitis	50-70
Myalgia	50-70
Diarrhea	50
Rash	40-80
Lymphadenopathy	40-70
Headache	30-70
Nausea	30-60

only become efficient 3-12 weeks after infection[98]. This is why tests based on the detection of the virus have also been developed. While plasma levels of p24 antigens are transient, they can also be detected by EIA and usually lead to detection of infection 2 weeks post-exposure[99]. Finally, 2-3 days prior to a positive p24 assay, the viral load in the plasma is sufficiently elevated that it can be detected via polymerase chain reaction (PCR) or hybridzation with oligomeric probes[97,100]. While capable of detecting infection

the earliest, viral load based tests are usually not used alone for diagnosis because of their increased rate of false positive results over serological tests[98].

Peak viral load, 1-10 million copies/ml of plasma, usually occurs 6-15 days after onset of symptoms and is transient[93]. With the appearance of adaptive immune responses, specifically HIV-specific CD8+ T cells, the viral load decreases and is controlled at a relatively stable plateau[101,102]. The level of this viral plateau is a strong correlate of the rate of disease progression[103]. However, CD4+ T cell count and, even more so, levels of immune activation are also strong predictors of rate of disease progression[103-105].

Within the first week of infection, there is substantial loss of the CD4+ T cell population (figure 10). It has been shown both in SIV and HIV infection that this loss occurs primarily in CD4+CCR5+ T cells of the gut associated lymphoid tissue (GALT) where the majority of CD4+ T cells are found[106-108]. Both direct killing of the target cells and bystander mechanisms have been implicated in depletion of CD4+ T cells[107,108]. As measured by plasma lipopolysaccharide (LPS; found in gram negative bacteria) levels, it was recently shown that this early damage to the gut leads to increased microbial translocation that may be responsible for inducing systemic immune activation[109]. In SIV natural hosts (African green monkeys and sooty mangabeys), which undergo a nonpathogenic course of infection, while there is GALT CD4+ loss in acute infection, for reasons that are still unclear, this is not followed by microbial translocation and chronic immune activation[110]. This central difference between nonpathogenic and pathogenic infection has lead to the current argument that HIV disease is a result of systemic immune activation that itself results from events in acute infection (see figure 11). While immune activation has the benefit of restoring the CD4+ T cell pool, it also provides the

virus with abundant target cells for replication in addition to reducing T cell half-life, inducing thymic dysfunction, clonal exhaustion, loss of the memory T cell pool and retention of effector T cells in the lymph nodes[111]. Overall, there is a gradual loss of CD4+ T cell and collapse of the immune system.

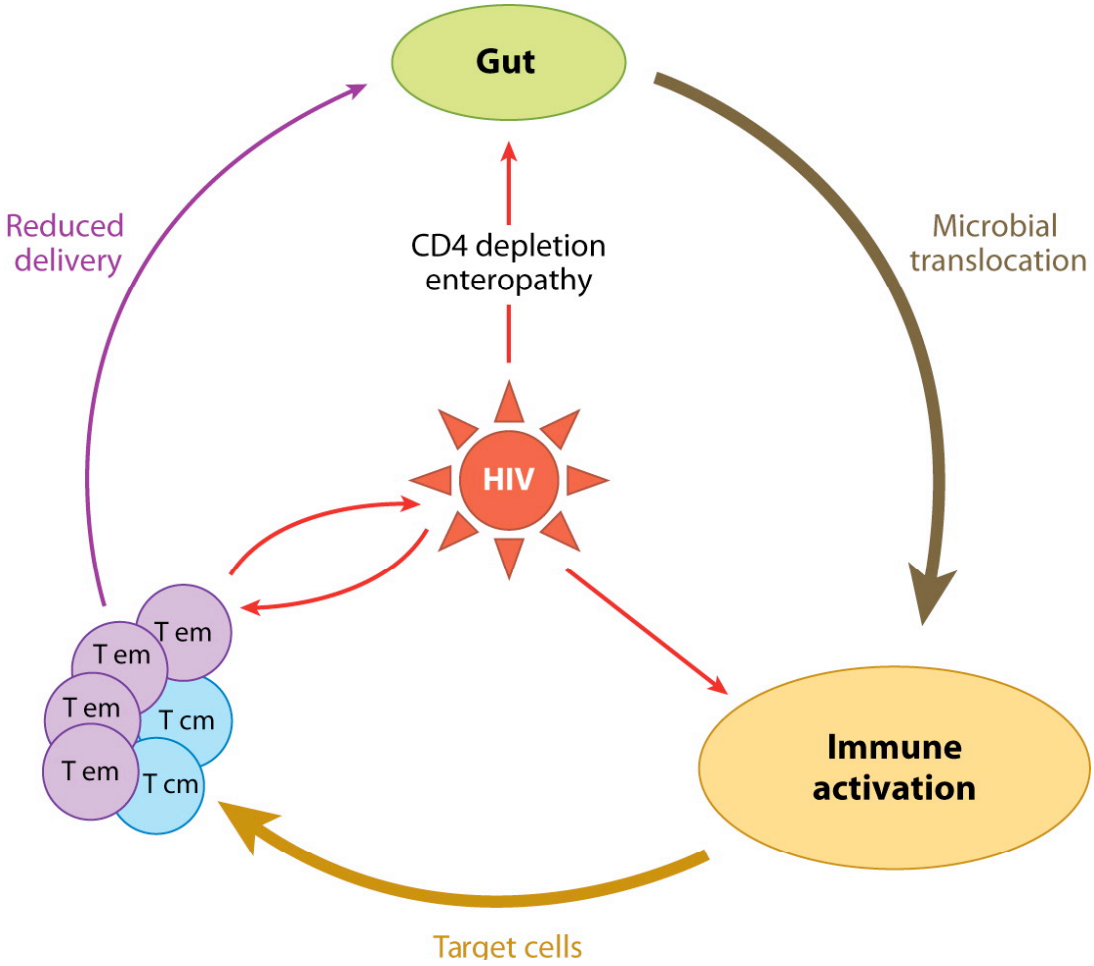


Figure 11[111]: Immunopathogenesis of HIV infection. (Reprinted, with permission, from the *Annual Review of Medicine*, Volume 60 © 2009 by Annual Reviews, www.annualreviews.org).

Asymptomatic phase

Approximately six months after infection, individuals enter an asymptomatic phase. However, despite the lack of severe clinical symptoms, there is ongoing viral

replication during this clinical phase[12]. In addition, due to mechanisms explained above, there is a gradual decline of 50-90 CD4+ T cells/ml/year[112,113]. This constant loss usually lasts ten years before immune system deteriorates to the point where AIDS is diagnosed.

AIDS

To be diagnosed with AIDS, an HIV infected individual must have a CD4+ T cell count below 200 cells/μL of blood or be affected by at least one AIDS-defining illness[114]. The list of AIDS-defining illnesses includes: esophageal candidiasis, Kaposi's sarcoma, pneumocystis carinii pneumonia, toxoplasmosis, wasting syndrome, neuropathy, etc[114]. These are caused by the HIV-induced breakdown of the immune system and usually result from opportunistic fungal, parasitic, viral and bacterial infections. The term opportunistic refers to the fact that these microorganisms are ubiquitous and generally harmless to immunocompetent individuals[20]. The survival time of an untreated individual diagnosed with AIDS is approximately 1 year but can vary according to the AIDS-defining condition[20,115].

H. CURRENT ANTIRETROVIRAL THERAPY

Only four years elapsed between the isolation of HIV-1 from patients affected with AIDS in 1983 and the FDA (US Food and Drug Administration) approval of the first HIV-specific antiretroviral, zidovudine (or azydothimidine - AZT), in 1987[5,6,116]. Before AZT became available for treatment, physicians were limited to the management of opportunistic infection affecting their seropositive patients. This course of action had little success, patients usually dying within a year after the diagnosis of an AIDS-defining

illness[20]. Access to AZT, which inhibits the reverse transcriptase viral enzyme, led to a sense of excitement in the medical field arising from the possibility that perhaps HIV could be controlled. However, it became rapidly clear that monotherapy was insufficient to control HIV replication long term. Its high rate of replication, the low fidelity of reverse transcription and its capacity for recombination lead to an elevated genetic diversity and the development of drug resistant strains[18]. This realization paved the way for a new therapeutic strategy: HAART (highly active antiretroviral therapy). A patient on HAART would receive several drugs that would target different steps in the viral life cycle. It is this strategy that has provided the impetus for the development of 32 antiretroviral drugs currently approved by the FDA, which can be divided in 6 different classes (see table 4).

HIV drug classes

The initial target of HIV drug design was the Reverse Transcriptase (RT). Nucleoside Reverse Transcriptase inhibitors (NRTI) was the first class of drugs introduced against HIV. NRTIs are structurally similar to nucleosides but lack the 3'-hydroxyl group required for DNA elongation[117]. They thus inhibit HIV replication by binding competitively to the active site of RT, then they are integrated in the viral DNA strand, resulting in chain termination. Most treatment regimens contain at least two NRTIs, chosen on the basis of efficacy and toxicity for each patient[118]. Also targeting RT, NNRTIs (non-nucleoside Reverse Transcriptase inhibitors) act as non-competitive

inhibitors since they bind a site on RT distal from the nucleotide binding site[119].

Multi-class Combination Products

Brand Name	Generic Names	Manufacturer Name	Approval Date
Atripla	efavirenz, emtricitabine and tenofovir disoproxil fumarate	Bristol-Myers Squibb and Gilead Sciences	12-July-06

Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

Brand Name	Generic Name(s)	Manufacturer Name	Approval Date
Combivir	lamivudine and zidovudine	GlaxoSmithKline	27-Sep-97
Emtriva	emtricitabine, FTC	Gilead Sciences	02-Jul-03
Epivir	lamivudine, 3TC	GlaxoSmithKline	17-Nov-95
Epzicom	abacavir and lamivudine	GlaxoSmithKline	02-Aug-04
Hivid	zalcitabine, dideoxycytidine, ddC	Hoffmann-La Roche	19-Jun-92
Retrovir	zidovudine, azidothymidine, AZT, ZDV	GlaxoSmithKline	19-Mar-87
Trizivir	abacavir, zidovudine, and lamivudine	GlaxoSmithKline	14-Nov-00
Truvada	tenofovir disoproxil fumarate and emtricitabine	Gilead Sciences, Inc.	02-Aug-04
Videx EC	enteric coated didanosine, ddI EC	Bristol Myers-Squibb	31-Oct-00
Videx	didanosine, dideoxyinosine, ddI	Bristol Myers-Squibb	9-Oct-91
Viread	tenofovir disoproxil fumarate, TDF	Gilead	26-Oct-01
Zerit	stavudine, d4T	Bristol Myers-Squibb	24-Jun-94
Ziagen	abacavir sulfate, ABC	GlaxoSmithKline	17-Dec-98

Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Brand Name	Generic Name	Manufacturer Name	Approval Date
Intelence	etravirine	Tibotec Therapeutics	18-Jan-08
Rescriptor	delavirdine, DLV	Pfizer	4-Apr-97
Sustiva	efavirenz, EFV	Bristol Myers-Squibb	17-Sep-98
Viramune	nevirapine, NVP	Boehringer Ingelheim	21-Jun-96

Protease Inhibitors (PIs)

Brand Name	Generic Name(s)	Manufacturer Name	Approval Date
Agenerase	amprenavir, APV	GlaxoSmithKline	15-Apr-99
Aptivus	tipranavir, TPV	Boehringer Ingelheim	22-Jun-05
Crixivan	indinavir, IDV,	Merck	13-Mar-96
Fortovase	saquinavir (no longer marketed)	Hoffmann-La Roche	7-Nov-97
Invirase	saquinavir mesylate, SQV	Hoffmann-La Roche	6-Dec-95
Kaletra	lopinavir and ritonavir, LPV/RTV	Abbott Laboratories	15-Sep-00
Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03
Norvir	ritonavir, RTV	Abbott Laboratories	1-Mar-96
Prezista	darunavir	Tibotec, Inc.	23-Jun-06
Reyataz	atazanavir sulfate, ATV	Bristol-Myers Squibb	20-Jun-03
Viracept	nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14-Mar-97

Fusion Inhibitors

Brand Name	Generic Name	Manufacturer Name	Approval Date
Fuzeon	enfuvirtide, T-20	Hoffmann-La Roche & Trimeris	13-Mar-03

Entry Inhibitors - CCR5 co-receptor antagonist

Brand Name	Generic Names	Manufacturer Name	Approval Date
Selzentry	maraviroc	Pfizer	06-August-07

HIV integrase strand transfer inhibitors

Brand Name	Generic Names	Manufacturer Name	Approval Date
Isentress	raltegravir	Merck & Co., Inc.	12--Oct-07

Table 4[120]**:** FDA approved drugs for the treatment of HIV**. (**Reproduced from FDA website, May 9, 2009: http://www.fda.gov/oashi/aids/virals.html)

The NNRTI-bound enzyme suffers a conformational change, which inhibits its enzymatic capacity[119]. NNRTI-based regimens are usually prescribed initially, having the advantage over protease-based regimens of established efficacy and lower pill burden[118].

Protease inhibitors (PI) prevent the viral enzyme from processing viral precursor proteins, a step required for the formation of new virions. PIs are part of many initial regimens and of salvage therapy, when multidrug-resistant HIV has developed[118].

While NRTIs, NNRTIs and PIs have been the most widely used drugs in the fight against HIV, three newer classes of drugs have provided the medical field with additional ammunition against the virus. The fusion inhibitor T-20, which is not orally bioavailable and must be injected, prevents entry of HIV into CD4+ cells by inhibiting fusion of the viral membrane with the target cell membrane[118]. Maraviroc is an entry inhibitor, preventing the interaction of HIV with the CCR5 co-receptor[118]. Finally, integrase inhibitors prevent viral DNA insertion into the host genome by inactivating Integrase and preventing binding to host DNA[121].

HAART: successes and pitfalls

Since the introduction of HAART for the clinical management of HIV disease, HIV infection has evolved from a fatal infection to a chronic disease. In the USA, 85-90% of individuals diagnosed with AIDS now survive more than 6 years[122]. In parallel, HAART has led to decreases in opportunistic infections and plasma viral loads as well as increases in CD4+ T cell counts[123-125]. However, while beneficial, HAART is not the ideal solution to the HIV epidemic, as there are drawbacks associated with its use.

Since HIV is capable of integrating into the host genome, it establishes a viral reservoir in long-lived resting CD4+ cells (e.g. memory T cells, macrophages). Current therapeutic strategies, including HAART, thus fail to eradicate the infection and are not cures for HIV.

In addition, like any drug regimen, HAART is associated with a number of adverse events (figure 12). NRTI are designed to interact with RT, but can also affect other nucleotide-binding proteins, such as DNA polymerase γ, the only enzyme involved in replication of human mitochondrial DNA[126]. Impaired function of this DNA polymerase has been linked to lactic acidosis, hepatic steatosis and myopathy[127]. While this adverse effect is specific to NRTIs, most drug classes have hepatic toxicities either through their interaction with liver enzymes, mitochondrial toxicity or host hypersensitive reactions to the drug[128]. Another common adverse effect of NRTIs and PIs is lipodystrophy, characterized by the loss of peripheral fat and the accumulation of central fat, which is linked to an increased likelihood of insulin resistance[126].

HAART drug regimens can be complex, sometimes requiring taking 6-12 pills per day. Furthermore its use is often accompanied by adverse side effects. For these reasons, patients taking HAART can fail to adhere strictly to treatment[129]. However, a sustained virological response is strongly correlated with drug adherence[130]. While adherence may need to be slightly less rigorous for NNRTI regimens, it needs to be greater than 95% in order to achieve viral suppression PI regimens[131,132]. Poor adherence and HIV's naturally elevated mutational capacity favor the emergence of drug resistant viruses and, eventually, drug failure. The ease with which HIV develops resistance depends on each drug, some such as Lamivudine require only a single point mutation[133]. Unfortunately,

development of resistance is almost inevitable and affects most patients. This was demonstrated by a study showing that among viremic individuals, 76% were resistant to at least one class of antiretrovirals[134].

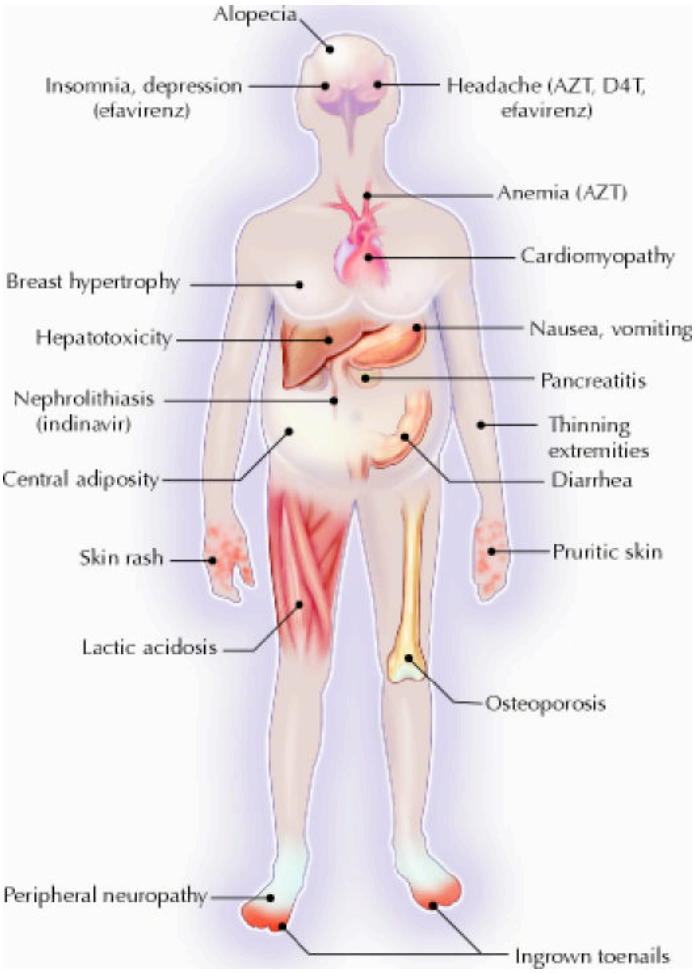


Figure 12[126]**:** Adverse events associated with HAART. *(© Canadian Medical Association. Reprinted by kind permissio from Montessori et al., CMAJ, 170 (2): 229-38, Jan 20 2004. Illustration by Chesley Sheppard. Copied under licence from Access Copyright. Further reproduction prohibited).*

Persistence of a viral reservoir, toxicities and development of resistance seem, for the moment, inseparable form HAART. There is little doubt that HAART remains the treatment approach of choice for HIV and increased efforts should be focused on

expanding its access globally. At the moment an estimated 80% of people with a clinical need for treatment do not have access to antiretrovirals because of licensing policies, elevated costs and lack of required infrastructure[18]. However, the better solution to the HIV pandemic remains the development of a preventative vaccine.

I. THE FIELD OF HIV VACCINES

While vaccination has lead to tremendous global successes for certain viruses such as polio, measles and smallpox, an effective vaccine against HIV remains elusive despite considerable dedication of scientific and monetary resources to this goal. In 2006 alone, public sector investment for the development of an HIV vaccine was evaluated at US$776 million[135]. The fact that an efficient vaccine is still unavailable reflects how challenging the quest has been.

Challenges in HIV vaccine design

HIV's genetic diversity not only allows it to develop drug resistance, but it also creates challenges for vaccine design. As discussed in section IB, HIV-1 can be divided into several subtypes and sequence diversity within a subtype can be as elevated as 20%[136]. Even within an individual, HIV sequence diversity "can exceed the variability generated over the course of a global influenza epidemic, the latter of which results in the need for a new vaccine each year"[137]. Which viral epitopes should be targeted in order to protect against all potential HIV strains in this context represents one of the current unanswered questions. The fact that HIV infects $CD4^+$ cells and results in massive loss of $CD4^+$ T cell in primary infection also impedes vaccine development (section IG)[137].

$CD4^+$ T cells are crucial in the modulation of the immune response. If these are lost before the response can be mounted, a vaccine relying on effective T-helper cell support may be difficult to design. Finally, the virus has evolved strategies to evade the immune response[138]. A notable example includes the Nef-mediated downregulation of MHC (major histocompatibility complex) molecules, which are required for T-cell recognition of HIV epitopes. Also, heavy glycosylation of envelope proteins masks epitopes targeted by neutralizing antibodies, which explains at least partially the failure of the VAX004 trial. This vaccine designed to induce neutralizing antibodies by immunizing against gp120 was tested in a phase III clinical trial, but failed to protect against infection despite the elicitation of anti-HIV antibodies[139].

In addition to these HIV-related challenges, the fact that the type of immune response that needs to be elicited (i.e. the correlates of immune protection) is still unclear, that attenuated viruses are unsafe for use in human, and that a good small animal model is still lacking all impede the development of a protective vaccine[137,138].

Failure of the STEP trial

In 2007, a T-cell based vaccine trial (STEP trial) was interrupted for futility in achieving its primary end-points. Although the vaccine, which used recombinant adenovirus 5 (rAd5) as a vector, induced HIV-specific T cell responses, no protection from infection was observed and individuals who became infected were not more likely to have lower viral loads when compared to the placebo arm[140,141]. Causing an even greater setback, individuals with pre-existing antibodies against Ad5 were at greater risk of contracting HIV than the placebo-vaccinated individuals, perhaps due to the activation

and increased infection susceptibly of T-cells following formation of Ad5 immune complexes[140,142]. The failure of this trial has led the scientific community to conclude that HIV immunology needs to be better understood in order to be able to design a vaccine[137,138,143,144]. While neutralizing antibodies and T-cell responses are still the major focus of vaccine design efforts, there is an increasing interest for the role of the innate immune system in HIV infection[137,144-146]. In the early stages of an infection, effectors of the innate arm, such as dendritic cells, macrophages, and NK cells, mediate viral recognition and control. These cells are also central in inducing and modulating subsequent T- and B-cell responses[147-149]. Thus, clarifying the interplay between HIV and innate immunity may provide clues for a better vaccine. While all cells of the innate immune system deserve attention, recent evidence that NK cells may have an influence on outcome of HIV infection has brought some of the spotlight on these cells[150-152].

II. NK Cells

In the late 1960s and early 1970s, several investigators observed the capacity of lymphocytes isolated from non-immunized human or mouse hosts to lyse certain tumor cells[153-157]. This phenomenon was termed 'natural immunity'[158]. At the time, it was unclear whether the effectors of natural immunity, 'natural killer (NK) cells', were a single or many cell types. However, within the human peripheral blood lymphocytes, most of natural immunity was eventually found within the morphologically distinct large granular lymphocytes (LGL), which were characterized by cytoplasmic granules and a high cytoplasmic:nuclear ratio[159]. LGL were later shown to contain non-MHC-restricted $CD3^+$ cells (i.e. a subset of T cells) and $CD3^-$ cells[158]. While both cell types were capable of natural immunity, the $CD3^-$ fraction of LGLs mediated most of the cytotoxicity against MHC-devoid target cells[158]. These are today known as NK cells[147].

A. Biological importance of NK cells

Approximately 5-15% of circulating peripheral blood lymphocytes are NK cells[160]. These are characterized by the absence of CD3 (a molecular component of the T-cell receptor complex) and the expression of CD56 and/or CD16. Defined by these molecules, there are two main types of NK cells: $CD56^{bright}CD16^{dim/-}$, which account for approximately 10% of circulating human NK cells, and $CD56^{dim}CD16^+$, which account for 90% of the NK cell population in the periphery[161]. A third type of NK cell, $CD56^-$ $CD16^+$, is 'anergic' and usually found at very low frequencies in healthy individuals, but can be expanded in some diseases, such as HIV infection[162,163]. Also found on a minority

of T cells, CD56 is an isoform of NCAM (neural cell adhesion molecule) and its function on NK cell is still unknown[164,165]. CD16, or FcγRIII, mediates antibody-dependent cellular cytotoxicity (ADCC) by binding the Fc portion of IgG antibodies coating the target cell and activating the NK cell via immunoreceptor tyrosine-based activation motif (ITAM) signaling (see section IIC)[161]. While the initial description of NK cells was made based on their ability to kill cancerous cells, they are also involved in controlling viral infections. The importance of NK cell in tumor and viral immunology is perhaps best illustrated by cases of NK cell deficiencies (NKD).

NK cell deficiencies

Several genetic defects result in altered NK cell function or phenotype (severe combined immunodeficiency, bare lymphocyte syndrome, Wiscott-Aldrich syndrome, etc.)[166]. However, in most cases, these syndromes alter several other components of the immune system and provide limited insight into the specific role of NK cell in human immunology. As of 2006, a few cases of isolated NKDs have been reported, although these best highlight the specific contribution of NK cells to maintenance of health[166]. Isolated NKDs are classified into three categories: absolute NKD (ANKD), classical NKD (CNKD) and functional NKD (FNKD) (table 5)[166].

ANKD, of which there are six reports, is defined by the complete absence of $CD56^+$ cells, including NK cells and $CD56^+$ T cells (NKT cells)[167-172]. Most individuals affected with ANKD suffered infection from viruses of the *Herpesviridae* family, such as varicella-zoster virus[170], Epstein-Barr virus[172], cytomegalovirus[169] and herpes simplex virus[167]. In one case, the deficiency was marked by mycobacterium avium infection[168].

Premature death (from 18 months to 21 years of age) was observed in approximately 40% of cases.

To date, there have been only two reports of CNKD[173,174]. Perhaps the strictest definition of NKD, it is described by the complete absence of NK cells with presence of NKT cells[166]. One individual died in his early 30s of human papilloma virus-associated malignancies while the other was treated for disseminated fungal infection. The limited number of cases of reported CNKD may be the result of under characterization of NKT cells in some patients with NKD[166].

Table 3 Classification of the isolated natural killer cell deficiencies

Diagnosis[a]	Natural killer cell function[b]	Natural killer T cells[c]	Natural killer cells[d]
ANKD	Absent	Absent	Absent
CNKD	Absent	Present	Absent
FNKD	Deficient	Present	Present[e]

ANKD, absolute natural killer cell deficiency; CNKD, classical natural killer cell deficiency; FNKD, functional natural killer cell deficiency.
[a] In natural killer cell deficiency, the observed defect must be consistent over time and non-natural killer cell immune components or non-natural killer cell-dependent immune components should be normal.
[b] Natural killer cell function as typically defined by cytotoxicity, but can include any function that can be attributed to natural killer cells. As many of these functions are also performed by other cells, it is important that the deficit be specifically attributed to the natural killer cell.
[c] Natural killer T cells as defined by the presence of $CD3^+$, $CD56^+$ cells. This includes the iNKT cell population expressing the $V\alpha24$ and $V\beta11$ combination of TCR genes.
[d] Natural killer cells as defined by, but not limited to $CD3^-$, $CD56^+$ cells.
[e] Although natural killer cells by definition are present in FNKD, there may be phenotypic abnormalities or absence of particular natural killer cell subsets.

Table 5[166]**:** Classification of isolated NK cell deficiencies. (Reprinted from Clinical Immunology, Vol.118, Orange JS, Ballas ZK, Natural killer cells in human health and disease, pages 1-10, 2006, with permission from Elsevier).

Finally, individuals affected with FNKD have NK cells, which are non-functional. Dysfunction of the NK cells is usually defined by their markedly decreased cytolytic capacity, although other functions may be affected[166]. FNKD also results in the increased likelihood of viral infections, cancer and death[175-178].

All types of isolated NKD highlight the importance of NK cells in control of infections, particularly viral infections usually suppressed by healthy individuals. Increased cancer rates in NKD, possibly directly related to viral infections, underlined the role of NK cells in controlling tumors, as highlighted by the *in vitro* killing of tumor cells by NK cells described above.

Additional evidence: NK cells and cancer

Evidence for the role of NK cells in tumor suppression is also provided by extensive mouse model experiments, human correlative studies and clinical trials. Mice depleted for NK cells via antibodies or with NK-cell related genetic deficiencies were often shown to develop more aggressive tumor growth[179-182]. However, given the lack of specificity of antibodies or genetic mutations, the role of other cells in tumor control cannot be excluded in these studies. Perhaps more convincingly, mice deficient for RAG2 (recombinase activation gene 2) and STAT1 (signal transducer and activator of transcription 1) spontaneously develop adenocarcinomas at higher rates than RAG2 only deficient mice[183,184]. While STAT1 is a gene involved in the immune response of both T- and NK cells, RAG2 specifically abrogate T-cells[182]. The increased rate in carcinomas observed in the mice deficient for both genes should be the result of the added NK cell deficiency and directly implicates these cells in tumor control[182].

In a Japanese study of over 3600 subjects, 154 of which developed cancers over the 11-year follow-up, low NK cell cytotoxic activity was linked to increased incidence of cancer[185]. Additionally, favorable prognosis of colorectal carcinoma, gastric carcinoma, and squamous cell lung cancer has been linked to increased NK cell tumor infiltration[186-188]. However, these remain correlative studies and more direct evidence of the importance of NK cells in cancer immunology is provided by a clinical trial where patients with acute myeloid leukemia were transplanted with allogeneic NK cells[189]. Survival of graft recipients was greatly increased if they lacked ligands specific for inhibitory receptors found on donor NK cells.

Additional evidence: NK cells and pathogens

The capacity of NK cell to kill cells infected with different pathogens, including CMV (cytomegalovirus), HSV (herpes virus simplex), HIV, vaccinia virus and *Mycobacterium tuberculosis* has been demonstrated *in vitro*, suggesting a role for NK cells in immunity against infectious agents[190-193]. This is further supported by observations in mouse models of infection. In mice, antibody-mediated neutralization of NK cell effectors such as interferon (IFN)-γ lead to increased murine CMV (MCMV) replication and severity of viral hepatitis[194]. In this system, NK cells were shown to be the only secretors of IFN-γ[194]. Additionally, murine adenovirus infection is marked by significant NK cell liver infiltration and hepatocellular damage, which is reduced if NK cells are depleted[195].

Several epidemiological studies have linked NK cell receptor alleles (KIR- killer immunoglobulin-like receptors) with outcome of certain infections, such as HIV and HCV[150,151,196]. These studies will be discussed further in section IIIB.

NK cells and pregnancy

While NK cells are important in tumor and pathogen immunity, their presence in the uterus seems to suggest a role for them in pregnancy. At their peak, in the first trimester, NK cells represent 70% of all lymphocytes in the uterus whereas they are not found at term[197]. NK cell deficient mice have a rate of fetal loss of 64% and reduced placental size, a phenotype that can be reversed by transplantation of NK cells[198-200]. Uterine NK (uNK) cells differ from their blood counterparts phenotypically, as the majority of uNK cells are $CD56^{bright}CD16^{dim/-}$ and poorly cytotoxic[201]. The exact role of NK cells in the uterus is still unclear, but they may control trophoblast invasion, regulate blood flow to the pregnant uterus via secretion of angiogenic factors, and/or protect the uterus from infection[202,203].

B. MISSING-SELF HYPOTHESIS

The first reports describing NK cells centered on their ability to kill cancerous (and eventually virally infected) cells without any prior sensitization[153-157]. How they were able to recognize and selectively eliminate aberrant cells over normal cells remained unclear. T-cells are able to recognize non-self antigens presented by MHC (major histocompatibility complex) molecules on the surface of transformed or infected cells[204]. However, soon after they were discovered, it became clear that NK cells were able to kill

certain tumor lines that expressed no or very low-levels of MHC class I molecules[205-208]. Thus, a distinct mechanism must be employed by NK cells to recognize their targets. Experiments in mice were able to show that rather than occurring *in spite* of MHC class I decreases on tumor cells, rejection was in fact dependent on this decrease[209]. In these experiments, murine lymphoma cells deficient for H-2 molecules (murine MHC class I proteins) were less malignant than $H\text{-}2^{+}$ cells and tumor rejection was dependent on NK cells[209,210]. Additionally, tumors or bone marrow transplants bearing an allogeneic H-2 molecule (i.e. an $H\text{-}2^{a/a}$ transplant into an $H\text{-}2^{b/b}$ host) were rejected[209]. These observation led to the 'missing-self' hypothesis, where NK cells were posited to become activated, among other mechanisms, by cells that have decreased surface expression of self-MHC molecules[204]. Given that several tumors and infections evade the T-cell response via a downregulation of MHC class I molecules, the 'missing-self' activation of NK cells would provide an immunological backup response to aberrant cells[204]. Because it is centered around the capacity of NK cells to sense surface expression of MHC class I molecules, the 'missing-self' hypothesis predicts that NK cells should have receptors for these molecules that would inhibit their function when the MHC class I is present.

An-MHC class I dependent mechanism of regulation of NK cell function is elegant because virtually all nucleated cells express MHC class I molecules. However, some normal tissues express little (neural tissue) or no (erythrocytes) MHC class I molecules[204]. In addition, NK cells from TAP-deficient (transporter associated with antigen processing) mice, which have no cell surface expression of MHC I, are not autoreactive[211]. These findings suggest that functional NK cell inhibition is not completely dependent on MHC class I sensing and/or that, in addition to loss of MHC

class I dependent inhibition, the presence of certain NK-cell activating ligands on target cells (which would be absent on erythrocytes) is also required for NK cell activation.

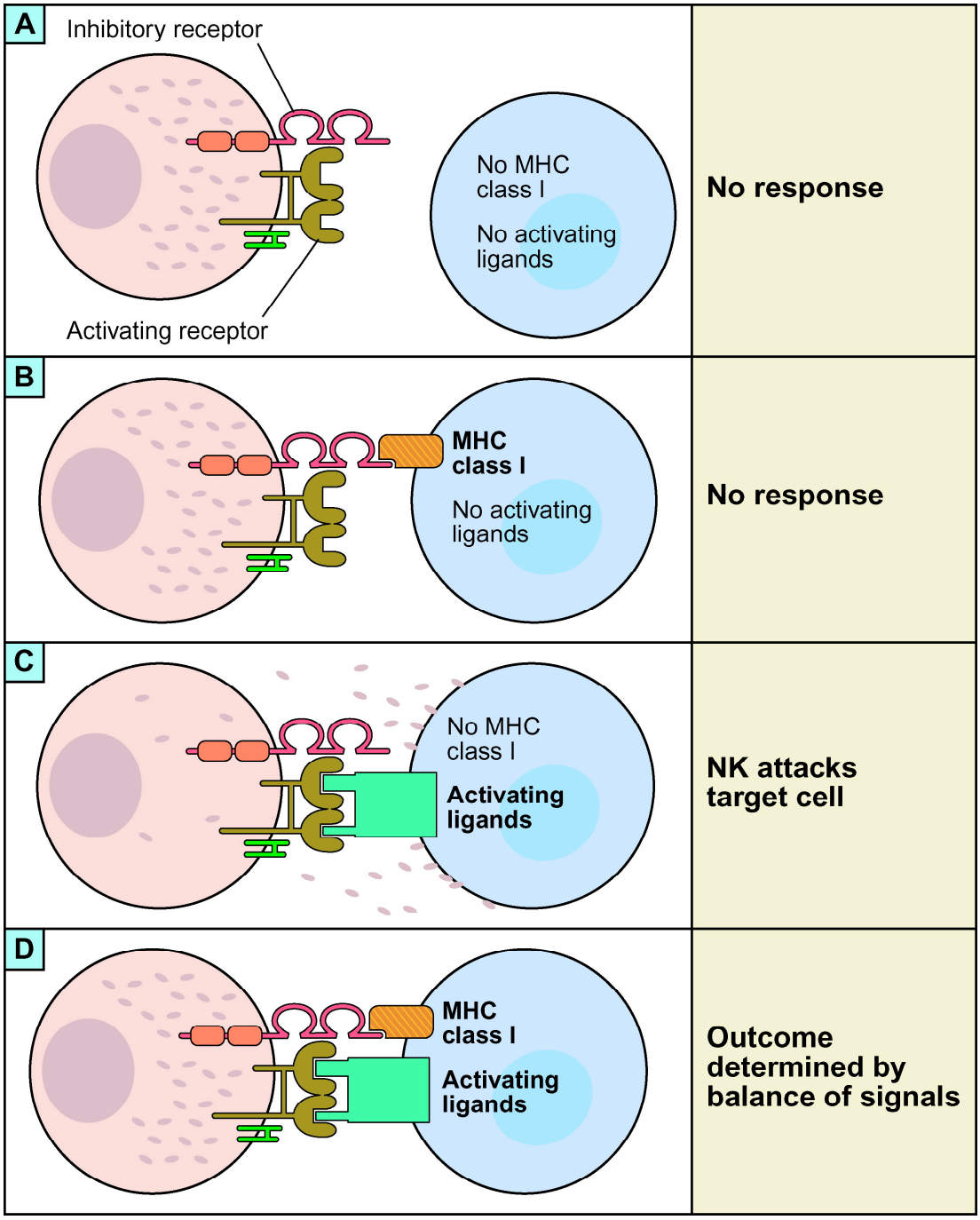


Figure 13[212]**:** NK cell activation is regulated by the integration of activating and inhibitory signals transmitted via cell surface receptors for MHC and non-MHC ligands. (Reprinted, with permission, from the *Annual Review of Immunology*, Volume 23 © 2005 by Annual Reviews, www.annualreviews.org).

Therefore, upon contact with a target cell, NK cell activation would be regulated by the integration of inhibitory and activating signals transmitted through NK cell receptors sensing the presence of both MHC class I and non-MHC class I ligands on the target cell (figure 13)[212].

C. NK CELL RECEPTORS

As demonstrated in RAG2-deficient mice, genetic rearrangement of NK cell receptors is not required for NK cell function[212]. Thus, NK cells are part of the innate arm of the immune system. However, given the complexity involved in distinguishing between healthy and diseased cells, the repertoire of receptors expressed on NK cells, although not adaptive and not exclusive to NK cells, is highly sophisticated. As predicted by the 'missing-self' hypothesis, several receptors regulating NK cells are dedicated to the recognition of classical and non-classical MHC class I molecules. These receptors can be divided into two classes: C-type lectin-like receptors and immunoglobulin (Ig) domain-containing receptors.

C-type lectin-like receptors

In mice, the Ly49 multigenic family regroups the main receptors for MHC class I molecules. These are transmembrane receptors with a carboxyl-CTLD (C-type lectin-like domain), which forms a protein-protein interaction with a peptide-loaded MHC class I molecule[212]. There are at least 23 Ly49 members, termed Ly49A through Ly49W, approximately half of which inhibit the activity of the NK cell while the other half transmit activating signals when they bind their MHC class I ligand[213]. The structure of Ly49 receptors is shown in figure 14.

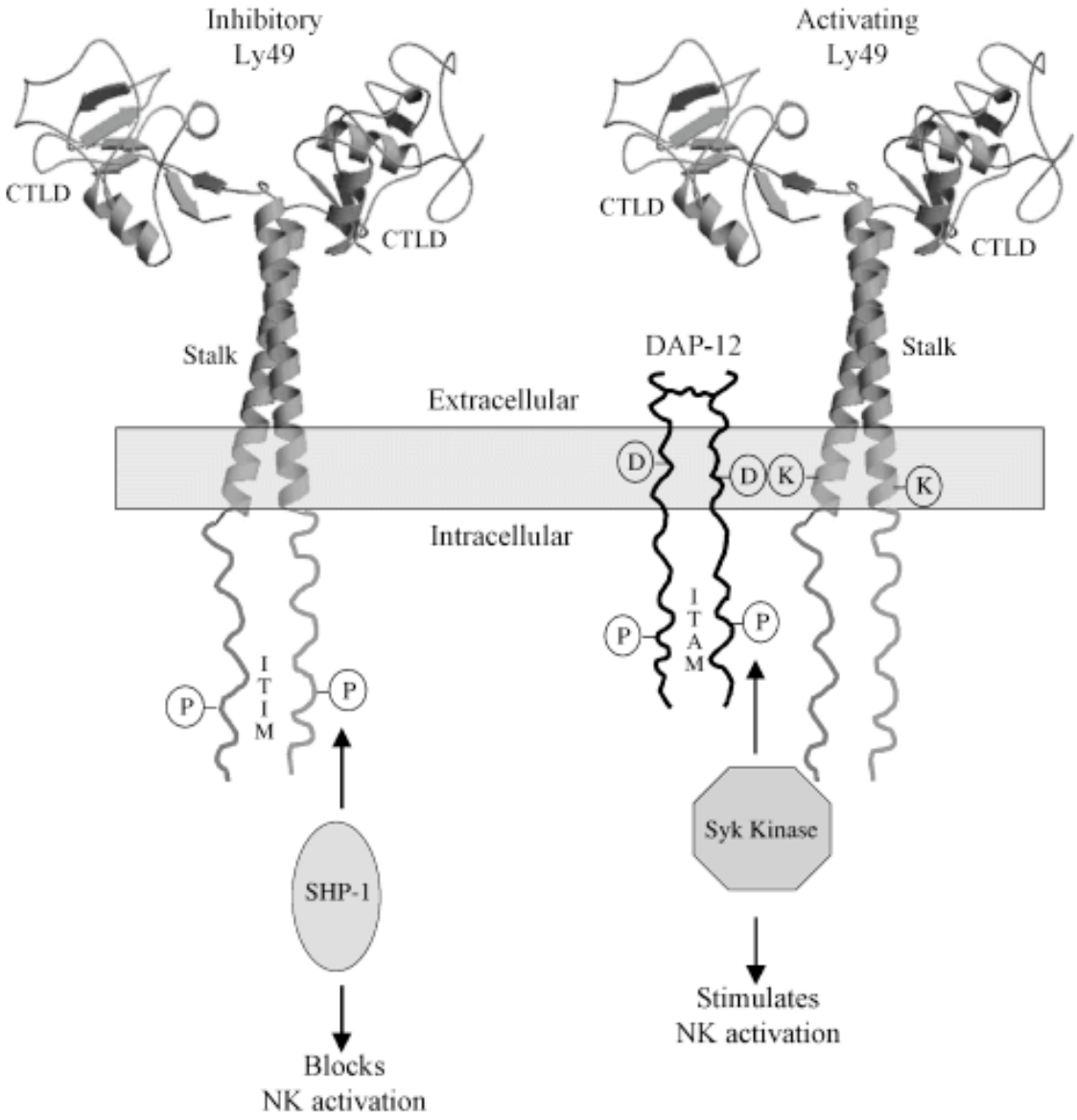


Figure 14[213]**:** Structure of inhibitory and activating Ly49 receptors and their downstream signaling events. CTLD: C-type lectin-like domain ITIM: immunoreceptor tyrosine-based inhibitory motif ITAM: immunoreceptor tyrosine-based activation motif. (Reprinted by permission from Macmillan Publishers Ltd: Immunology & Cell Biology, 83:1-8, 2005).

Like all other inhibitory NK cell receptors, inhibitory Ly49 receptors have an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tail. This

peptide motif, with the Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val consensus sequence, allows for the phosphorylation of tyrosine residues upon receptor-ligand interaction by a Src family kinase[212]. The phosphorylated tyrosine residues allow the recruitment of SHP (SH2-containing protein-tyrosine phosphatase)-1, SHP-2 or SHIP (SH2-containing inositol phosphate 5-phosphatase). These phosphatases lead to the dephosphorylation and deactivation of signaling proteins (ZAP-70, Syk, Vav-1, etc.) and decreased function of the cell[213]. Activating Ly49 molecules, as well as other NK cell receptors, lack ITIM sequences. Rather, their cytoplasmic domain associates with ITAM-containing adaptor molecules, such as DAP12[212]. The ITAM prototypical sequence is Asp/Glu-x-x-Tyr-x-x-Leu/Ilex_{6-8}Tyr-x-x-Leu/Ile[212]. Upon binding, the tyrosine residue of the ITAM is phosphorylated, leading to the recruitment of SH2-containing tyrosine kinases ZAP-70 and Syk. Downstream events include calcium influx, transcription of cytokine and chemokine genes, and displacement of granules to the cell surface (figure 15)[212].

Found in the genome of mouse, rat and humans, the CD94/NKG2 receptors are also C-type lectin-like transmembrane receptors. Five different NKG2 molecules have been shown to interact with CD94: NKG2A and NKG2B have an intracellular ITIM while NKG2C, E and H associate with the ITAM-containing DAP-12[214]. While CD94 can be expressed at the cell surface as a homodimer, it is thought than only CD94/NKG2 heterodimers are capable of interacting with their ligand (the non-classical MHC class I HLA-E molecule in humans) and transmit intracellular signals[212]. Unlike classical MHC class I molecules, HLA-E is genetically stable. This could explain the relatively low polymorphism of the

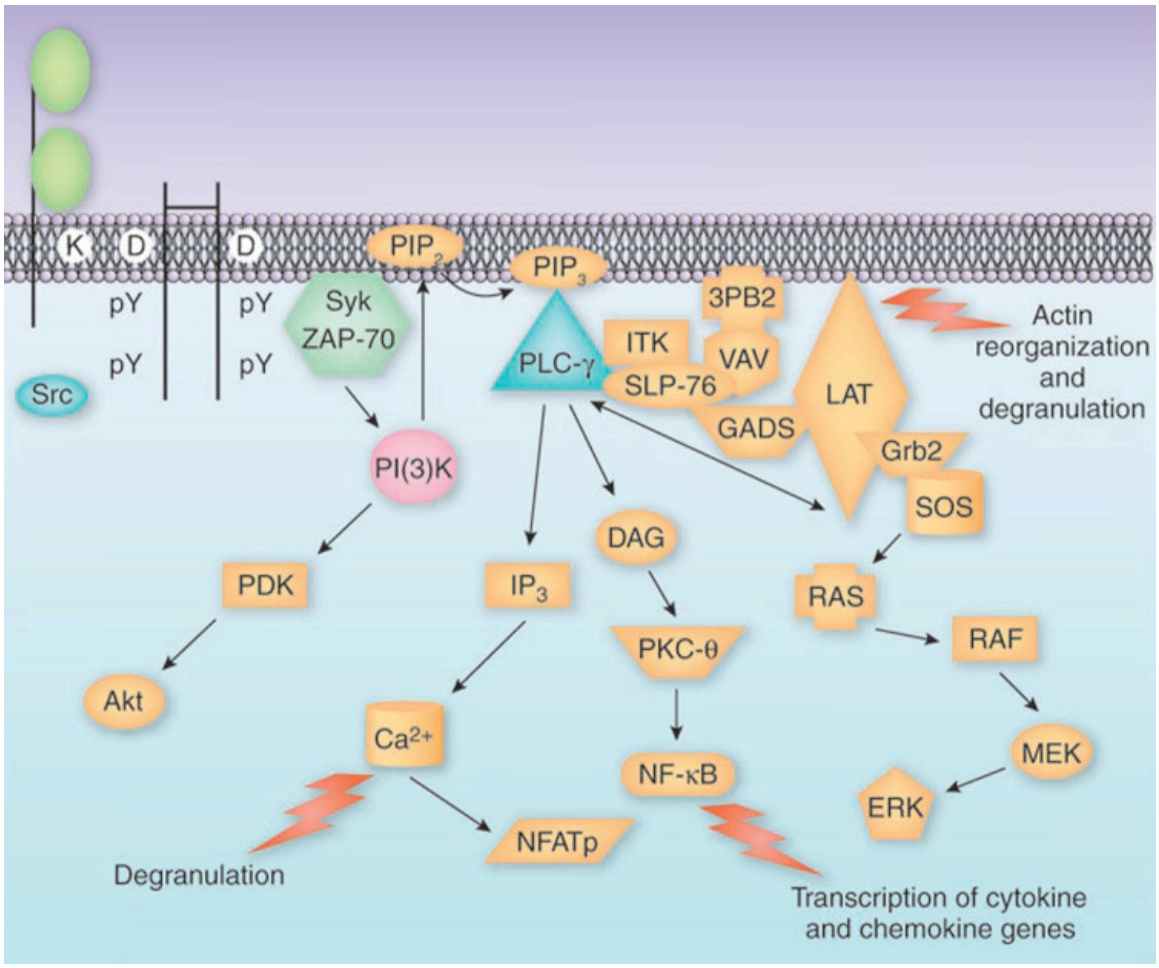


Figure 15[215]**: S**ignaling cascade of ITAM-containing receptors. (Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology, 9:495-502, 2008).

CD94/NKG2 family of receptors. Given that HLA-E usually has a self MHC class I-derived peptide in its peptide-binding groove, CD94/NKG2 receptors are involved in primordial self/non-self discrimination, as well as sensing the status of MHC I proteins[212]. However, HLA-E can also bind stress peptides, derived from heat shock proteins and, perhaps, certain pathogens. Evidence suggests that the peptide bound by HLA-E may affect the binding affinity of different CD94/NKG2 heterodimers, differentially modulating the outcome of the NK cell response. For example, NKG2A

complexed with CD94 has a greater affinity for HLA-E loaded with peptides derived from self-MHC class I molecules than the CD94/NKG2C receptor[216]. In addition, CD94/NKG2A is unable to bind HLA-E associated with hsp60 (heat shock protein 60)[217]. Perhaps the origin of the peptide associated to HLA-E could favor inhibition or activation of NK cell function through NKG2A/B or NKG2C/E/H, respectively.

Whereas other members of the NKG2 family are closely related to each other, NKG2D only has 28% amino-acid identity in the CTLD with other NKG2 receptors[218]. This sequence divergence translates into a functional difference. The ligands for NKG2D are a family of proteins with structural homology to MHC class I molecules, including MICA, MICB and ULBP1-5[212]. In addition, NKG2D does not interact with CD94, is expressed at the cell surface as a homodimer and associates intracellularly with DAP-10, a signalling chain that does not have an ITAM[218,219]. Instead, DAP-10 has a site involved in the recruitment of phosphatidylinositol-3-kinase or the adaptor Grb2 (growth factor receptor bound protein 2), eventually leading to calcium influx, actin reorganization and activation of the cell[218].

Ig domain-containing receptors

Recognition of MHC class I molecules by NK cells is also mediated by two types of Ig-like domain containing receptors: LILR (leukocyte Ig-like receptors) and KIR (killer cell immunoglobulin-like receptors)[212]. Also known as ILT (immunoglobulin-like transcripts) or CD85, the *LILR* family of genes is located on human chromosome 19[212]. Of the 13 LILR genes, only one encodes for an MHC class I receptor that is expressed on NK cells[212,220]. LILRB1 is a glycoprotein with four extracellular Ig-like domains and four

cytoplasmic ITIMs and binds with low affinity to conserved regions of all human MHC class I molecules[221,222]. Because it is expressed more broadly on B cells and monocytes, this receptor may have a more significant role in regulating the function of these leukocytes, although it has been shown that lytic activity of NK cell clones could be suppressed by the specific ligation of LILRB1[212,223,224].

Although human KIRs and mouse Ly49 receptors are structurally different, they share several common features. Both are expressed by NK and T-cells, both use MHC class I molecules as ligands, both can be either activating or inhibitory through ITAM or ITIM signaling and both are encoded by regions that are polygenic and genes that are polymorphic[212,225]. LILR and CD94/NKG2 receptors have limited polymorphism such that they can only monitor overall expression of MHC class I on cell surfaces[225]. On the other hand, KIR/Ly49 molecules represent a refined mechanism for surveying expression of autologous MHC class I through several levels of diversity: individual genomes differ in KIR/Ly49 gene content; KIR/Ly49 genes show allelic variability and ligand specificity; each NK cell clone expresses only a certain combination of KIR/Ly49 molecules[225].

As shown in figure 16, KIR nomenclature is based on receptor structure and reflects the number of extracellular Ig-like domains (either 2D or 3D) and the length of the intracellular cytoplasmic tail (L or S for long or short tails, P denotes putative pseudognes)[225]. KIRs with similar structure but with a sequence divergence that exceeds 2% are then numbered in sequence (e.g. KIR2DL1 and KIR2DL2)[226]. The function of KIRs is predicted by the length of its cytoplasmic tail. Long tails (L) contain one or two ITIMs and are inhibitory[225]. Short tails (S) do not have ITIMs, but a charged amino acid

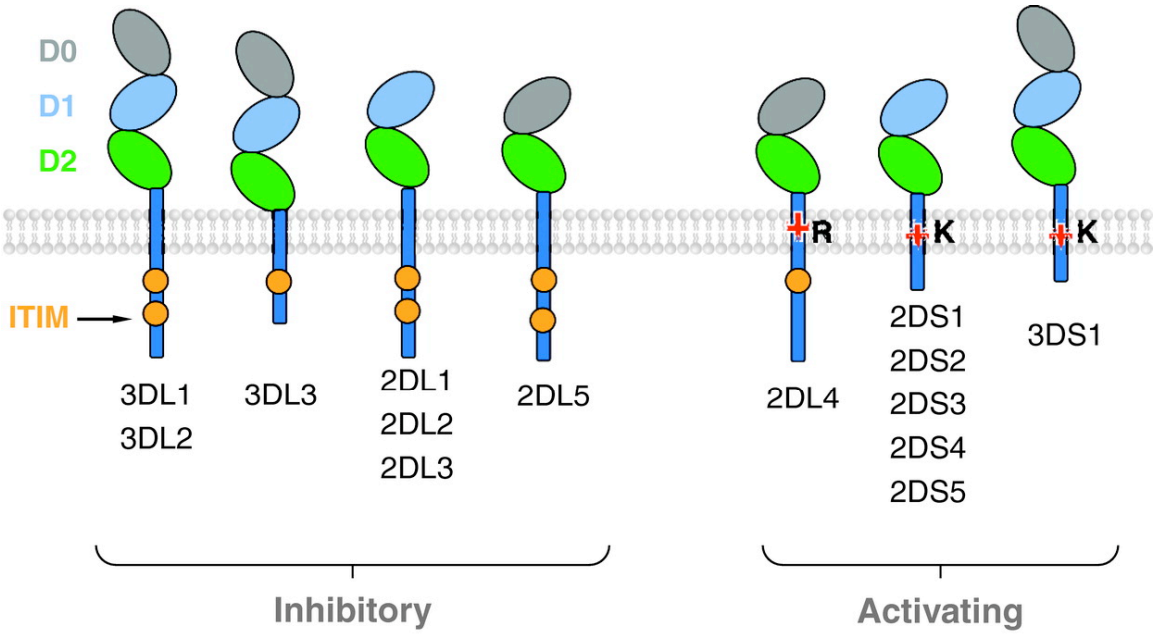


Figure 16[225]**:** Relationship between KIR nomenclature, structure and function. D0, D1, D2 designate Ig-like domains. ITIM are shown as orange circles. R and K indicate positively charge amino acids, which interact with DAP-12 or FcεRI-γ. (Reprinted, with permission, from the *Annual Review of Genomics & Human Genetics*, Volume 7 © 2006 by Annual Reviews, www.annualreviews.org).

in the transmembrane domain that mediates interaction with DAP-12[225]. KIR2DL4 is the only exception to this rule, having both a charged amino acid and an ITIM. Recent studies have shown that it associates with ITAM-containing adaptor protein FcεRI-γ and sends a stimulatory signal[227].

There are 15 known KIR genes and 2 pseudogenes closely linked in the leukocyte receptor complex of chromosome 19q13.4[212]. However, a typical genome will only carry 7-11 different KIR genes[228]. While KIR genes are highly homologous and differ only by a few nucleotides from one another, the proteins encoded by these genes can differ significantly in terms of ligand binding, cell surface expression, intracellular signaling and protein folding[225]. Although there is great variability of KIR gene content,

segregation analyses and sequencing data have revealed systemic features in organization of the KIR complex[228,229]. Four KIR genes, KIR3DL3, 3DP1, 2DL4 and 3DL2 are present on virtually all haplotypes and have been termed framework loci (figure 17). In addition, most genomes contain KIR2DL2 or KIR2DL3, which segregate as alleles of a single locus, as do KIR3DL1 and KIR3DS1. Therefore, these loci (KIR2DL2/3 and KIR3DS/L1) are also often considered as framework loci[225,230]. The KIR complex is also characterized by the existence of two basic groups of haplotypes, based on their gene content[226]. Haplotype A contains nine KIRs, only one of which, KIR2DS4, contains a short cytoplasmic tail (figure 17). While haplotype A is fixed in terms of the number and types of gene present, some variability is introduced by extensive polymorphisms at some loci[225]. The KIR3DL1 locus, for example, has over 50 known allotypes, which can differ in surface expression, ligand affinity, and inhibitory signal strength[151,231-234]. Haplotype B exhibits more diversity in terms of gene content and allelic polymorphism. Over 20 B haplotypes have been described, most of which have at least one activating KIR[225].

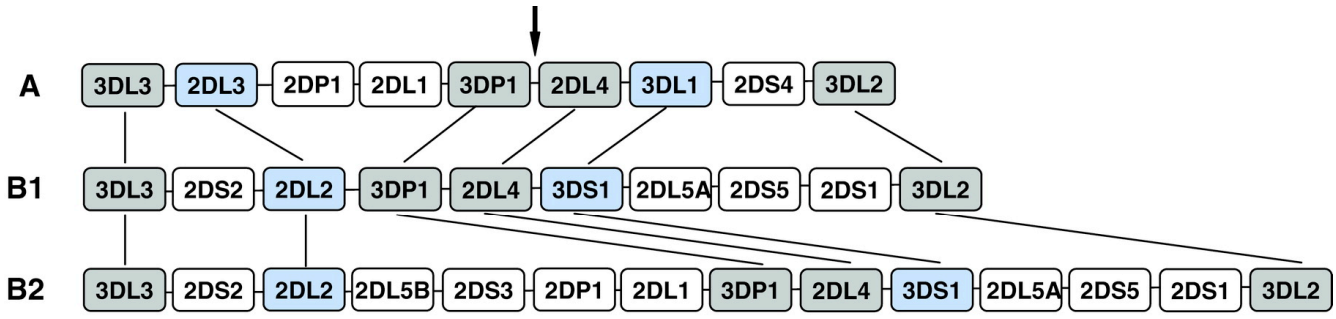


Figure 17[225]**:** Example of one KIR A haplotype and two B haplotypes. Framework loci are shown in gray. Blue loci are often also considered framework loci. (Reprinted, with permission, from the *Annual Review of Genomics & Human Genetics*, Volume 7 © 2006 by Annual Reviews, www.annualreviews.org).

Only MHC class I molecules have been identified as ligands for KIRs and many remain without a known ligand (see known KIR ligands in table 6). The sequence diversity between alleles translates into varying ligand affinities[225]. KIRs are usually specific for an HLA family (e.g. HLA-C1, HLA-Bw4), but may have greater affinity for some allotypes within an HLA family. For example, KIR3DL1, which is known to interact with HLA alleles bearing the serological motif Bw4, preferentially interacts with HLA-Bw4 alleles that have an isoleucine amino acid at position 80 over those that have a threonine at the same position[235]. Additionally, as recently demonstrated in experiments using NK cell clones expressing a single KIR allele, the KIR-HLA interaction is modulated by the type of peptide bound in the HLA peptide-binding groove[232]. In these studies, HLA-A24 tetramers loaded with HIV-derived peptides were capable of binding KIR3DL1. This was not the case if the peptide was from CMV or Dengue virus.

KIR	Ligand
2DL1, 2DS1	HLA-C group 2 (C*02, *04, *05, *06)
2DL2, 2DL3, 2DS2	HLA-C group 1 (C*01, *03, *07, *08)
3DL1 (3DS1 - ?)	HLA-B Bw4
3DL2	HLA-A3, HLA-A11
2DL4	HLA-G
2DS4	HLA-Cw4

Table 6[225]: Known human KIR ligands. (Reprinted, with permission, from the *Annual Review of Genomics & Human Genetics*, Volume 7 © 2006 by Annual Reviews, www.annualreviews.org).

Recognition of host non-MHC class I ligands

In addition to the evidence presented in section IIB, two other observations suggest that the MHC class I-dependent 'missing-self' hypothesis is insufficient to explain how NK cells are regulated. Firstly, in mice, some NK cells are devoid of any of the known MHC class I receptors and yet to not react to autologous cells[236]. Secondly, during maturation, NK cells can become functional, but not autoreactive, before they express KIR or Ly49 receptors[237]. There are several human non-MHC class I-specific NK cell receptors, a summary is provided in table 7[211,212,238]. The contribution of these receptors to the activation state of the NK cell is still unclear. Often described as 'co-receptors', they could increase or decrease signaling from other unidentified receptors, rather than independently determine the NK cell response[212]. Alternatively, they could be

Table 7: Human NK receptors for host-encoded non MHC ligands

Receptor	Function	Cellular Ligand
NCR1	Activation	HSPGs
NCR2	Activation	HSPGs
NCR3	Activation	HSPGs
NKR-PIA	?	?
DNAM1	Activation	CD155, CD112
2B4	Activation*	CD48
NTB-A	Activation*	NTB-A
CS1	Activation*	CS1
NKp80	Activation	AICL
IRp60	Inhibition	?
LAIR-1	Inhibition	Collagens
Siglec7	Inhibition	(α2,8) saccharides (α2,8) or (α2,8) saccharides

HSPG: heparan sulfate proteoglycans; AICL: activation-induced C-type lectin; * receptors of the SLAM (signaling lymphocytic activation molecules) family are usually activating, but they inhibit NK cell function in patients with X-linked lymphoproliferative disease.

part of a fail-safe system whereby the threshold for activation would be determined by a delicate balance between the absence of MHC class I and of secondary inhibitory ligands (e.g. collagens) on target cells and the presence of co-stimulatory signals (e.g. heparan sulfate proteoglycans)[212]. Individually, co-receptor signaling would be insufficient in determining the outcome of an interaction between the NK cell and its target, but in a system of checks and balances they would minimize the chance of an accidental response against a healthy cell.

Receptor repertoire

Cell surface expression of NK cell receptors encoded by multiple loci, such as KIRs, NKG2 receptors and Ly49 receptors, is stochastic; the product of their individual frequencies can calculate the probability of co-expression of two distinct receptors[239,240]. On a single cell, not all KIR receptors encoded by an individual's genome are expressed. However, each gene is usually expressed on at least one NK cell clone and KIR2DL4 is expressed on all clones[241]. The mechanism controlling transcriptional KIR expression is still unclear. Studies have shown that methylation in the 5' area of a KIR gene correlates with silencing of KIR transcription and that treatment with a demethylating agent resulted in *de novo* KIR expression[242,243]. Whether a specific KIR allele gene becomes methylated could depend on the state of its bi-directional promoter (figure 18). KIR genes have two promoters: one proximal to the start codon and another distal, further upstream from the start codon[244]. Via reverse transcription of NK cell RNA, in addition to sense transcripts originating from both proximal and distal promoters, antisense transcripts corresponding to the intergenic sequence found between the proximal and distal promoters were

detected[245]. This suggests that the proximal promoter is bi-directional. Given that antisense KIR transcripts were only detected in cells that lack protein expression, it was proposed that the proximal promoter acted as a probabilistic switch for the activation/inactivation of KIR genes[244,245]. If transcription of the proximal promoter occurred in the antisense direction, double stranded RNA (dsRNA) would form from the interaction with sense RNA transcribed from the sense distal promoter. This dsRNA would then lead to methylation and silencing of the KIR gene[244,246]. KIR2DL4 is unique and has a third promoter that may bypass this system and allow expression of this receptor on all NK cells[244]. Once established, the repertoire of receptors on an NK cell clone is stable through subsequent cell division and unaffected by environmental factors[243,247].

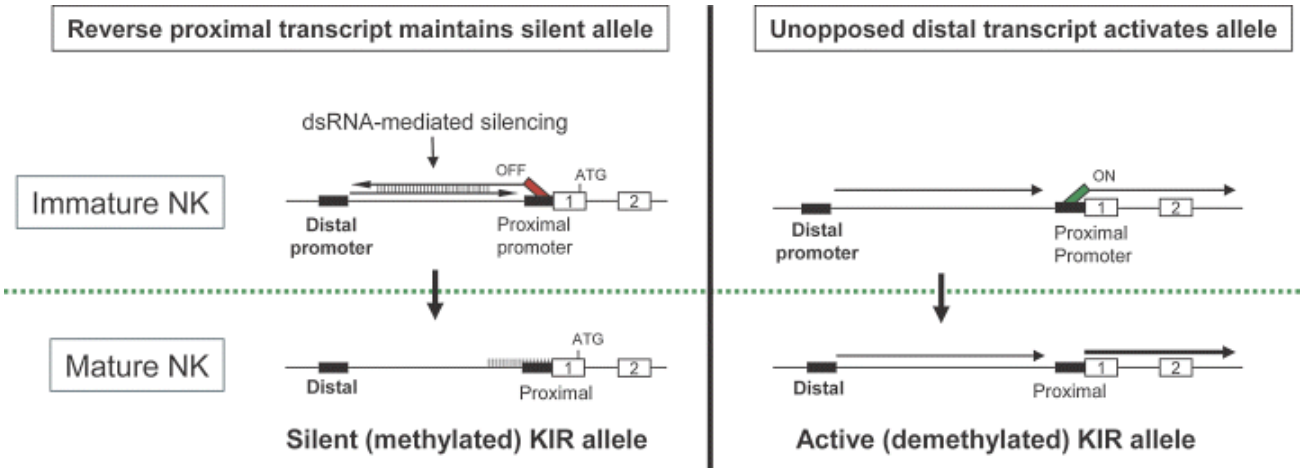


Figure 18[244]**:** Mechanism of KIR silencing. (Reprinted by permission from Wiley- Blackwell: Pascal et al., Immunological Reviews, 214: 9-21, 2006).

The 'missing-self' hypothesis leads to the 'at least one' model, where at least one inhibitory receptor specific for a self-MHC class I had to be expressed on each NK cell in

order to maintain tolerance to self[248]. However, because expression of inhibitory receptors is stochastic, this model requires that the HLA repertoire somatically influences NK cell KIR expression. Analyses have demonstrated that a dynamic process occurring during NK cell development leads to the sequential accumulation of inhibitory molecules until the cell expresses one or more self-specific receptor[237,249]. Additionally, the

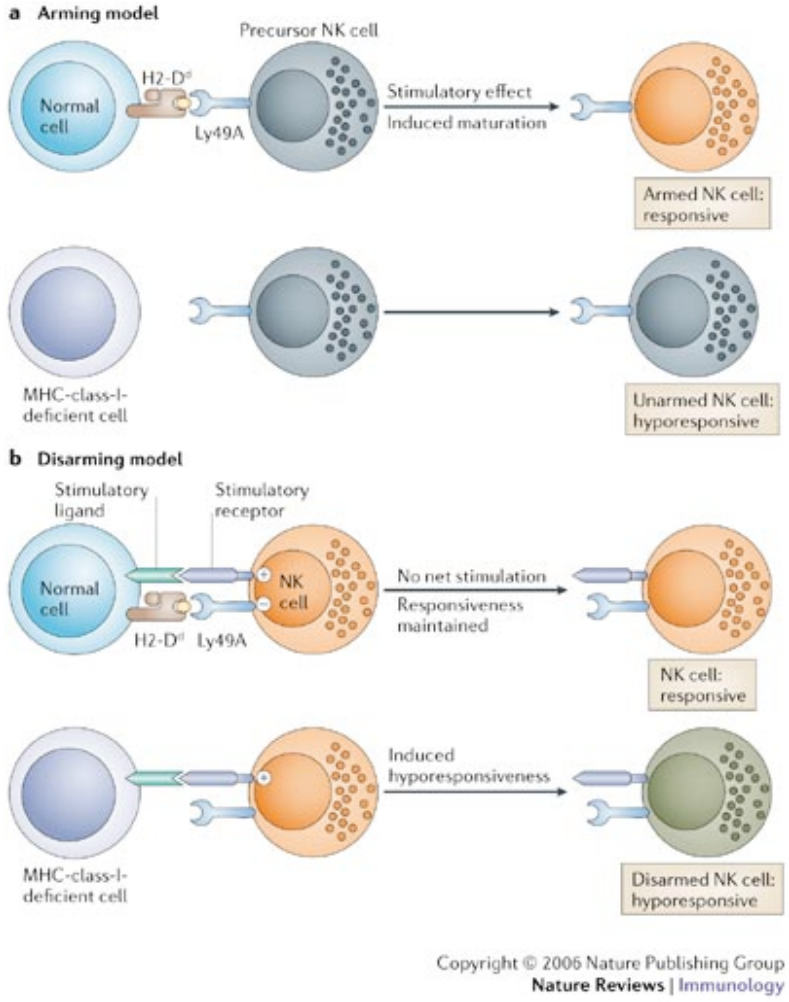


Figure 19[250]**:** Graphical depiction of the arming and disarming models of self-tolerance for MHC-class-I-deficient NK cells. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, 6:520-531, 2006).

accumulation of multiple self-MHC inhibitory KIRs confers increasing effector capacity to the NK cell, such that an NK cell with only one self-MHC inhibitory receptor will be less potent than a cell bearing two distinct receptors[251]. These results clearly support a link between MHC molecules and the KIR repertoire. However, the 'at least one' model is not supported by mouse and human studies where a subset of NK cells do not express any self-specific MHC class I inhibitory receptors[236,251]. In mice, approximately 10-15% of mature NK cells lack expression of any self-specific Ly49 or NKG2A receptors[236]. Interestingly, these cells were found to be hyporesponsive, failing to attack MHC class I deficient targets, insuring a form of tolerance. Similar findings have been reproduced in human NK cells failing to express self-receptors[251,252]. Two models have been proposed to explain why self-specific inhibitory signaling seems to be required for the development of a functional NK cell (figure 19)[253]. The 'disarming' model proposes that NK cells that fail to receive inhibitory signals are over stimulated through their activating receptors, leading to a phenotype similar to T-cell anergy. This phenotype would be caused by a decreased capacity to transmit activating signals by stimulatory receptors. Another model, 'licensing' or 'arming', proposes that inhibitory signals induce the cell to undergo a final maturation step, rendering them functional. Both these models are still under investigation.

D. NK CELL FUNCTION

While NK cells were first experimentally discovered as a result of their tumor suppressive capacity, NK cells are not only cytotoxic but can also have an immunoregulatory role. Phenotypically, the functional profile of a given NK cell

correlates with the surface expression levels of CD56, even though the role of this adhesion molecule in shaping NK cell function is unknown (figure 20)[161]. $CD56^{dim}CD16^{+}$ NK cells are usually cytotoxic, although they are also capable of secreting IFN-γ, while the $CD56^{bright}CD16^{dim/-}$ cells are immunoregulatory and secrete cytokines (IFN-γ, GM-CSF, TNF-α, TGF-β, IL-5, IL-10) and chemokines (CCL3, CCL4, and CCL5). However, either cell type does not exclusively perform these functions.

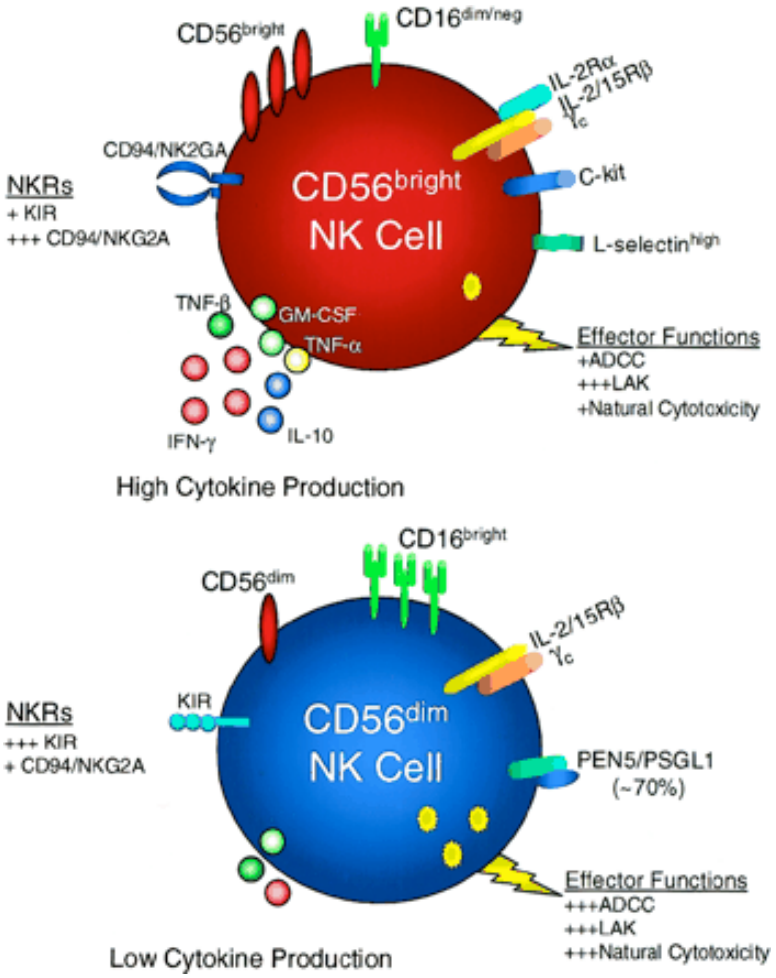


Figure 20[254]**:** Phenotype of immuno regulatory (top) and cytotoxic (bottom) NK cells. (This research was originally published in Blood. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, Carson WE, Caliguri MA. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001; 97: 3146-51. © the American Society of Hematology).

Cytotoxicity

$CD56^{dim}CD16^{+}$ NK cells have increased cell surface expression of KIRs compared to their immunoregulatory counterparts and are mostly located in the periphery[161]. They traffic to sites of inflammation via chemotactic receptors such as CXCR1 and ChemR where, following an encounter with a target cells, they perform their cytotoxic function by two distinct mechanisms[255,256].

Specialized organelles, termed lytic granules, are involved in the secretion of lytic effector molecules following interaction with a target cell. The site of interaction between the NK cell and the target cell is known as the immunological synapse, a site shaped by a certain number of receptors, signaling molecules, cytoskeletal elements and cellular organelles[257]. It is at the immunological synapse that NK cell receptors involved in self-recognition or ADCC determine the outcome of the encounter with the target cell. If this encounter results in NK cell activation, exocytosis of lytic granules, which contain granzyme, perforin and granulysin, will occur[258]. Lytic granules are directed to the synapse by moving along microtubules to the MTOC (microtubule-organizing center), which is itself then directed to the synapse[257]. Granular contents can be delivered to the target cell once they fuse with the plasma membrane.

There are five known granzyme proteins in humans (Grz A, B, H, K and M)[259]. Grz A, B and M promote apoptosis by directly cleaving DNA or activating proteins involved in the apoptotic cascade, such as caspases and BID (BH3-interacting domain death agonist)[258]. No pro-apoptotic function for Grz H and K has been found yet. Granzyme's capacity to induce cell death by cleaving intracellular substrates is dependent on perforin, as demonstrated by the loss of cytolytic capacity of perforin deficient

mice[256,260]. Two mechanisms have been proposed for perforin function, both of which have to do with its membranolytic capacity[256]. The first suggests that perforin forms a pore in the cellular membrane, allowing granzyme to diffuse into the cell. The alternative theory proposes that perforin and granzyme, once released from the lytic granule, are rapidly internalized into the target cell via endosomes. Perforin is then thought to disrupt these endosomes, allowing granzyme release. Also found in lytic granules, granulysin can lead to the lysis of tumors and certain pathogens, such as *Mycobacterium tuberculosis* and *Plasmodium falciparum*[258]. The mechanism of action of granulysin is still unknown, but it is dependent on the presence of mitochondria in the target cell and can be inhibited by Ca^{2+}, K^{+} and Na^{+} transport inhibitors[261].

Members of the tumor necrosis factor (TNF) family of cytokines, TRAIL (TNF related apoptosis-inducing ligand) and FasL (Fas ligand), can be induced on NK cells by interferons, IL-2 and IL-15[258]. The receptors for these apoptotic cytokines (TRAIL-R1, TRAIL-R2 and Fas), known as death receptors, contain a death domain. Their aggregation on the target cell leads to apoptosis through the recruitment and activation of caspases, providing a second mechanism for NK-cell mediated killing[256].

The cytotoxic capacity of NK cells has traditionally been evaluated by incubating NK cells with radio-labeled MHC class I deficient target cells and then measuring radioactive release. While effective, methods employing radioactivity are limited in terms of providing quantitative measures, have a number of safety concerns and are often subject to elevated background noise. Lining the membranes of lytic granules is the lysosomal-associated-membrane-protein 1 (LAMP-1, also known as CD107), a protein whose function is still unclear[262]. Upon fusion of the lytic granule with the plasma

membrane, CD107 is then found at the cell surface. Using fluorescently labeled antibody specific for CD107, flow-cytometry based methods have recently measured cell surface increases of this molecule as a surrogate marker for cytotoxicity of T cells and NK cells[263,264]. In addition to avoiding the use of radioactive isotopes, CD107 detection allows simultaneous cell phenotyping by flow-cytometry.

Immunoregulation

Immunoregulatory NK cells are less effective mediators of cytotoxicity. However, treatment with IL-2 brings their cytotoxic capacity to levels similar to that of $CD56^{dim}CD16^{+}$ NK cells[265]. $CD56^{bright}CD16^{dim/-}$ NK cells express low levels of KIRs and high levels of NKG2A, but exclusively express CCR7 (CC-chemokine receptor 7) and CD62L, two molecules implicated in the homing of NK cells to secondary lymphoid organs, where modulation of other immune cells is favored by their proximity[161]. In addition, immunoregulatory NK cells constitutively express the high affinity receptor for IL-2 and the receptor kinase c-kit, which augment their sensitivity and proliferative capacity in response to IL-2[161].

NK cells interact extensively with DCs (dendritic cells) and can act on them in two distinct ways. *In vitro,* KIR-deficient $NKG2A^{+}$ NK cells are capable of killing immature DC (iDC)[266]. It is thought that only iDCs expressing sufficient levels of MHC class I and co-stimulation molecules will survive an encounter with NK cells[147]. This editing process would select DC capable of priming an effective immune response. Secondly, IFN-γ and TNF-α secretion favors DC maturation[267,268]. In turn, the mature DC secretes IL-12 and IL-18, which can stimulate NK cells to proliferate, secrete cytokines

or prime for cytolysis[269,270]. This 'NK-DC crosstalk', which is also dependent on NK cell receptors such as NCRs and NKG2A, creates a favorable environment for the development of a T_H1 (T-helper 1) immune response ideal for controlling the proliferation of intracellular pathogens. IFN-γ secreted by NK cells also directly polarizes naïve CD4+T cells towards a T_H1 profile[271,272]. B-cells can also be modulated by NK cells, which have been noted to induce immunoglobulin class switching and suppress autoimmune B-cells[273,274]. Finally, NK cells can participate in a feedback loop inhibition of inflammatory responses by secreting anti-inflammatory cytokines IL-10 and TGF-β[275].

Some cytokines secreted by NK cells, such as IFN-γ and TNF-α, have antiviral/antibacterial effects. IFN-γ effectively upregulates MHC class I and II antigen-presentation pathways to increase the quantity and diversity of peptides presented on the cell surface, promoting cell-mediated responses[276]. In addition, it leads to the increased secretion of chemoattractants for T-cells and macrophages[276]. Finally, IFN-γ increases the synthesis of proteins with direct antiviral effects: PKR (protein kinase dsRNA regulated) and OAS (2',5'-oligoadenylate synthetases)[277]. PKR requires dsRNA (double stranded RNA), a product common in the replication cycle of some viruses but not of eukaryotes, for its activation. Once activated, it leads to inhibition of both viral and cellular protein synthesis by inhibiting the elongation factor eIF-2α (elongation Initiation Factor 2α), preventing the virus from hijacking the cellular machinery. Also activated by dsRNA, OAS leads to the activation of RNase L, which cleaves RNA in the cytoplasm, effectively inhibiting protein expression.

Evidence of the antimicrobial effects of TNF-α can be found in mice depleted of TNF-α or genetically deleted for the TNF receptor. These have a decreased capacity to

control infections with intracellular bacteria, such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*[278,279]. As a pro-inflammatory cytokine, TNF-α can mediate the recruitment of immune cells, increase antigen processing and act on the hypothalamus to increase body temperature[280]. Body temperature directly influences the capacity of replication of pathogens. Finally, TNF-α can initiate the synthesis of acute-phase proteins, such as C-reactive protein, which can activate the complement pathway[280].

III. NK cells in HIV infection

The current model for the immune control of viral infections proposes that innate effector mechanisms hinder viral dissemination in the early stages of the infection until an adaptive response can be mounted (figure 21)[160]. NK cells have several characteristics that allow them to be implicated in the early stages of viral infection: preformed lytic granules, proliferation in response to DC-secreted cytokines and chemokine receptors promoting their migration to inflamed tissue. As suggested by an expansion of NK cells in the early stages of infection, these innate immune cells are also important in controlling HIV[281].

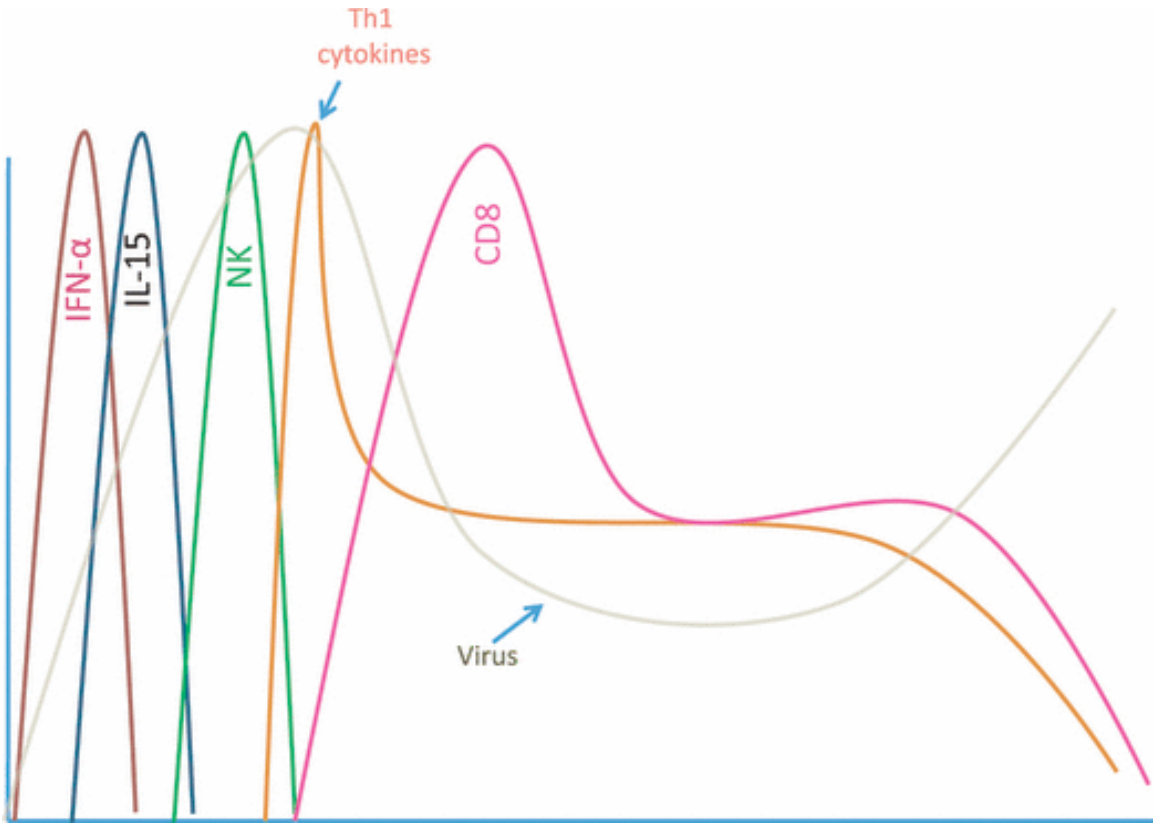


Figure 21[282]**:** Temporal sequence of immune effectors in response to a viral infection. (Reprinted by permission from Wiley-Blackwell: Alter et al, Journal of Internal Medicine, 265 (1) 29-42, 2009).

Based on evidence for the importance of T-cells in controlling HIV infection, strategies for the development of a vaccine have recently centered on the induction of HIV-specific T-cells. The importance of T-cells in HIV infection is demonstrated by temporal associations; first between the appearance of HIV-specific CD8+ T cells and a decrease in viral replication rates in human studies and, second, in macaque models, between the depletion of $CD8^+$ T-cells and increases in viral load[102,283]. Additionally, viral epitopes targeted by $CD8^+$ T-cells are often susceptible to escape mutations, indicating a selective pressure applied by these lymphocytes[284]. Finally, certain MHC class I alleles are linked to slower HIV disease progression, perhaps due to their increased capacity to present conserved viral peptides[285].

The recent failure of a T-cell based vaccine (see section IJ) underlines that an effective immune response to HIV is most likely composed of several elements. Perhaps the temporal association between viral replication and HIV-specific $CD8^+$ T cell rather reflects an association with several components of the immune response, including those deployed early in infection. Supporting this, studies in macaques have found that viral replication levels differed significantly between animals in the first seven days post-inoculation with SIV, before an adaptive response could be mounted, suggesting that host-specific factors acting early in infection also determined outcome[286]. It must also be noted that NK cells and DCs bear cell surface receptors that recognize MHC class I molecules, such that associations between HLA (human leukocyte antigen, the human family of MHC class I molecules) and HIV disease progression may not be exclusively due to T-cell responses.

As will be discussed in this section, there is increasing evidence for an intimate relationship between NK cells and HIV. Infection with HIV alters the phenotype of NK cells. Alternatively, NK cells can exert pressure on the virus. This interplay may provide insights into new HIV therapeutic and vaccine design strategies.

A. THE INFLUENCE OF HIV INFECTION ON NK CELLS

There are several factors related to HIV infection that can interact to deregulate NK cells. The events leading to a state of generalized immune activation (section IG) also affect NK cells. Additionally, although only a minority of NK cells bears the CD4 receptor, HIV may directly infect them[45]. The majority of NK cells, which lack CD4, still carry the HIV co-receptors CCR5 and CXCR4[287]. While the virus is unlikely to infect these, it could still induce intracellular signaling by binding either chemokine receptor. These mechanisms combine, in ways that are still unclear, to alter the phenotype of NK cells in HIV infection.

NK cell surface molecules

Initial analyses evaluating the effect of HIV infection on NK cell population numbers found conflicting results. While some studies reported a reduction in NK cell counts, other reported no change[162,288,289]. As demonstrated by a recent study evaluating NK cell populations during different stages of the disease, these conflicting conclusions are likely the result of changes in NK cells dynamics over the course of HIV infection. Utilizing three markers to define three populations of NK cells ($CD3^-CD56^{bright}CD16^{dim/-}$, $CD3^-CD56^{dim}CD16^+$, $CD3^-CD56^-CD16^+$), Alter et al demonstrated that there was a

dramatic increase in total NK cells in the acute phase of infection, mirroring viral load levels[163]. However, as viral load decreased to a plateau and the infection entered the chronic phase, there was a sequential decrease of $CD3^{-}CD56^{bright}CD16^{dim/-}$ and $CD3^{-}CD56^{dim}CD16^{+}$ NK cell subsets with a concomitant increase in the anergic $CD3^{-}CD56^{-}CD16^{+}$ population. Thus, while there were no overall changes in the contribution of NK cells to the total lymphocyte population, there were increases and decreases in different NK cell subsets.

There are contradictory reports as to the specific increase or decrease of the total expression of each individual NK cell receptors (NKR) during HIV infection[162,289-292]. These contradictions are again probably due to observations performed on cells isolated at different disease stages. Overall, however, there is an agreement that inhibitory NKR (e.g. inhibitory KIRs or LIR1) expression is increased during chronic infection. A notable exception is the decrease of NKG2A[293]. Since the $CD3^{-}CD56^{bright}CD16^{dim/-}$ subset expresses this receptor at high levels, the loss of the regulatory NK cell population likely explains the observed decrease of NKG2A. Along with an increase in iNKR, there are consistent reports of decreases in expression of all members of the activating NCR family, including NKp30, NKp44 and NKp46[162,294]. These variations in NKR expression result in NK cells that are highly sensitive to inhibitory signals and require strong activating signals. This inhibitory phenotype may decrease the capacity of NK cells to contribute to control of HIV replication and of other infections or tumors that can arise during AIDS.

Changes in NK cell functionality

The capacity of NK cells isolated from HIV infected individuals to mediate a normal response once stimulated is subject to some debate. Some groups report a decrease in the capacity of these NK cells to secrete IFN-γ and TNF-α or mediate cytolysis[162,288,290,295,296]. Other groups report that, with increasing viremia, NK cells increase in functional capacity[163,289,297-299]. These discrepancies are attributable to a number of methodological differences. Experimental settings relying on non-specific mitogen stimulation, such as phorbol myristate acetate, to measure cytokine secretion or induced degranulation usually find that cells from HIV-infected individuals have decreased function[163,290]. Conversely, experiments that specifically measure the function of $CD3^{-}CD56^{+}$ cells (i.e. excluding the 'anergic' NK cells) in response to stimulation with the MHC-devoid K562 myelogenous leukemia cell line generally find that NK cells in HIV infection have an increased functionality[163,297,299]. This has led to the suggestion that increased KIR expression on NK cells from HIV^{+} subjects may favor a vigorous response to MHC-devoid cells[160]. Some studies also differ from each other in terms of the sample population targeted. Variations in exposure to treatment, viral load and CD4 T cell counts can all potentially influence NK functionality. Additionally, cytotoxic function is measured either by CD107 staining or radioactive release of labeled targets. The advantages of CD107 staining are discussed in section IID, but it remains an indirect measure of cytotoxicity.

There are several other features of NK cell function that are affected by HIV infection, including CC chemokine secretion, ADCC and modulation of the interaction between NK cells and DCs[271]. Many of these defects are related to the expansion of the anergic $CD3^{-}CD56^{-}CD16^{+}$ NK cell subset. Because CC chemokines such as RANTES

and MIP-1α bind co-receptors required for HIV entry, they can competitively inhibit viral replication, providing NK cells with a cell-to-cell contact independent mechanism of viral suppression. The capacity of NK cells to secrete CC chemokines is inversely correlated with plasma viral load[287]. This explains at least in part the fact that NK cells isolated from individuals with elevated plasma viral loads have a decreased capacity to suppress *in vitro* HIV replication[287].

The ability of NK cells to mediate ADCC is decreased as the disease progresses[300]. This occurs in spite of the fact that there is an increase in $CD16^+$ NK cell subsets. The capacity of NK cells to lyse target cells through the use of HIV-specific antibodies is decreased because microtubular rearrangements that usually occur upon encounter of a target cell do not take place in NK cells from HIV-infected individuals[301]. Additionally, there is a decreased delivery of granular contents[302].

Some of the molecules involved in 'NK-DC crosstalk', NKG2A, NCRs, IFN-γ and TNF-α, as discussed above, were reported to be decreased in HIV infection in several studies. For this reason, it is thought that the interaction between these cells may be defective in the setting of infection with HIV[271]. However, this is still an active area of investigation.

B. NK CELL-MEDIATED IMMUNITY AGAINST HIV

Despite the dysregulation of NK cells in HIV infection, there is substantial evidence that these cells can mediate immunological pressure on the virus and modulate disease outcome. Understanding how NK cells fail in an infectious setting, as discussed in the previous section, provides a number of possible therapeutic targets designed to

restore NK cell function. However, before these cells are investigated for therapeutic considerations, it is also important to know if and how these are capable of affecting the outcome of an HIV infection.

Lysis of infected cells

The capacity of NK cells to lyse HIV-infected targets was first established by measuring the increased lysis of infected over uninfected tumor $CD4^+$ T cell lines[303,304]. However, cell line susceptibility to NK cell-mediated lysis varied considerably, suggesting that certain phenotypic characteristic of different target cells could inhibit cytolysis. Interestingly, NK cells isolated from healthy individuals were subsequently found to be unable to lyse HIV-infected autologous T-cell targets, despite a significant decrease in measured surface MHC class I expression[305]. It is known that HIV leads to the specific decrease in expression of cell surface HLA-A and -B molecules, but not HLA-C and -E molecules, through a Nef-dependent mechanism[306]. Thus, HIV has developed a mechanism to avoid elimination by both $CD8^+$ T cells and NK cells. Limited expression of MHC class I molecules on infected cells suggests that a subpopulation of circulating NK cells are capable of eliminating these. Further investigations have shown that NK cells lacking inhibitory receptors for HLA-C and -E were capable of killing HIV-infected autologous T cells[191]. However, given the increased cell surface expression of inhibitory NKR, including receptors for HLA-C, in HIV-infected individuals the number of NK cells capable of *in vivo* lysis of HIV^+ cells is limited[162,271,307].

Inhibition of viral replication

Incubating HIV-infected $CD4^+$ T cells with NK cells leads to a decrease in viral replication[307-310]. In many instances, the supernatant collected from NK cell culture is sufficient to mediate strong inhibition, suggesting that secretory molecules are involved. Most of the supernatant's inhibitory capacity can be abrogated by the use of anti-CC chemokine antibodies[307,309]. In NK cell supernatants treated with anti-CC chemokine antibodies, residual inhibition of viral replication can still be observed, however[309]. The contribution of other soluble factors secreted by NK cells to *in vitro* inhibition of replication is still unclear. Additionally, the observation that separation of NK cells from infected targets by a semi-permeable membrane leads to dramatic decreases in viral replication suggests that cell-to-cell contact-dependent mechanisms also contribute to the NK cell's capacity to limit the production of HIV virions[310]. These mechanisms are probably not limited to cytolysis, given the ability of HIV to escape NK-cell mediated killing of its host cell.

KIR3DS/L1

Perhaps the most convincing evidence that NK cells are important in the control of HIV infection comes from recent studies linking the KIR3DL1 and KIR3DS1 genes to HIV disease outcome[150,151,311-313]. The KIR3DS/L1 locus can encode for either the activating (KIR3DS1) or the inhibitory receptor (KIR3DL1) and is often considered as a framework locus given that it is found on almost all haplotypes. In a Caucasian population, KIR3DL1 frequency is approximately 0.78, while KIR3DS1 frequency is 0.22[150]. Because the two receptors are allelic, in a typical Caucasian population

approximately 61% of the individuals are KIR3DL1 homozygous, 34% KIR3DL1/KIR3DS1 heterozygous and 5% KIR3DS1 homozygous.

While KIR3DL1 and KIR3DS1 significantly differ intracellularly, their extracellular domains share 97% sequence homology[314]. Thus, these receptors could share common ligand affinities. All HLA-B molecules express one of two serologically defined epitopes, termed Bw4 and Bw6. These are determined by a string of five amino acids located at positions 77-83[315]. The Bw6 epitope is found on approximately two-thirds of all HLA-B alleles and is exclusive to HLA-B molecules. The Bw4 epitope is found on several HLA-A alleles and the remaining third of HLA-B alleles. The latter are known ligands for KIR3DL1. There is some controversy as to the capacity of KIR3DL1 to bind HLA-A molecules bearing the Bw4 epitope, but it seems that a subset of these can interact with KIR3DL1[316,317]. HLA-B molecules with the Bw4 epitope (HLA-B Bw4) can further be subclassified according to the amino acid at position 80. This amino acid can either be threonine (T or Thr) or isoleucine (I or Ile). HLA-B Bw4 molecules with an isoleucine at position 80 (Bw4-80I) are better ligands for KIR3DL1 than those with a threonine[235]. Because of its high degree of homology, it was thought that the ligand for KIR3DS1 would also be found within the HLA-B Bw4 or Bw4-80I family. However, to date, no interaction has been demonstrated.

i- KIR3DS1 and HIV

Despite the lack of any physical evidence linking KIR3DS1 to its ligand, a large epidemiological study published in 2002 demonstrated an epistatic interaction between KIR3DS1 and HLA-B Bw4-80I[150]. By tracking the HIV-disease progression of over a

thousand individuals, those carrying both KIR3DS1 and HLA-B Bw4-80I

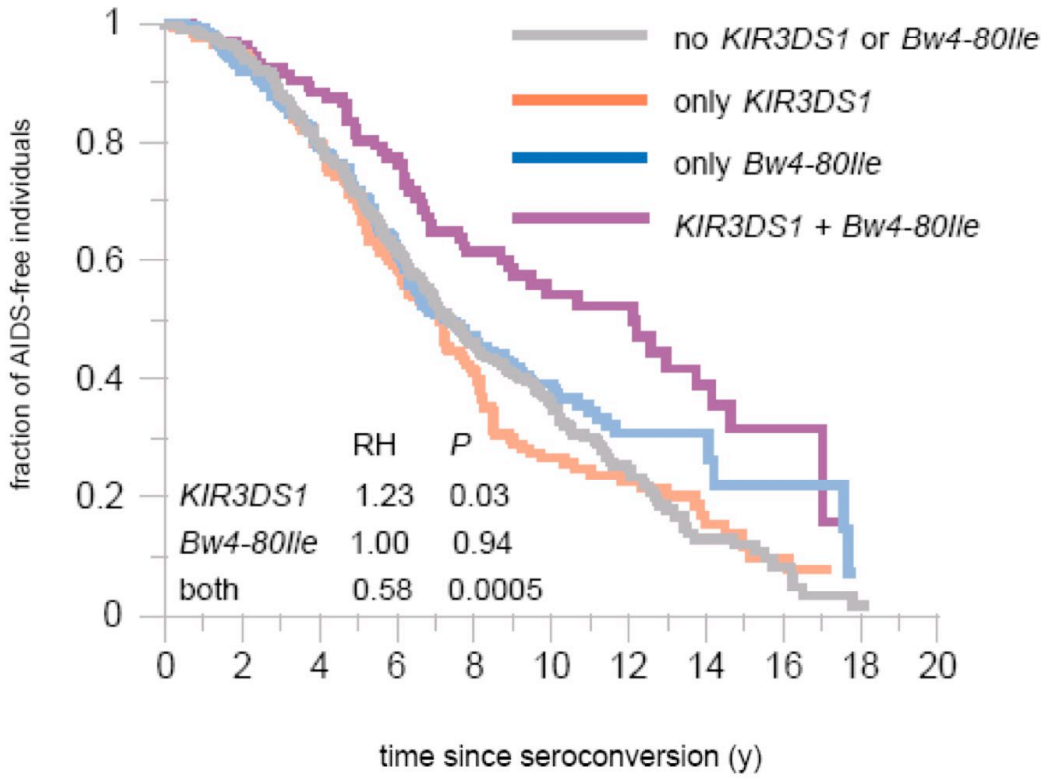


Figure 22[150]**:** KIR3DS1 and HLA-B Bw4-80I combine to slow down time to AIDS. (Reprinted by permission from Macmillan Publishers Ltd: Nature Genetics, 31:429-434, 2002).

were shown to progress to AIDS more slowly than those carrying only one or none of these genes (figure 22). This genetic combination was suggested to lead to early containment of HIV viral load and diminish susceptibility to opportunistic infections several years following infection[311]. However, subsequently, Barbour et al attempted to determine whether KIR3DS1 and HLA-B Bw4-80I affected markers of disease progression in the early stages of infection[312]. They concluded that KIR3DS1 and HLA-B Bw4-80I acted independently on CD4+ T cell counts and HIV viral loads, respectively. The synergistic effect of these genes on progression to AIDS observed after several years

of follow up has yet to be clearly linked to clinical markers measured in the early stages of infection.

The dramatic effect of this KIR-HLA combination on HIV disease progression has led to the hypothesis that NK cells with KIR3DS1 should have a distinct functional capacity. *In vitro* experiments have shown that NK cells from individuals who are both KIR3DS1 and HLA-B Bw4-80I positive had an increased capacity to inhibit HIV replication in a contact-dependent manner[310]. This effect was shown to be specific to KIR3DS1^{+} NK cells and is linked to their capacity to effectively lyse HIV-infected HLA-B Bw4-80I targets[310]. Additionally, NK cells isolated from HIV-infected individuals with at least one copy of the KIR3DS1 gene were found to have greater effector functions than those without the NK cell receptor[318].

The epidemiological and *in vitro* interactions between KIR3DS1 and HLA-B Bw4-80I strongly suggest that these may form a receptor-ligand pair. Because KIR3DS1 is an activating receptor, it is presumed that it would function by stimulating NK cells once it binds it putative ligand. As KIR3DS1 seems to be effective in conditions where HIV is present, it is possible that the ligand for KIR3DS1 is HLA-B Bw4-80I with an HIV peptide in its peptide-binding groove. The possible impact of peptides on KIR-HLA interactions has already been discussed in section IIIC. However, binding assays using a variety of HIV-bound HLA tetramers have failed to identify a ligand for KIR3DS1[319]. Unfortunately, these experiments included a limited number of HIV-derived peptides and HLA-B Bw4-80I molecules and must be expanded.

ii- KIR3DL1 and HIV

After studying the epidemiological link between KIR3DS1 and HLA-B Bw4-80I, Martin et al performed a follow-up study in order to determine the effect of KIR3DL1 on HIV disease progression[151]. Compared to KIR3DS1, the KIR3DL1 alleles are highly polymorphic[320]. There are currently over 50 known KIR3DL1 alleles, which differ in cell surface expression levels[231,233,234,321]. Cell surface expression of KIR3DL1 alleles is defined by measuring the signal intensity emitted by a fluorescently labeled DX9 antibody, which binds specifically KIR3DL1[321]. Not all KIR3DL1 alleles have been tested for their DX9 fluorescence intensity and, for the moment, there are at least four categories of KIR3DL1 alleles defined by their cell surface expression levels: high (*h; KIR3DL1*001, *002, *008, *015, *020), intermediate (*i; KIR3DL1*005, *006, *007), low (*l; KIR3DL1*028, *053) and null (KIR3DL1*004)[239]. KIR3DL1*028 and 053 have only recently been identified however, thus much of the literature refers to KIR3DL1*005, *006 and *007 as the low expressing alleles and considers three categories of KIR3DL1 alleles.

In terms of percentage of NK cells expressing KIR3DL1 and the level of KIR3DL1 expression at the cell surface, KIR3DL1 homozygote individuals with two *h alleles (or *h/*h) show significant increases when compared to individuals with at least one copy of a low expressing allele (*h/*l or *l/*l)[151,233]. To test whether the high-expression alleles differed from the low-expression alleles, Martin et al divided their population into two groups: those with at least one *l allele and those with a *h allele (and no *l alleles)[151]. The first group was termed KIR3DL1*l/*x where *l is any low expressing allele and *x is any other allele (*h, *l or KIR3DL1*004). The second group

was termed KIR3DL1*h/*y where *h is any high expressing allele and *y is either another *h or KIR3DL1*004.

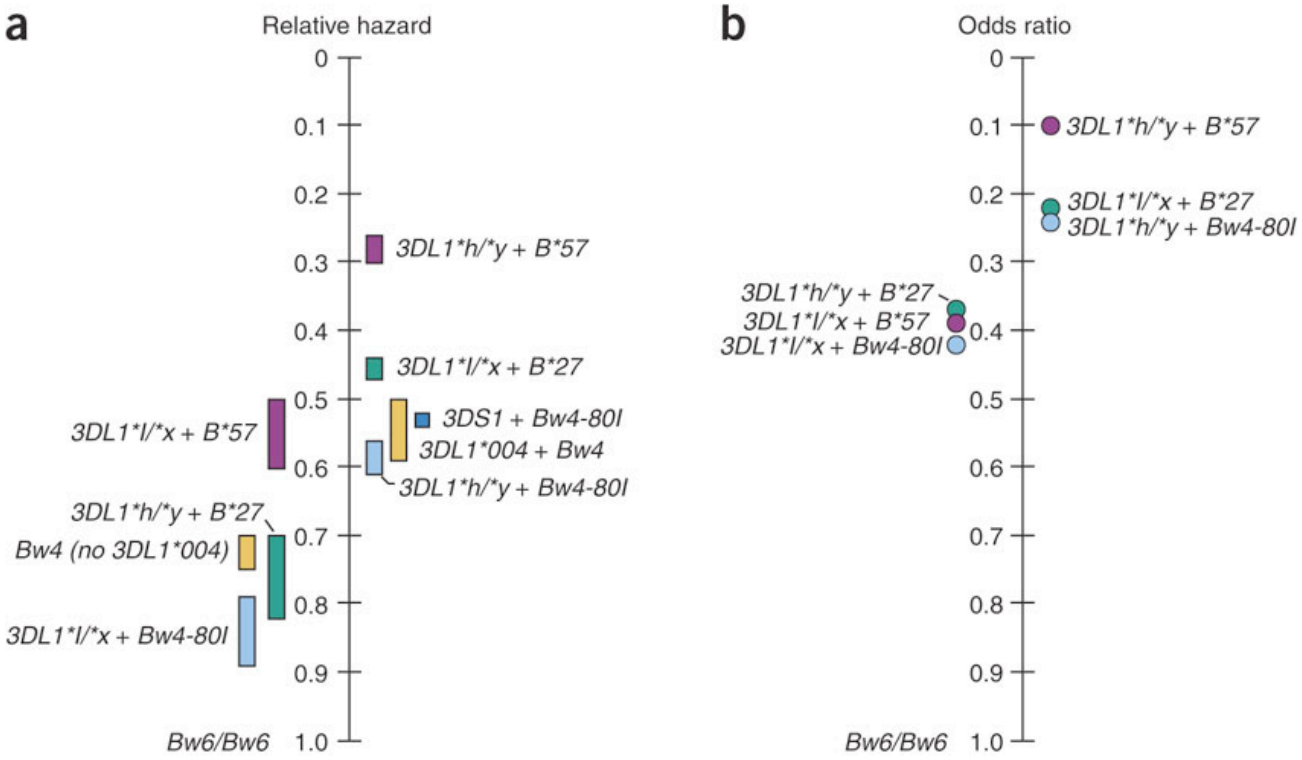


Figure 23[151]**:** KIR3DL1 and Bw4 combined genotypes are ordered by degree of protection in terms of (a) HIV disease progression and (b) control of viral load in relation to Bw6 homozygote individuals. Repinted by permission from Macmillan Publishers Ltd: Nature Genetics, 39:733-740, 2007.

The goal of their epidemiological study, performed in a sample size of over 1500 HIV^+ individuals, was to determine the effect of receptor-ligand pairs (i.e. KIR3DL1 and HLA-B Bw4 pairs) on rate of progression to AIDS and plasma viral loads. Individuals with two copies of HLA-B Bw6 (Bw6 homozygotes) served as controls because they lack any ligands for KIR3DL1, rendering this NK cell receptor ineffective. Their findings suggest that distinct allelic combinations of the KIR3DL1 and HLA-B loci influence progression to AIDS and plasma HIV RNA levels (figure 23)[151]. The KIR-HLA

combination influencing both disease outcomes most potently is KIR3DL1*h/*y with HLA-B*57 (an HLA-B Bw4-80I allele), a receptor-ligand pair that is linked to longer time to AIDS and decreased viral loads when compared to Bw6 homozygous individuals. Several other pairings had positive effects on HIV-associated measures, notably KIR3DL1*004 with HLA-B Bw4. How KIR3DL1*004 mediates an effect on HIV infection despite the fact that it is not expressed at the cell surface is still unknown.

It is surprising that an inhibitory NK cell receptor is linked with better HIV prognosis. Because a partnership between KIR3DL1 and its ligands is required to affect progression to AIDS or HIV viral load, it has been proposed that 'licensing' of $KIR3DL1^{+}$ NK cells in a HLA-B Bw4 genetic background predisposes these cells to become active[151]. Given that HIV-infected cells downregulate cell surface expression of HLA-B, an NK cell 'licensed' through KIR3DL1 could no longer be inhibited in the presence of an infected cell (figure 24), therefore resulting in NK cell activation and ability to mediate cytolysis of the target cell[322].

Evidence from unique populations

While most patients infected with HIV normally progress to AIDS in an average time of 10 years if they are not treated, some individuals remain healthy with CD4 counts above 500/mm^3 and AIDS free with no treatment for more than 13 years[323]. These are defined as long-term non-progressors (LTNP) and have been extensively studied in order to understand the mechanism(s) underlying their favorable HIV disease course. Recently, NK cells isolated from LTNPs were shown to be more cytotoxic than those from individuals with a typical disease progression[324]. However, in the same study, LTNP NK

cells showed an increase in iNKR and a decrease in NCR, as seen in all HIV-infected individuals. Thus, the increased cytotoxicity of NK cells from LTNP is not due to an activation phenotype shaped by surface NK cell receptors, but perhaps to increased perforin and granzyme contents or to a unique cytokine milieu[324].

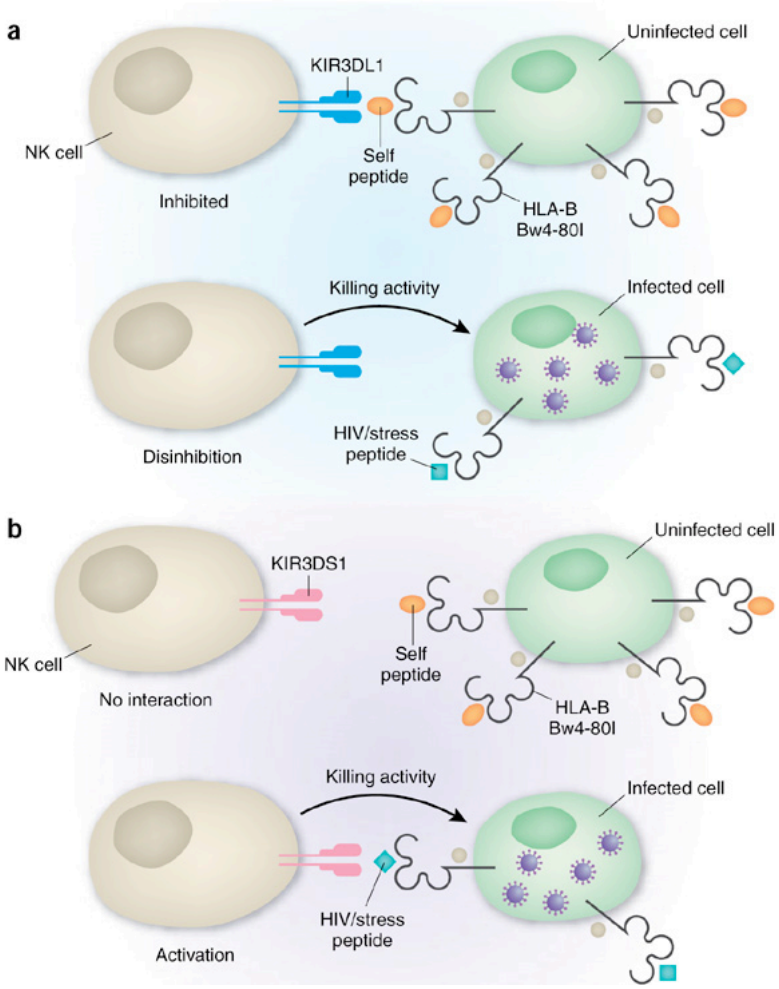


Figure 24[322]**:** Proposed model of interaction of KIR3DL1 (a) and KIR3DS1 (b) with their known or putative ligands allowing for favorable clinical outcome of HIV infection. Reprinted by permission from Macmillan Publishers Ltd: Nature Genetics, 39:708-710, 2007.

While LTNP provide clues as to how HIV replication and disease progression may be controlled, another population, exposed uninfected (EU) individuals may also provide clues for the design of a preventative vaccine. These individuals, and the characteristics of their NK cells will be discussed in the following section.

IV. Exposed Uninfected Individuals

In 1989 T-cell responses against HIV envelope and core proteins were detected in five HIV antibody- and antigen-negative sexual partners of HIV seropositive individuals[325]. This phenomenon was soon confirmed in other groups of individuals at high-risk of contracting HIV, including healthcare workers parenterally exposed to HIV by accidental needle sticks and infants born to HIV-infected mothers[326,327]. The theory that these exposed uninfected (EU) individuals could have a natural 'resistance' to HIV gained strength. It was strongly supported by the observation that approximately 15% of commercial sex workers followed between 1985 and 1994 in Nairobi, Kenya (the HIV resistant Pumwani cohort), did not seroconvert despite several years of repeated sexual exposure[328]. In 1986, the seroprevalance in the Nairobi sex clientele was 12% and grew thereafter. Based on behavioral data and HIV prevalence in the area, the calculated number of yearly exposures to HIV of women in the HIV resistant Pumwani cohort increased from 24 in 1984 to 64 in 1994. Given the level of HIV exposure, if all of the women in the Pumwani sex worker cohort were equally susceptible to HIV, statistically all should have seroconverted in the course of follow-up[328]. When these results were published, the possibility that some individuals were protected from infection drew considerable attention; perhaps clues to a vaccine could be found if the mechanisms of their resistance were understood.

Several cohorts of individuals with high-risk behavior have since been organized. These include commercial sex workers (CSW), injection drug users (IDU), serodiscordant couples, men having sex with men (MSM) and children born to HIV^+

mothers. Amongst the different cohorts, there is no strict definition used to categorize someone as an EU. A minimal number of exposures to HIV can determine inclusion into some cohorts. Alternatively, other cohorts define EUs based on a minimum duration of seronegativity despite repeated exposure. In addition, studies attempting to determine mechanism(s) underlying resistance in EUs often include a relatively small sample size due to problems inherent in identifying highly exposed seronegative individuals and/or the rarity of the phenotype. These factors may explain why several different mechanisms have been proposed to explain the resistance to HIV observed in some individuals. These include genetic factors, HIV-specific adaptive immune responses, and characteristics of the innate immune system. It is also likely that several factors combine to provide EUs with an immunological advantage and create a state of reduced susceptibility to infection, making it difficult to narrow down a single determinant of EU status.

A. GENETIC CORRELATES

By targeted genotyping of individuals in EU cohorts, several loci have been correlated with reduced susceptibility to infection. Because host-virus interactions are required for viral reproduction, host genetic factors can influence the probability of a productive infection by acting at virtually every stage of HIV's replication cycle. Additionally, the genetic background of the host will shape its immune responses.

CCR5Δ32

In an effort to determine their *in vitro* susceptibility to infection, cells isolated from two EUs were found to be particularly resistant to M-tropic but not to T-tropic

strains of HIV[329]. Because M-tropic HIV requires CCR5 as a co-receptor for entry into the host cells, this chemokine was investigated for its possible role in resistance to infection. Genotyping revealed that both individuals had an identical homozygous defect in their CCR5 gene[330]. The 32 base pair deletion (Δ32) result in a truncated protein that fails to reach the cell surface and therefore cannot be used by HIV for cellular entry. However, HIV infection, mostly with T-tropic viruses, has been found in CCR5Δ32 homozygote individuals, indicating that this phenotype does not confer absolute resistance to infection[331-334]. While heterozygosity for the 32 base pair deletion is not protective against HIV infection, it has been linked to delayed HIV disease progression and lower viral loads when compared to individuals with 2 copies of the wild-type gene encoding CCR5[335].

The frequency of the CCR5Δ32 mutation is 1.4-20.9% in individuals of European, Middle Eastern and Indian descent[336]. However, this mutation is virtually absent from African and East Asian populations[336]. Additionally, the frequency of the homozygous CCR5Δ32 phenotype in screened EU cohorts ranges from 1.7% to 13%, and is sometimes not detected at all[329,337-341]. This suggests that, while CCR5Δ32 homozygosity provides a level of resistance to infection, it does not provide a satisfactory answer to the EU phenomenon, particularly in African cohorts such as the HIV resistant Pumwami cohort.

Nonetheless, knowledge gained by studying the CCR5Δ32 in EUs has recently been successfully exploited to develop new treatment strategies for HIV infection. A seropositive 40-year old man diagnosed with acute myeloid leukemia successfully received an allogeneic stem cell transplant from a donor homozygous for the CCR5Δ32 mutation[342]. For the moment, HIV viral load is undetectable in peripheral blood and

rectal biopsies and HAART has been discontinued. In addition, while anti-HIV antibodies are still detectable, those targeting viral polymerase and capsid proteins were lost after transplant, suggesting that the individual may eventually revert to a seronegative status.

HLA alleles

HLA alleles that are very similar in peptide-binding affinity can be grouped into supertypes[343]. One such supertype, termed A2/6802, has been linked to HIV resistance. The A2/6802 supertype includes HLA-A*0202, A*0205, A*0214 and A*6802. In the Pumwani cohort of commercial sex workers, possession of alleles belonging to this supertype was linked to an increased probability of remaining seronegative (study n=232)[344]. This finding was confirmed in mother-to-infant perinatal transmission in another cohort located in Nairobi (n=171) as well as in a cohort of North American homosexual males (n=284)[345,346]. Therefore, the link between the A2/6802 supertype and resistance has been determined in populations of different genetic backgrounds and different risk factors for HIV acquisition. In the North American cohort, the effect of resistance was largely mediated by the A*0205 allele.

Other HLA alleles have been linked with resistance to infection in several EU cohorts. These include the HLA class II molecules HLA-DRB1*01 in the Pumwani cohort, HLA-B*53 in North American Hispanic individuals, HLA-DR5 in an Irish study and HLA-A*11 in a cohort of sex workers in Thailand[344,347-349]. However, these results have not been confirmed in other populations, limiting the significance of the findings

until molecular or other epidemiological studies corroborate the effect of these HLA molecules on resistance.

CCL3L1

CCL3L1 (CC chemokine ligand 3 like-1 or MIP-1αP) is the most potent agonist of the CCR5 HIV co-receptor[350]. Its gene is found on chromosome 17q and duplications result in varying copy numbers (from 0 to 14 copies) of CCL3L1 among individuals[351]. Additionally, increasing copy numbers of the gene is associated positively with CCL3L1 secretion levels[351]. In a study performed in several populations across the world, it was determined that individuals with CCL3L1 copy numbers that were below the population average were at increased risk of being infected with HIV[351]. In another study, infants born to HIV^+ mothers with increased gene copy numbers were less likely to become infected[352]. As discussed in section IIIB, CC chemokines can competitively inhibit HIV replication, which provides a mechanistic explanation for the importance of CCL3L1 in resistance to infection. However, more recent studies have failed to reproduce these findings and the effect of CCL3L1 on HIV infection remains controversial[353].

Polymorphisms

Polymorphisms in several genes influencing the immune system have been linked to decreased susceptibility to HIV infection. However, few of these have been confirmed in more than one study. Nonetheless, polymorphisms in the genes for SDF-1 (stromal cell-derived factor 1), DC-SIGN, IRF-1 (interferon regulatory factor 1), and vitamin D receptor were all found in increased proportions in at least one EU cohort[354]. Each of

these could lead to the development of new therapeutic strategies if their effects can be confirmed.

B. ADAPTIVE IMMUNE RESPONSES

Two reasons explain the considerable attention that has been given to the capacity of EUs to mount an HIV-specific T-cell or humoral immune response. Historically, EUs were first described as individuals capable of mounting an HIV-specific adaptive immune response[325]. Secondly, the ultimate goal of an HIV preventative vaccine is to induce adaptive immune responses capable of controlling the virus. However, the type of immune response capable of mediating HIV resistance being unknown, perhaps clues helping vaccine design could be found in EUs.

T-cell immune responses

Both $CD8^+$ and $CD4^+$ HIV-specific T-cell responses have been reported in EUs. These are usually evaluated by comparing the HIV-induced responses of lymphocytes isolated from highly-exposed seronegative individuals to those isolated from low-risk controls and/or HIV^+ subjects.

$CD8^+$ responses have been evaluated by measuring the percent lysis of radioactive HIV-labeled target cells or the IFN-γ secretion in response to stimulation with short HIV-peptides. Using one of the two methods, responses have been detected in peripheral and mucosal tissues of EU from the Pumwani cohort[355,356], in injection drug users[357,358], in occupationally exposed health care workers[326], in children born to HIV-infected mothers[327,359,360] and in sexual partners of seropositive individuals[340,341,361]. Importantly,

low-risk controls were not found to respond to HIV stimulation. Despite methodological and analytical differences between the studies, consistent detection of HIV-specific responses in cohorts recruiting individuals that differ in risk factor for HIV infection and genetic background supports an association between $CD8^+$ T-cell function and resistance to HIV infection. When comparing the target specificity of T-cell responses in EUs versus their seropositive sexual partners, a differential immunodominance is noted[362]. While the number of HIV-peptides recognized by EUs is smaller than that in their seropostive partners, EU responses more often target the Vif protein and sequences known to be associated with a slower rate of disease progression. In the Pumwani cohort, epitopes recognized by lymphocytes from EUs were found to be more often restricted to HLA molecules A2, A*6802, A24, B14 and B18[363]. The latter finding seems to confirm previous epidemiological studies performed in this particular cohort linking these MHC class I molecules to resistance from infection[344]. Finally, the loss of $CD8^+$ HIV-specific responses was linked to the seroconversion of eleven Kenyan commercial sex workers that previously qualified as resistant to infection[364]. A break from sex-work was associated with the loss of HIV-specific responses, suggesting that continuous antigenic exposure may be required to maintain these. There are thus several lines of evidence supporting the role of cytotoxic T lymphocytes in resistance to HIV infection.

Data concerning the presence of HIV-specific $CD4^+$ T cells in EUs is perhaps less convincing than that for $CD8^+$ T cells. Initially, several studies reported increased IL-2 in the culture supernatant or increased proliferation (evaluated by tritiated thymidine incorporation) by cells isolated from EUs following stimulation of lymphocytes with HIV fragments[341,365-368]. However, in some studies, depending on the statistical algorithm

employed to distinguish between a positive and a negative response, 2-16% of low-risk control individuals also had a measurable $CD4^+$ T cell response to HIV stimulation[341,365,366]. While this is consistently a smaller proportion than in EUs, it seems to suggest that initial assays, based on surrogate markers for CD4 responses, lacked specificity. In a more recent study, flow cytometry was used to specifically measure $CD4^+$ T cell responses[369]. Thirty-eight percent of EUs were found to have IFN-γ secreting $CD4^+$ T cells in response to Gag stimulation. None of the low-risk controls were found to respond. The role for $CD4^+$ T cells in EU is also supported by *in vitro* evidence that CD4 clones from EUs secrete more CC chemokines, such as RANTES, MIP-1α, and MIP-1β in response to HIV peptides than uninfected controls[370]. This explains how supernatant collected from HIV-peptide stimulated EU clones, but not clones from uninfected subjects, is able suppress viral replication[370]. Similarly to $CD8^+$ T cell responses, HIV-specific $CD4^+$ T cell responses in EUs seem to require frequent exposure to the virus to be maintained[369,371].

Humoral responses

Humoral responses that lead to protection from infection in EUs can be of two types: those directed against cellular proteins involved in the initial steps of HIV infection and those directed directly against viral proteins. The *in vitro* anti-viral activity of sera isolated from EUs has, in some cases, been associated with the fraction that interacts with CD4 or CCR5[372,373]. Because these are receptors required for viral entry into the host cell, anti-CD4 and anti-CCR5 antibodies could interfere with the viral life cycle either by steric hindrance or by leading to the decreased cell surface expression of

the receptors. In both cases, HIV attachment to the host cell could be prevented. How these antibodies are generated is still unclear, but could be the result of a secondary response mounted to self-antigens associated with viral particles following exposure[354].

Despite being seronegative by conventional testing methods that usually rely on the detection of HIV-specific IgG antibodies, the presence of HIV-specific IgA antibodies has been detected in the mucosa of several cohorts of sexually exposed EUs[341,365,374,375]. These antibodies have been shown to be capable of preventing entry into $CD4^+$ T cells in experiments using *in vitro* models of infection[376-378]. Additionally, IgA from EUs was shown to neutralize primary HIV isolates from different clades[376]. However, the presence of HIV-neutralizing antibodies has not been confirmed by all studies; some studies performed on African and North American EUs were unable to detect mucosal HIV-specific IgA[379,380]. Another caveat regarding the role of humoral responses in resistance to infection is that HIV-specific IgA molecules have been detected in unexposed uninfected individuals[341,365]. In all studies describing the neutralizing capacity of IgA antibodies isolated from EUs, none has been able to isolate the clone secreting this antibody. Because HIV epitopes are highly variable, the development of neutralizing antibodies that are capable of targeting multiple clades has been difficult. Uncovering the exact mechanism of the HIV neutralizing capacity in EUs could provide clues to better understand HIV immunology.

C. INNATE IMMUNE RESPONSES

The early events following HIV exposure are likely to determine whether the outcome of the exposure results in a productive infection. Acting prior to the

development of an adaptive immune response, innate effectors are possibly involved in the resistance of EUs to HIV infection.

APOBEC3G

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3G (APOBEC3G) are proteins with antiretroviral capacity. They inhibit retroviral replication through their cytidine deaminase activity and other nonenzymatic mechanisms[381]. APOBEC3G can be incorporated into viral particles upon budding from the host cell and therefore be associated with the viral genome when the particles infect a new target cell. Upon reverse transcription, APOBEC3G recognizes nascent single stranded DNA and mediates cytidine deamination, yielding uracil, as found in RNA. Two fates await the uracil-containing genome (figure 25). It can either be recognized by uracil-DNA glycolase and endonucleases and be degraded or the uracils on the 'minus' DNA strand can result in adenine on the 'plus' DNA strand during subsequent genome synthesis, resulting in a net guanine to adenine (G to A) mutation on the 'plus' strand. This G to A hypermutation can be lethal to the virus. However, APOBEC3G mutants deficient for deamination activity retain some antiviral activity, suggesting that other mechanisms may be involved[382]. It is possible that APOBEC3G binds viral DNA and physically blocks reverse transcription. In order to counteract the action of APOBEC3G, HIV Vif can target the deaminase complex for degradation by ubiquitination[381].

In a cohort of sexually exposed uninfected individuals, levels of APOBEC3G were shown to be elevated in monocytes isolated from EUs when compared to both

infected and uninfected low-risk controls[383]. In addition, increased APOBEC3G levels were linked to a reduced susceptibility to HIV infection *in vitro*.

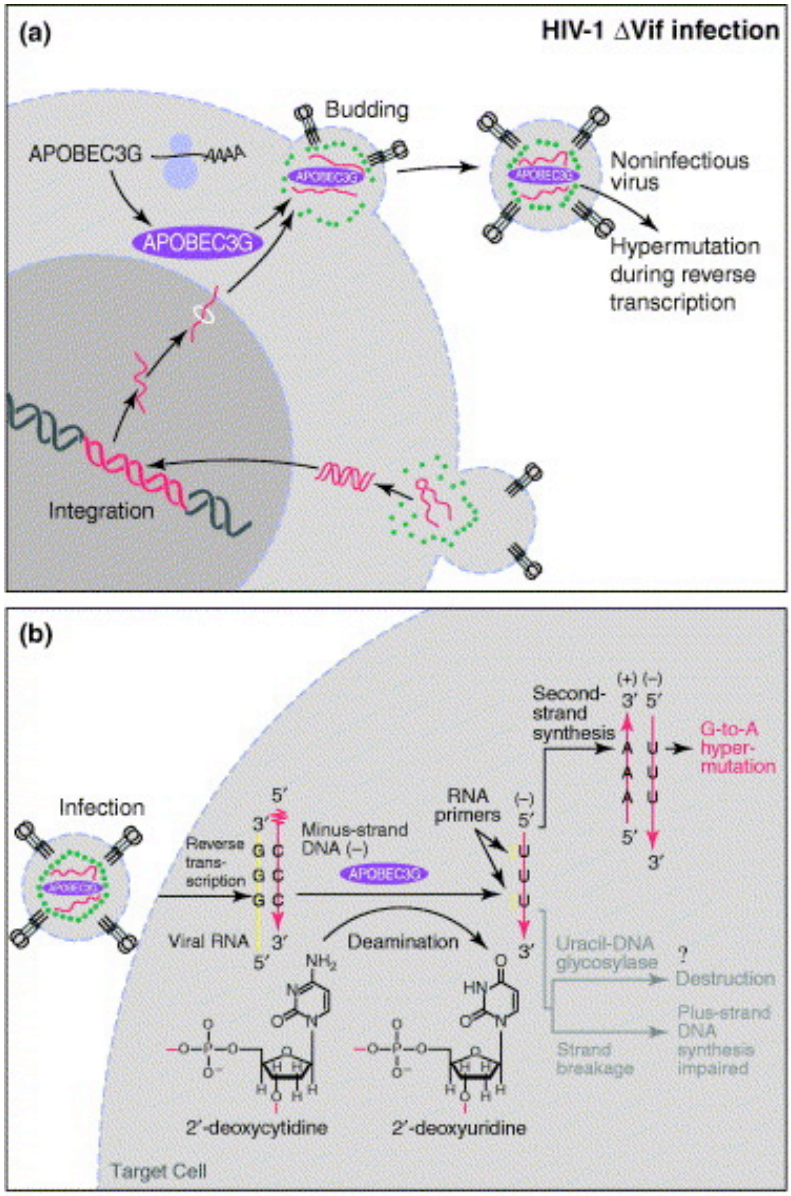


Figure 25[381]**:** APOBEC3G deamination anti-viral mechanism. (Reprinted from Trends in Immunology, Vol. 27, Chiu YL, Greene WC, Multifaceted antiviral actions of APOBEC3 cytidine deaminases, 291-297, 2006, with permission from Elsevier).

IL-22

IL-22 (inteuleukin-22) mediates the increased production of a number of innate effectors, including acute-phase amyloid A protein (A-SAA)[354]. A-SAA mediates downregulation of CCR5 on the surface of dendritic cells by favoring its phosphorylation, which could explain the *in vitro* anti-HIV function of A-SAA[384]. Both IL-22 and A-SAA were shown to be increased EUs[384].

NK cells

The relationship between NK cells and HIV has been extensively covered in section III. Given this relationship, it is therefore not surprising that a possible link between NK cells and EUs has been investigated. In 2003, Scott-Algara et al published observations comparing the activity of NK cells in 37 Vietnamese high-risk injection drug users (IDU) to those of low-risk controls or seroconverters[152]. NK cells isolated from EUs demonstrated increased activity as measured by lysis of radiolabeled targets, secretion of IFN-γ and TNF-α cytokines and secretion of CC chemokines. In a follow-up study, it was demonstrated that NK cells from IDU EUs had a receptor repertoire consistent with activation[385]. For example, there was an increased NKG2C/NKG2A ratio and an increased expression of the activation marker CD69 on NK cells isolated from EUs. Interestingly, in KIR3DS/L1 heterozygous individuals (n=7), an increased ratio of KIR3DS1/KIR3DL1 mRNA (messenger RNA) was observed in EUs. This ratio was particularly elevated in three individuals. Given the known relationship between the KIR3DS/L1 locus and HIV viral load and disease progression in infected individuals, this observation seems to suggest a role for this NK cell receptor in resistance to infection as

well[150,151,385]. However, because of the small number of individuals analyzed for KIR3DS1 and KIR3DL1 transcript levels, the role of KIR3DS/L1 locus in decreased HIV susceptibility remains unclear.

Observations performed in a cohort of 21 female sex workers from Côte d'Ivoire also support a role for NK cells HIV resistance[386]. Genetic analyses demonstrated that EU blood donors were more likely to possess KIR B haplotypes (which contains more activating KIR molecules, see section IIC). EUs in Abidjan were also more likely to posses inhibitory KIRs in the absence of their HLA ligand. Consequently, HIV resistant female sex workers in this cohort were more likely to posses KIR2DL2 and KIR2DL3, but not their ligand HLA-C1. Similarly, KIR3DL1 homozygosity was often accompanied by the absence of HLA-Bw4. The absence of ligands for inhibitory KIRs was proposed to lower the threshold for activation of NK cells. Thus, data obtained from two distinct cohorts points to a role of NK cells in decreased HIV susceptibility.

V. Rationale

The immunological mechanism behind the decreased susceptibility to HIV infection observed in some individuals may provide clues for new therapeutic approaches or preventative vaccine strategies. NK cells are known to be important for the control of viral infections and a role for these cells in exposed uninfected individuals has recently emerged. Given the known link between the NK cell receptor KIR3DS/L1 locus and HIV disease outcome, we sought to determine whether this locus could also be linked to resistance from infection, perhaps through effects on NK cell function.

Chapter 2: Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals

Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals

Running head: Innate immunity in HIV exposed uninfected individuals

Salix Boulet*, Saeid Sharafi*, Nancy Simic*, Julie Bruneau†, Jean-Pierre Routy*, Christos M Tsoukas*, Nicole F. Bernard*

* Research Institute of the McGill University Health Centre, Montreal, Canada

†Centre de recherche du Centre Hospitalier de l'Université de Montréal, Montreal, Canada

Mailing address: Dr. Nicole F. Bernard, Research Institute of the McGill University Health Center, Montreal General Hospital, 1650 Cedar Ave. Rm C10-160, Montreal, Quebec, Canada, H3G 1A4.
Tel: +1 (514)934-1934 ext: 44584
Fax: +1 (514)937-1424
E-mail: nicole.bernard@mcgill.ca

Funding: This study received support from the Canadian Institutes for Health research #MOP-79515, the Fonds de la Recherche en Santé du Québec (FRSQ). S Boulet was supported by a Ph.D. scholarship from the Canadian Network for Vaccines and Immunotherapeutics (CANVAC) Network Centre for Excellence. J.P. Routy is a scientific scholar receiving support from FRSQ.

Word count: 1910

I. Abstract

Objectives: Natural killer (NK) cell activity is increased in individuals who remain uninfected despite repeated exposures to HIV. Given that a combined major histocompatibility complex (MHC) class I and killer immunoglobulin-like receptor (KIR) KIR3D genotype has been linked to rate of HIV disease progression, we assessed whether these genotypes played a role in protection from infection.

Design: The study genotyped 80 HIV exposed uninfected (EU) and 304 subjects in HIV primary infection (PI) at the MHC class IB and KIR3DS/L1 loci.

Methods: KIR3D genotyping was performed by sequence-specific primer PCR using 2 pairs of specific primers for each locus. The MHC class IB locus was typed by sequence specific oligonucleotide PCR and sequencing to resolve Bw4 and Bw6 alleles and the amino acid present at position 80.

Results: Comparison of the genetic distribution of KIR3D, HLA Bw4 and HLA Bw4-I80 genotypes in EU versus PI subjects reveal an increased proportion of KIR3DS1 homozygotes in EU (11/80, 13.8%) compared to subjects in PI (16/304, 5.3%). Analyses of combined MHC class I and KIR3D expression show no differences between the two populations.

Conclusions: Homozygosity for the activating NK receptor KIR3DS1, may contribute to the more active NK cell function observed in EU and their relative resistance to HIV infection.

II. Introduction

Certain individuals remain seronegative despite repeated exposures to HIV. The study of these exposed uninfected (EU) individuals may help identify features of natural resistance to this virus. A recent study of Vietnamese injection drug users (IDU) demonstrated that NK cells are more activated in EU than in seroconverters and low-risk controls [1]. This suggests that NK cells may be involved in conferring protection from HIV infection seen in EU.

NK cells play an important role in innate immunity by providing protection against viruses and tumor cells [2]. Their function is regulated by the integration of activating and inhibitory signals transmitted through cell surface receptors [3,4]. Killer Immunoglobulin-like receptors (KIR) are a family of activating and inhibitory receptors that participate in the activation of NK cells. Although the gene content of a KIR genome is highly variable, a few framework loci, such as KIR3DS/L1, are found on nearly all haplotypes [5]. KIR3DS1 is an activating KIR gene with an unknown ligand and is allelic with KIR3DL1, an inhibitory receptor that binds to HLA Bw4 [6].

In HIV infected individuals, co-expression of KIR3DS1 with a subset of HLA Bw4 alleles having an isoleucine at position 80 (HLA Bw4-I80) is correlated with slower disease progression [7]. Given that NK cells may be involved in the resistance demonstrated by EU, we sought to determine whether either KIR3DS1 or HLA Bw4 or a combination of these alleles were also associated with protection from infection. Previous

work examining NK receptor expression in EU from an African population and a Vietnamese IDU group found patterns reflecting increased NK activation potential compared with controls, but no increase in KIR3DS1 or link between KIR3DS1 and HLA Bw4-I80 [8,9]. However, the small sample size of the populations studied together with the frequency of KIR3DS1 expression in these populations may have precluded detecting an association between KIR3DS1 expression with or without co-expression of HLA-Bw4 alleles and protection from infection [8,9]. Here we compared the distribution of KIR3DS/L1 and HLA B genotypes of a larger cohort of North American EU and subjects in HIV primary infection (PI) and report a significantly increased proportion of KIR3DS1 homozygosity in these EU versus the HIV susceptible PI population.

III. Methods

Study Subjects

Eighty EU and 304 individuals in PI were included in this study. Subjects in PI were participants of the Montreal PI cohort, which recruits individuals who are within 1 year of infection as determined using the criteria established by the Acute HIV Infection Early Disease Research Program sponsored by the National Institutes of Health and follows them over 2 years [10]. EU were recruited from a prospective cohort of active HIV-negative IDU at high risk for HIV acquisition [11], and among HIV negative partners of serodiscordant couples followed in medical clinics in Montreal [12]. Subjects were followed longitudinally every 6 months. Follow up included assessment of the frequency of high-risk behavior for HIV acquisition, blood draws and monitoring of HIV serostatus. All EU subjects maintained a negative HIV enzyme immunoassay (HIV EIA) test despite at least 5 documented exposures. Parenteral exposure was defined as sharing needles with known HIV infected partners while mucosal exposure was defined as unprotected sex with a known HIV infected partner. None of the EU subjects were CCR5Δ32 homozygotes. Informed consent was obtained from all study subjects, and the research conformed to ethical guidelines of all the authors' institutions.

Genotyping

Genomic DNA was extracted from PBMC or EBV-transformed cells using a QIAamp DNA blood kit (QIAGEN, Inc., Mississauga, Ontario, Canada). KIR3DS/L1 genotyping was performed by PCR with sequence-specific primers based on previously

published studies [7,13]. Two pairs of specific primers for each locus were used for amplification of KIR3DL1 and KIR3DS1. Primers for NKG2A were also included as a positive control. All primer sequences and amplification conditions are available upon request. Subjects were typed for MHC class I expression using the line probe assay (Innogenetics Inc, Alpharetta, GA) and by sequencing (Atria Genetics, South San Francisco, CA) if high resolution typing was needed to resolve the assignment of B alleles to the Bw4 or Bw6 public specificities based on amino acids at positions 77 to 83.

Statistics

Statistical analysis and graphical presentation were performed using GraphPad InStat 3.05 and GraphPad Prism 4.01. Fisher's exact test was used to compare proportions of selected genotypes between EU and PI. A two-tailed chi-square (χ^2) test was used to examine differences in the distribution of genotypes between EU and PI subjects. Hardy Weinberg equilibrium was evaluated using a χ^2 test with frequencies of each allele derived from the combined population (KIR3DS1 = 0.22). A *p*-value of less than 0.05 was considered significant.

IV. Results

The EU and PI populations were mainly composed of Caucasian (93%) living in the same geographic region (Montreal) and there was no significant between-group differences in racial composition (data not shown). All individuals were genotyped for KIR3DS/L1 and MHC class IB locus alleles. While the distribution of the three KIR3DS/L1 genotypes: KIR3DS1 homozygote (3DS1hmz), KIR3DS1/KIR3DL1 heterozygote (3DS/L1htz) and KIR3DL1 homozygotes (3DL1hmz) at this locus in the PI population did not deviate statistically from the Hardy-Weinberg (H-W) equilibrium (χ^2=2.17), the same distribution in the EU population diverged from H-W equilibrium (χ^2=13.20). The deviation from H-W equilibrium of EU but not of PI was reflected in a statistically significant difference in the distribution of the 3 KIR3DS/L1 genotypes between the two groups (χ^2 test, p=0.0283, Table I). When each of the frequencies for 3DS1hmz, 3DS/L1htz and 3DL1hmz were compared in EU vs. PI subjects, we found that there was a significant increase of 3DS1hmz individuals in EU (OR=2.87, CI=1.28-6.46, p=0.0132) as shown in Table I. However, the proportions of 3DS/L1htz and 3DL1hmz were similar. Thus these results suggest an increase in the proportion of individuals with the activating genotype KIR3DS1hmz in our EU population.

While the known ligand for KIR3DL1 is HLA Bw4, a molecular interaction between KIR3DS1 and either HLA-Bw4 or HLA Bw4-I80 has yet to be demonstrated experimentally despite an association of the KIR3DS1 and HLA Bw4-I80 genes with slower HIV disease progression [7,14-16]. The possible interaction of HLA Bw4

supertype or of the subset of alleles expressing an isoleucine at position 80 (HLA Bw4-I80) with KIR3DS/L1 prompted us to assess the distribution of these HLA alleles in EU and PI subjects. No significant differences or trends in the between group distribution of these alleles were found (Table I).

We next examined whether there were between group differences in the distribution of KIR3DS/L1 genotypes in association with their known or putative HLA ligand (HLA Bw4 or HLA Bw4-I80). Figure 1A shows that the proportion of individuals having at least one copy of KIR3DS1 in the presence of HLA Bw4-I80 was similar in EU and subjects in PI.

A recent study found an increased proportion of 3DL1hmz EU that were HLA Bw6 homozygotes (i.e. lacked the HLA Bw4 receptor for 3DL1)[8]. However, as shown in Figure 1B, we could not confirm this observation in our cohort. For all analyses testing for associations of KIR3DS/L1 and HLA Bw4 or HLA Bw4-I80, no significant differences were found between EU and PI except those reflecting the increased frequency of KIR3DS1hmz in EU described above (data not shown). This suggests that HLA genotypes, alone or in combination with KIR3DS1, implicated in modulating rate of HIV disease progression in infected subjects do not appear to be a factor in preventing HIV infection of the EU population studies here.

V. Discussion

We have studied the distribution of KIR3DS/L1 genotypes and of their known or possible ligands in HIV-resistant EU and HIV-PI subjects. Subjects in PI were selected as a control population for these studies because they are susceptible to HIV infection and eliminate any effect that disease progression could have on skewing KIR or HLA genotypes. We have shown that KIR3DS/L1 genotypes were not in H-W equilibrium in EU due to an increased proportion of 3DS1hmz in these subjects. The presence of this activating KIR gene (or the absence of the inhibitory KIR3DL1) could contribute to the increased level of NK cell activation in EU described in previous studies [1,9,17]. Whether the KIR3DS1hmz genotype translates into more activated NK cell function remains to be demonstrated. It is unclear whether it is the absence of an inhibitory KIR3DL1 gene or the presence two copies of KIR3DS1 is more important for resistance to HIV infection. In the former case, expression levels of KIR3DL1 alleles, their inhibitory capacity or whether they are expressed at all (such as KIR3DL1*004) may modulate susceptibility to HIV in heterozygous individuals expressing one copy of KIR3DS1 [18-21]. A recent report provides support for a role for high expression KIR3DL1 allotypes in combination with HLA-Bw4 in protection from HIV disease progression [22]. Selection of greater NK receptor inhibitory potential during the NK functional maturation process termed licensing may permit superior activation when signaling through the receptor is disrupted such as during viral infection [22,23]. Therefore an analysis of the distribution of KIR3DL1 allotypes in EU and PI subjects is warranted to determine whether these alleles also play a role in HIV resistance. It is also

possible that two copies of KIR3DS1 are required for increased potency of NK cells for limiting virus spread before infection can become established. Finally, we cannot exclude that a gene in linkage disequilibrium with KIR3DS1 mediates the protective effect.

Although we cannot rule out that the size of our sample was too small to observe between group differences, we did not observe even a trend towards an effect of expression of MHC class I alleles HLA Bw4 or HLA Bw4-I80 either alone or in combination with KIR3DS/L1 genotypes when we compared our study groups. Although, interactions between KIR3DS1 and HLA-Bw4 molecules have not been experimentally demonstrated, it has recently been shown that KIR3DL1/HLA-Bw4 binding is improved by the presence of peptide [14,15,21,24]. In EU subjects, in whom a productive infection is never established, the impact of HIV peptide would have relevance limited to a time interval between transmission and control/clearance of infection. In this scenario, the interaction of KIR3DS/L1 with its ligand may be less important than possessing NK cells with a greater potential for activation in the context of all MHC class I types expressed on potential HIV infected donors. In addition, the impact of co-expression of KIR3DS1 and HLA Bw4-I80 on HIV disease progression has recently been called into question as some investigators have failed to demonstrate synergy between expression of these molecules in relation to markers of clinical HIV disease progression [25,26]. The difference between these results and those reported by Martin et al may be explained by the differences in patient status, clinical outcome measures used as markers of disease progression or statistical methodology used to analyze results [7,25-27].

In an African EU population, female sex workers had decreased genetic pairing of an inhibitory KIR with its HLA ligand, which would reduce signaling through inhibitory receptors [8]. For example, they found more KIR3DL1hmz/HLA Bw6hmz (i.e. Bw4 -/-) in their EU group than controls. Although this result was not confirmed in this study, the overall concept that potential for improved NK activation or reduced NK inhibition is linked with resistance to HIV infection is a consistent finding [8]. Neither of the studies that have examined NK receptor patterns in EU found an increase in KIR3DS1hmz in the EU subjects [8,9]. The discrepancy between these findings and ours could be explained by differential frequency of KIR3DS/L1 in African, Vietnamese and North American populations but also by study population sample size.

VI. Acknowledgments

We would like to acknowledge Drs. M. Martin and M. Carrington for providing the sequence for the primers used for KIR3DS/L1 genotyping, Drs. G. Alter and M. Altfeld for helpful discussion of the results, Mr. Mario Legault and Mr. Martin Rioux for coordination of the Montreal PI and IDU cohort, respectively. We would also like to acknowledge the investigators of the Montreal PI cohort for subject recruitment and clinical follow up.

VII. Figure Captions

Table 1: Three genetic factors were evaluated for the differences in distribution between EU and PI (χ^2test) as well as the proportion of homozygotes and heterozygotes in each population (OR, odds ratio; CI, confidence interval): KIR3DS/L1, HLA Bw4 and HLA Bw4-I80. Only a different distribution in KIR3DS/L1 and an increase in KIR3DS1hmz in EU were found to be significant. N/a = not applicable.

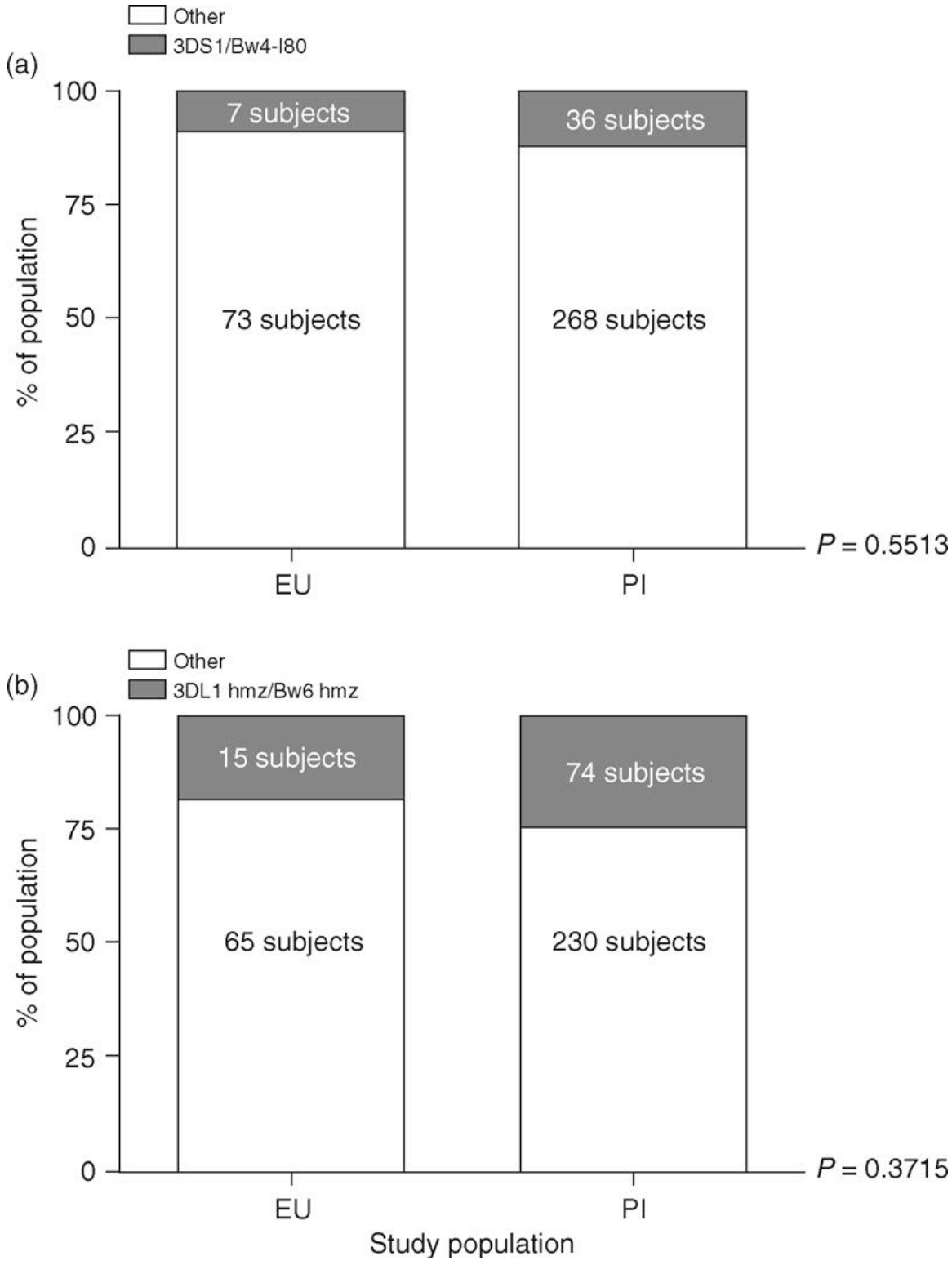
Figure 1. Combined distribution of HLA Bw4 or Bw4-I80 and KIR3DS/L1 in HIV exposed uninfected subjects (EU) and individuals undergoing primary HIV infection (PI). A) Percentage of individuals with at least one copy of HLA Bw4-I80 and one copy of KIR3DS1 (grey bars) relative to other possible combinations for these alleles (white bars) in EU and PI. B) Percentage of individuals 3DL1hmz and HLA Bw6hmz (grey bars) relative to other possible combinations for these alleles (white bars) in EU and PI.

VIII. Tables and Figures

Table 1: Overrepresentation of KIR3DS1 homozygotes in EU

Genetic factor	EU (%) (n= 80)	PI (%) (n= 304)	OR	95% CI	p
KIR3DS1					
3DS1/3DS1	11 (13.8)	16 (5.3)	2.87	1.28-6.46	**0.0132**
3DS1/3DL1	24 (30.0)	93 (30.6)	0.97	0.57-1.66	1.0000
3DL1/3DL1	45 (56.3)	195 (64.1)	0.72	0.44-1.19	0.1974
3X2 χ^2 test	n/a	n/a	n/a	n/a	**0.0283**
HLA Bw4					
Bw4/Bw4	13 (16.3)	43 (14.1)	1.18	0.60-2.32	0.5983
Bw4/Bw6	34 (42.5)	149 (49.0)	0.77	0.47-1.26	0.3165
Bw6/Bw6	33 (41.3)	112 (36.8)	1.20	0.73-1.99	0.5174
3X2 χ^2 test	n/a	n/a	n/a	n/a	0.5821
HLA BW4-I80					
Bw4-I80/Bw4-I80	3 (3.8)	8 (2.6)	1.44	0.37-5.57	0.7048
Bw4-I80/not Bw4-I80	22 (27.5)	83 (27.3)	1.01	0.58-1.75	1.0000
not Bw4-I80/not Bw4-I80	55 (68.8)	213 (70.1)	0.94	0.55-1.60	0.8912
3X2 χ^2 test	n/a	n/a	n/a	n/a	0.8637

Figure 1


Other
3DS1/Bw4-I80
(a)
100
75
50
25
0
% of population
7 subjects
36 subjects
73 subjects
268 subjects
P = 0.5513
EU
PI
Other
3DL1 hmz/Bw6 hmz
(b)
100
75
50
25
0
% of population
15 subjects
74 subjects
65 subjects
230 subjects
P = 0.3715
EU
PI
Study population

IX. References

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

2. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. Nat.Rev.Immunol. 2001; **1:** 41-49.

3. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu.Rev.Immunol. 2002; **20:** 217-251.

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

5. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D *et al.* Plasticity in the organization and sequences of human KIR/ILT gene families. Proc.Natl.Acad.Sci.U.S.A 2000; **97:** 4778-4783.

6. Uhrberg M. The KIR gene family: life in the fast lane of evolution. Eur.J.Immunol. 2005; **35:** 10-15.

7. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ *et al.* Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat.Genet. 2002; **31:** 429-434.

8. Jennes W, Verheyden S, Demanet C, Adje-Toure CA, Vuylsteke B, Nkengasong JN *et al.* Cutting edge: resistance to HIV-1 infection among African female sex

workers is associated with inhibitory KIR in the absence of their HLA ligands. J.Immunol. 2006; **177:** 6588-6592.

9. Ravet S, Scott-Algara D, Bonnet E, Tran HK, Tran T, Nguyen N *et al.* Distinctive NK cell receptor repertoires sustain high-level constitutive NK cell activation in HIV-exposed uninfected individuals. Blood 2007.

10. Alter G, Tsoukas CM, Rouleau D, Cote P, Routy JP, Sekaly RP *et al.* Assessment of longitudinal changes in HIV-specific effector activity in subjects undergoing untreated primary HIV infection. AIDS 2004; **18:** 1979-1989.

11. Boileau C, Bruneau J, Al-Nachawati H, Lamothe F, Vincelette J. A prognostic model for HIV seroconversion among injection drug users as a tool for stratification in clinical trials. J.Acquir.Immune.Defic.Syndr. 2005; **39:** 489-495.

12. Bernard NF, Yannakis CM, Lee JS, Tsoukas CM. Human immunodeficiency virus (HIV)-specific cytotoxic T lymphocyte activity in HIV-exposed seronegative persons. J.Infect.Dis. 1999; **179:** 538-547.

13. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B *et al.* Human diversity in killer cell inhibitory receptor genes. Immunity. 1997; **7:** 753-763.

14. Carr WH, Rosen DB, Arase H, Nixon DF, Michaelsson J, Lanier LL. Cutting Edge: KIR3DS1, a gene implicated in resistance to progression to AIDS, encodes a DAP12-associated receptor expressed on NK cells that triggers NK cell activation. J.Immunol. 2007; **178:** 647-651.

15. Thananchai H, Gillespie G, Martin MP, Bashirova A, Yawata N, Yawata M *et al.* Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. J.Immunol. 2007; **178:** 33-37.

16. Gillespie GM, Bashirova A, Dong T, McVicar DW, Rowland-Jones SL, Carrington M. Lack of KIR3DS1 Binding to MHC Class I Bw4 Tetramers in Complex with CD8(+) T Cell Epitopes. AIDS Res.Hum.Retroviruses 2007; **23:** 451-455.

17. Montoya CJ, Velilla PA, Chougnet C, Landay AL, Rugeles MT. Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. Clin.Immunol. 2006; **120:** 138-146.

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

19. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. J.Immunol. 2005; **175:** 5222-5229.

20. Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J.Exp.Med. 2006; **203:** 633-645.

21. O'Connor GM, Guinan KJ, Cunningham RT, Middleton D, Parham P, Gardiner CM. Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells. J.Immunol. 2007; **178:** 235-241.

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

23. Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class I. Immunol.Rev. 2006; **214:** 143-154.

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

25. Gaudieri S, DeSantis D, McKinnon E, Moore C, Nolan D, Witt CS *et al.* Killer immunoglobulin-like receptors and HLA act both independently and synergistically to modify HIV disease progression. Genes Immun. 2005; **6:** 683-690.

26. Barbour JD, Sriram U, Caillier SJ, Levy JA, Hecht FM, Oksenberg JR. Synergy or Independence? Deciphering the Interaction of HLA Class I and NK Cell KIR Alleles in Early HIV-1 Disease Progression. PLoS.Pathog. 2007; **3:** e43.

27. Qi Y, Martin MP, Gao X, Jacobson L, Goedert JJ, Buchbinder S *et al.* KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. PLoS.Pathog. 2006; **2:** e79.

Bridge from Chapter 2 to 3

By genotyping the KIR3DS/L1 locus in chapter 2, we uncovered a relationship between KIR3DS1 homozygosity and EU status. The KIR3DL1 allele did not seem to confer protection from infection. As discussed in section IIC of chapter I, the KIR3DL1 gene is highly polymorphic with over 50 known alleles. A study published by Martin et al., after we had completed the KIR3DS/L1 genotyping of our populations, demonstrated a link between certain HLA/KIR3DL1 allotypes and HIV disease progression[151]. We therefore undertook the task of allotyping the KIR3DL1 locus for both the EU and PI population for whom we already had information on HLA typing. The goal was to determine whether the HLA/KIR3DL1 allotypes linked to slower HIV disease progression could also be linked to decrease susceptibility to HIV infection.

Chapter 3: A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection

---

Concise Communication

A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection

Running head: KIR3DL1 subtypes, HLA and HIV resistance

Salix Boulet*, Marianna Kleyman*, Jenice YJ Kim*, Philomena Kamya*, Saeid Sharafi*, Nancy Simic*, Julie Bruneau†, Jean-Pierre Routy*, Christos M Tsoukas*, Nicole F. Bernard*

* Research Institute of the McGill University Health Centre, Montreal, Canada
†Centre de recherche du Centre Hospitalier de l'Université de Montréal, Montreal, Canada

Mailing address: Dr. Nicole F. Bernard, Research Institute of the McGill University Health Centre, Montreal General Hospital, 1650 Cedar Ave. Rm C10-160, Montreal, Quebec, Canada, H3G 1A4.
Tel: +1 (514)934-1934 ext: 44584
Fax: +1 (514)937-1424
E-mail: nicole.bernard@mcgill.ca

Funding: This study received support from the Canadian Institutes for Health research #MOP-79515, the Fonds de la Recherche en Santé du Québec (FRSQ). S Boulet was supported by a Ph.D. scholarship from FRSQ. J.P. Routy is a scientific scholar receiving support from FRSQ.

Word count: 1977

I. Abstract

Objectives: Co-expression of certain combinations of NK cell receptor KIR3DL1 and HLA-B alleles is associated with slower time to AIDS. The strongest protection in terms of disease outcome in KIR3DL1 homozygotes (3DL1hmz) is co-expression of HLA-B*57 and a set of KIR3DL1 genotypes (3DL1*h/*y) lacking alleles expressed at low levels on NK cells. We questioned whether this allele combination could also influence resistance to infection.

Design: The genetic distribution of 3DL1*h/*y and HLA-B*57 was compared in 41 HIV exposed uninfected (EU) and 186 recently HIV-infected 3DL1hmz.

Methods: KIR3DL1 subtyping was performed by sequencing the exons 3, 4, 5, 7-9. The MHC class IB locus was typed by sequence specific oligonucleotide PCR and sequencing to resolve Bw4 and Bw6 alleles and the amino acid present at position 80.

Results: Percent carriers of HLA-B*57 in EU and subjects in a primary infection (PI) cohort was 12.2% and 4.3%, respectively (p=0.0631), whereas that of 3DL1 *h/*y was similar in both populations (p=0.221). The 3DL1*h/*y-HLA-B*57 combined genotype was more frequent in EU (12.2%) than PI subjects (2.7%) (p= 0.019, OR=5.03, 95% confidence intervals, 1.38-18.3)

Conclusions: Co-expression of 3DL1*h/*y and B*57, which has been associated with a reduced risk of progressing to AIDS in HIV infected individuals also lowers the risk of HIV infection in EUs.

II. Introduction

Natural Killer (NK) cells are part of the innate immune response and function in tumor and viral control [1]. They are regulated by the integration of signals transmitted internally by activating and inhibitory surface receptors such as killer immunoglobulin-like receptors (KIRs) [2]. A number of KIR molecules have been linked to infectious disease outcomes, including alleles encoded by the KIR3DS/L1 framework locus [3-6].

KIR3DL1 (hereafter 3DL1) alleles can be divided in three groups based on their expression levels on the NK cell surface [7-10]: the 3DL1*h (high expression) group, the 3DL1*l (low expression) group and 3DL1*004, which is not expressed at the cell surface. Using these categories, it is possible to classify 3DL1 homozygotes (3DL1hmz) as having a 3DL1*h/*y genotype when they do not express an *l allele or as having a 3DL1*l/*x genotype when they express at least one *l allele. Given that the ligands for 3DL1 are MHC class I-B alleles of the Bw4 serotype, Martin *et al* measured the effect of these 3DL1 genotypes in combination with different MHC class I molecules on HIV disease progression [6]. The strongest protective effect was found for the 3DL1*h/*y-B*57 combination.

NK cell function [11-13] and KIR [13-15] have not only been linked to HIV disease progression, but also resistance to HIV infection in certain populations of exposed uninfected (EU) individuals who remain seronegative despite repeated exposure to HIV. Given that NK cells have the potential to mediate anti-viral activity at the earliest

phase of HIV infection and that there is genetic evidence for KIR involvement in resistance to HIV, we sought to determine whether co-expression of the subtypes of 3DL1 and their receptors that are associated with a slower disease progression would occur more frequently in EU than in recently infected HIV susceptible individuals. To this end we compared the 3DL1 and HLA genotype distribution in EU versus that of subjects enrolled in an HIV primary infection (PI) cohort.

III. Methods

Study Subjects

A total of 227 3DL1hmz (41 EU and 186 PI subjects) were included in this study. These individuals were a subset of previously described populations [15]. Informed consent was obtained from all study subjects, and the research conformed to all ethical guidelines of all the authors' institutions.

Cell isolation

PBMCs were isolated from blood by density gradient centrifugation (Ficoll-Paque; Pharmacia, Uppsala, Sweden) and cryopreserved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Mo.) with 90% fetal calf serum (FCS; Canadian Life Technologies, Burlington, Ontario, Canada).

Genotyping

Genomic DNA was extracted from PBMC or EBV-transformed cells using a QIAamp DNA blood kit (QIAGEN, Inc., Mississauga, Ontario, Canada). 3DL1hmz were 3DL1 subtyped at the allele level by gene sequencing as previously described [16]. Single nucleotide polymorphisms (SNP) corresponding to the 3DL1 *h subtypes (3DL1*001, 3DL1* 002, 3DL1*008, 3DL1*009, 3DL1*015, 3DL1*020), the 3DL1*l subtypes (3DL1*005, 3DL1*007) and 3DL1* 004, were identified by aligning the sequenced DNA

to a reference consensus sequence consisting of a contig of 3DL1 cDNA sequences. All primer sequences and amplification conditions are available upon request. Subjects were typed for MHC class I expression using the line probe assay (Innogenetics Inc, Alpharetta, GA) and by sequencing (Atria Genetics, South San Francisco, CA) if high resolution typing was needed to resolve the assignment of B alleles to the Bw4 or Bw6 public specificities based on amino acids at positions 77 to 83.

Statistics

Statistical analysis and graphical presentation were performed using GraphPad InStat 3.05 and GraphPad Prism 4.01. Fisher's exact test was used to compare proportions of selected genotypes between EU and PI. A p-value of less than 0.05 was considered significant. For comparisons of 3DL1 subtype frequency, a Bonferroni correction was performed by multiplying the p-value by the number of 3DL1 alleles tested (n=9) to give the corrected p-value, which was considered significant if less than 0.05.

IV. RESULTS

There were no significant between-group differences in racial composition: 92.7% and 91.9% of EU and PI, respectively were Caucasian, 2.4% and 2.7% were African, 0% and 0.5% were Asian, 0% and 3% were Hispanic, 0% and 0.5% were Native American and 4.9% and 1% reported themselves of mixed race. All individuals sequenced for 3DL1 subtype were 3DL1hmz in order to be able to classify them as 3DL1*h/*y (where *y is *004 or *h) or 3DL1*l/*x (where *x is *004, *l or *h) [6]. The frequencies of each 3DL1 subtype for both populations are shown in Figure 1. The only significant difference was in the frequency of *009 which was higher in EU (2.4%, 2/82 alleles) compared to PI (0%, 0/372 alleles) (p=0.0323, OR=23.14, CI=1.10-486.90, Fisher's exact test). However, this difference was attributable to a single EU individual *009hmz and was no longer significant after performing Bonferroni's correction (p=0.2907). When alleles were grouped based on expression into *h and *l we found no significant between-group differences in the distribution of these categories (*h: 59.8% and 57.3% and *l: 13.4% and 22.0% in EU and PI, respectively) although there was a non-significant trend (p=0.096) towards lower frequency of *l alleles in the EU population (Figure 1 and data not shown).

The ligands for 3DL1 are alleles belonging to the HLA Bw4 serotype. HLA Bw4 alleles can be split into HLA-Bw4 80I and 80T alleles based on whether an Isoleucine (I) or Threonine (T) is present at aa 80 of the HLA heavy chain. In a recent publication, 3DL1 subtypes with their ligands conferred various degrees of protection in terms of time to AIDS and viral load, the strongest protection being associated with 3DL1*h/*y and

HLA-B*57 co-expression [6]. We thus compared the distribution of each of these alone and in combination in our study populations in order to determine whether they were differentially distributed. As shown in Figure 2A, we found a higher proportion of 3DL1*h/*y-HLA-B*57 in EU than in PI subjects (5 of 41 [12.2%] versus 5 of 186 [2.7%] expressed this combined genotype, respectively; p=0.019, OR=5.03, CI=1.38-18.3, Fisher's exact test). Considered on its own, expression of the 3DL1*h/*y haplotype was not significantly different between the two groups (68.3% [28/41] and 57.0% [106/186] for EU and PI subjects, respectively; p=0.221, Fisher's exact test) (Figure 2B). Despite not being statistically significant, the percent carriers of the HLA-B*57 allele tended to be higher in the EU than PI population (5 of 41 [12.2%] and 8 of 186 [4.3%] were HLA-B*57 in these 2 groups, respectively; p=0.0631, Fisher's exact test) (Figure 2C). All other between group comparisons of KIR3DL1 alleles or genotypes (3DL1*h/*y, 3DL*l/*x, 3DL1*004) and their receptors (HLA-Bw4, HLA-Bw4-80I, HLA-B*57 or HLA-B*27) taken individually or in combination were not significantly different (data not shown).

V. Discussion

In this report, we assessed the distribution of 3DL1 subtypes and of their MHC class I ligands in HIV-resistant EU and HIV-PI subjects. The latter were selected as a control population for these analyses because they are susceptible to HIV infection and because, in a recently infected population, any effect that disease progression would have on skewing the distribution of KIR or MHC class I allele expression has yet to occur. For example, as time infected increases, individuals with alleles associated with rapid progression would be lost, increasing the frequency of allele associated with slow progression in survivors. The combined effect of high expression NK receptor 3DL1 subtypes in individuals expressing a 3DL1*h/*y genotype with its HLA-B*57 ligand, which has been shown to confer protection from disease progression and viral load control in HIV infected individuals [6], is also associated with a reduced risk of infection as an increased proportion of 3DL1hmz EU compared to 3DL1hmz PI subjects express this combined genotype. Limiting the study to homozygous individuals restricts the scope of our findings, but eliminates possible confounders associated with its allele, 3DS1, such as its effect on disease outcome, possible effects on 3DL1 expression levels or conflicting reports as to whether 3DS1 serves as a receptor for HLA-Bw4 80I or B*57 [3,15,17-21]. While the frequency of 3DL1*h/*y was similar between groups, the difference in HLA-B*57 percent carriers approached significance (p=0.0631). Given the sample size of the EU population and the lack of statistical significance of the observation, caution is warranted in concluding any effect of B*57 on its own. Thus, only co-expression of 3DL1*h/*y and HLA-B*57 was significantly linked to protection from HIV infection.

However, it is possible that the combined effect of 3DL1*h/*y and B*57 is driven by the HLA allele. The small population size precludes making a definitive conclusion on this and confirmation of this observation in other EU cohorts is warranted.

The mechanism underlying the synergistic protective effect of 3DL1*h/*y and HLA-B*57 in terms of HIV resistance may be related to the licensing process in NK cell development. During maturation, NK cells accumulate inhibitory receptors until they are able to quench autoreactivity upon encountering normal cells expressing self MHC class I ligands [18-20,22-24]. NK cells from individuals with a 3DL1*h/*y genotype encode alleles expressed on the NK cell surface at high levels in terms of abundance per cell and the percentage of NK cells expressing the allele [9]. In addition, the 3DL1*h alleles tested thus far have a higher affinity for HLA-Bw4 80I alleles such as HLA-B*57 than HLA-Bw4 80T alleles [7,9]. Therefore, individuals expressing this combination of alleles would have NK cells that are potently inhibited under normal circumstances. As HIV target cells are exposed to the virus, MHC class I downregulation will lead to interruption of the inhibitory signal mediated by 3DL1 receptors resulting in NK activation. Thus, in the case of the receptor ligand combination 3DL1*h HLA-B*57 which mediate potent inhibitory signals, disruption of the interaction by viral infection has the potential to result in strong NK activation and anti-viral activity that may play a role in preventing the establishment of an HIV infection. Functional studies evaluating the impact of different KIR-ligand combinations on NK cell activity are warranted to investigate the mechanisms underlying the association of HIV resistance with co-expression of KIR and MHC class I alleles, such as those reported here [25].

While Martin et al [6] were able to show a gradient of protection from HIV disease progression and viral load control for different 3DL1 and HLA combinations, we were only able to show a significant effect on protection from infection for the combination conferring the most potent protection vis-à-vis disease progression, i.e. 3DL1*h/*y and B*57 in EU. While other combinations influencing disease progression may also lower the risk of HIV infection, the sample size to which we had access was too small to detect the potentially more subtle effects of these interactions. It is also possible that 3DL1*h/*y and B*57 is the only 3DL1/HLA combination able to influence the risk of HIV infection.

We also evaluated the distribution of the 3DL1 subtypes in the two study populations. Although the 3DL1*009 allele had a higher frequency in the EU population, a single 3DL1*009hmz among the EU group accounted for this between-group difference. Furthermore, following Bonferroni's correction for multiple comparisons, this difference was no longer significant such that 3DL1 allele frequencies did not differ between the two populations. The similar racial composition of the study populations likely was not responsible for differences in allele or combined genotype frequency between populations.

In conclusion, we have found that 3DL1*h/*y-HLA-B*57 individuals were more frequent in the EU population when compared to a PI population. This finding further supports a role of NK cells in the protection from HIV in the early stages of infection. However this conclusion does not preclude a role for the full KIR genotype in HIV

infection. Given the many possible combinations of KIR genes in a genotype and their variegated expression on NK cells, a complete picture of the influence of these molecules on disease outcome may not be defined by a single allele. Defining a role for KIR receptors in HIV susceptibility will contribute to our knowledge of immune defenses to this virus and may suggest new avenues for the design of HIV vaccines.

VI. Acknowledgments

We would like to acknowledge Mr. Mario Legault and Mr. Martin Rioux for coordination of the Montreal PI and IDU cohort, respectively, as well as the investigators of the Montreal PI cohort for subject recruitment and clinical follow up. JB, JPR and CMT are responsible for the cohorts from which clinical samples, behavioral and clinical information were obtained, MK, JK, PK SS, and NS performed KIR and HLA genotyping; SB analyzed results and prepared the manuscript; NFB designed the research and prepared the manuscript.

VII. Figure Captions

Figure 1. Frequency of each KIR3DL1 subtypes in the HIV exposed uninfected (EU) and subjects in primary HIV infection (PI) populations that are KIR3DL1 homozygotes. Grouped high (*h) and low (*l) expressing alleles are also shown for each population.

Figure 2. Distribution of KIR3DL1*h/*y and B*57 in the HIV exposed uninfected (EU) and subjects in primary HIV infection (PI) populations that are KIR3DL1 homozygotes. Pie charts depict for each population: A) the proportion of combined KIR3DL1*h/*y and B*57 carriers, B) the proportion of KIR3DL1*h/*y carriers and C) the percent carriers of the HLA-B*57 allele.

VIII. Tables and Figures

Figure 1

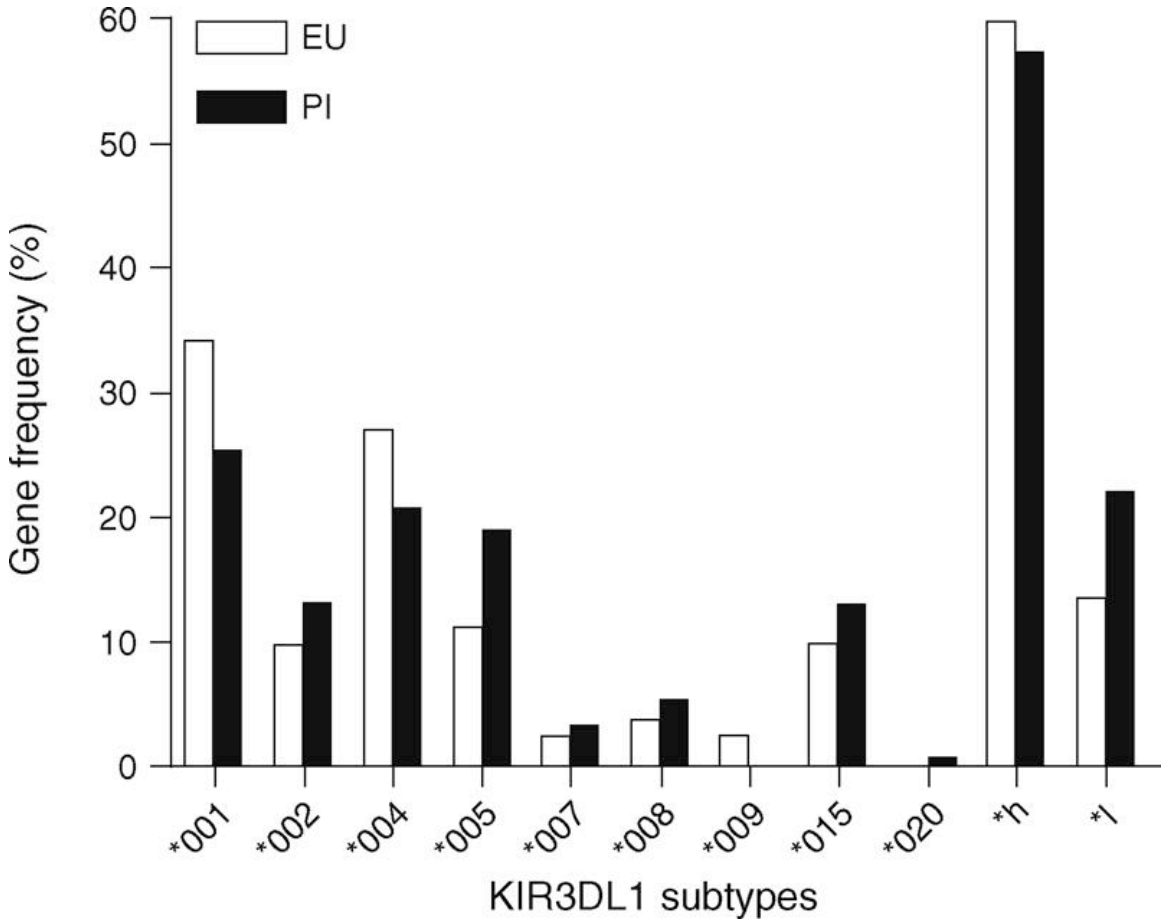
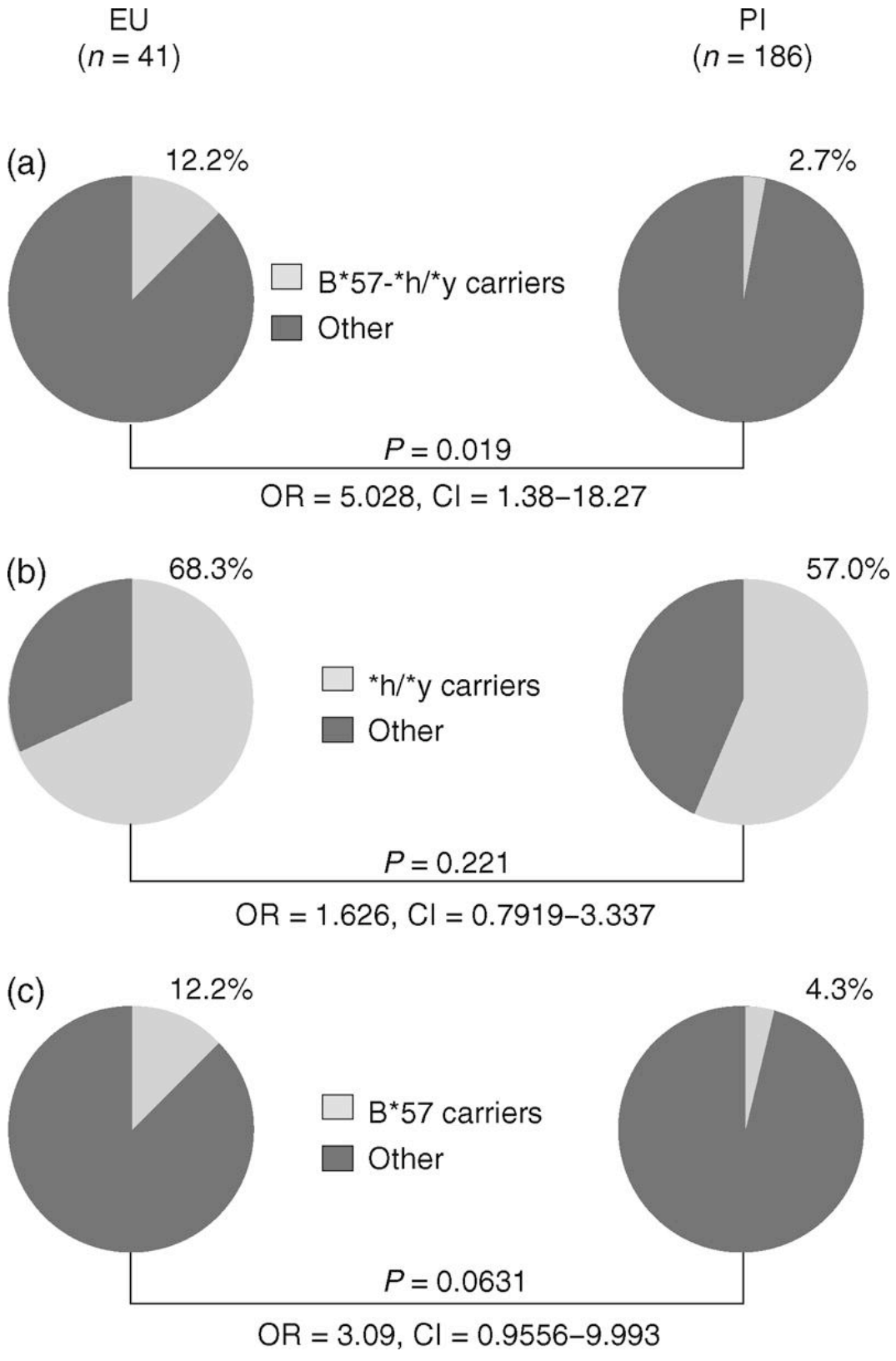


Figure 2


EU
(n = 41)
PI
(n = 186)
(a)
12.2%
2.7%
B*57-*h/*y carriers
Other
P = 0.019
OR = 5.028, CI = 1.38–18.27
(b)
68.3%
57.0%
*h/*y carriers
Other
P = 0.221
OR = 1.626, CI = 0.7919–3.337
(c)
12.2%
4.3%
B*57 carriers
Other
P = 0.0631
OR = 3.09, CI = 0.9556–9.993

IX. References

1. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. Nat.Rev.Immunol. 2001; **1:** 41-49.

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

3. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ *et al.* Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat.Genet. 2002; **31:** 429-434.

4. Parham P. Immunogenetics of killer cell immunoglobulin-like receptors. Mol.Immunol. 2005; **42:** 459-462.

5. Carrington M, Martin MP. The impact of variation at the KIR gene cluster on human disease. Curr.Top.Microbiol.Immunol. 2006; **298:** 225-257.

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

7. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. J.Immunol. 2005; **175:** 5222-5229.

8. Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R *et al.* Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. J.Immunol. 2001; **166:** 2992-3001.

9. Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J.Exp.Med. 2006; **203:** 633-645.

10. Pascal V, Yamada E, Martin MP, Alter G, Altfeld M, Metcalf JA *et al.* Detection of KIR3DS1 on the cell surface of peripheral blood NK cells facilitates identification of a novel null allele and assessment of KIR3DS1 expression during HIV-1 infection. J.Immunol. 2007; **179:** 1625-1633.

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

12. Montoya CJ, Velilla PA, Chougnet C, Landay AL, Rugeles MT. Increased IFN-gamma production by NK and CD3+/CD56+ cells in sexually HIV-1-exposed but uninfected individuals. Clin.Immunol. 2006; **120:** 138-146.

13. Ballan WM, Vu BA, Long BR, Loo CP, Michaelsson J, Barbour JD *et al.* Natural killer cells in perinatally HIV-1-infected children exhibit less degranulation compared to HIV-1-exposed uninfected children and their expression of KIR2DL3, NKG2C, and NKp46 correlates with disease severity. J.Immunol. 2007; **179:** 3362-3370.

14. Jennes W, Verheyden S, Demanet C, Adje-Toure CA, Vuylsteke B, Nkengasong JN *et al.* Cutting edge: resistance to HIV-1 infection among African female sex

workers is associated with inhibitory KIR in the absence of their HLA ligands. J.Immunol. 2006; **177:** 6588-6592.

15. Boulet S, Sharafi S, Simic N, Bruneau J, Routy J-P, Tsoukas C *et al.* Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. AIDS 2007.

16. Thananchai H, Gillespie G, Martin MP, Bashirova A, Yawata N, Yawata M *et al.* Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. J.Immunol. 2007; **178:** 33-37.

17. Barbour JD, Sriram U, Caillier SJ, Levy JA, Hecht FM, Oksenberg JR. Synergy or Independence? Deciphering the Interaction of HLA Class I and NK Cell KIR Alleles in Early HIV-1 Disease Progression. PLoS.Pathog. 2007; **3:** e43.

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

19. Raulet DH, Vance RE. Self-tolerance of natural killer cells. Nat.Rev.Immunol. 2006; **6:** 520-531.

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

21. Lopez-Vazquez A, Mina-Blanco A, Martinez-Borra J, Njobvu PD, Suarez-Alvarez B, Blanco-Gelaz MA *et al.* Interaction between KIR3DL1 and HLA-B*57 supertype alleles influences the progression of HIV-1 infection in a Zambian population. Hum.Immunol. 2005; **66:** 285-289.

22. Held W, Dorfman JR, Wu MF, Raulet DH. Major histocompatibility complex class I-dependent skewing of the natural killer cell Ly49 receptor repertoire. Eur.J.Immunol. 1996; **26:** 2286-2292.

23. Dorfman JR, Raulet DH. Acquisition of Ly49 receptor expression by developing natural killer cells. J.Exp.Med. 1998; **187:** 609-618.

24. Parham P. Taking license with natural killer cell maturation and repertoire development. Immunol.Rev. 2006; **214:** 155-160.

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

Bridge from Chapter 3 to 4

Epidemiological evidence supporting a role for the KIR3DS/L1 locus in protection against HIV infection presented in chapters 2 and 3 complements strong findings linking this locus to slower HIV disease progression[150,151]. However, in order to demonstrate a role for NK cells, and specifically for the KIR3DS/L1 locus, in control of HIV, genetic associations are insufficient. Protective KIR3DS/L1 genotypes must result in a measurable phenotype that confers an advantage against HIV infection.

In order to support our epidemiological observations, we sought to determine whether NK cells isolated from individuals with KIR3DS/L1 or HLA/ KIR3DS/L1 genotypes were associated with slower HIV disease progression and/or resistance from infection had functional characteristics in a manner that could explain superior control of HIV.

Chapter 4: HIV protective KIR3DL1 and HLA-B genotypes influence natural killer cell function following stimulation with HLA-devoid cells

HIV protective KIR3DL1 and HLA-B genotypes influence natural killer cell function following stimulation with HLA-devoid cells

Regular article: Immunobiology

Running head: KIR3DL1 and HLA-B effect on NK cells function

Salix Boulet[1], Julie Bruneau[2], Christos M. Tsoukas[1], Nicole F. Bernard[1]

[1]Research Institute of the McGill University Health Centre, Canada. 1650 Cedar avenue room C10-160, H3G 1A4, Montreal, Quebec, Canada.

[2]Centre Hospitalier de l'Université de Montréal, Canada. Hôpital Saint-Luc, 1058 Saint-Denis, H2X 3J4, Montréal, Quebec, Canada.

Corresponding author: Dr. Nicole Bernard, nicole.bernard@mcgill.ca
1650 Cedar avenue, room C10-160
H3G 1A4, Montreal, Quebec, Canada
Tel: 1-514-934-1934 x 44584; Fax: 1-514-937-6961

Text word count: 4432
Abstract word count: 174
Number of tables and figures: 3
Number of references: 60

I. Abstract

Epidemiological studies have implicated carriage of combinations of genes encoding certain KIR3DL1 (Killer Immunoglobulin-like Receptors) and their HLA-Bw4 ligands in slower progression to AIDS, lower viral load and protection from infection. To assess whether the KIR/HLA compound genotypes linked to better HIV outcome favor increased NK cell function, we measured by multiparametric flow cytometry cytokine secretion and degranulation in response to stimulation with HLA-devoid cells by NK cell from individuals with defined KIR/HLA genetic combinations. We found that in subjects with two genotypes associated with favorable HIV outcomes, KIR3DL1*h/*y/HLA-B*57 and KIR3DL1*004/HLA-Bw4, NK cells had increased functionality. In KIR3DL1*h/*y/HLA-B*57 subjects, these NK cells were more likely to be KIR3DL1^{+} while this was not the case in KIR3DL1*004/Bw4 individuals, perhaps because KIR3DL1*004 is not expressed at the cell surface. These results support a model where the potency of the NK cell response varies according to the carriage of KIR3DL1 and Bw4 receptor ligand combinations, which could provide an explanation for the observation that some KIR/HLA combinations are associated protective phenotypes in the context of host-HIV interactions.

II. Introduction

As cells of the innate arm of the immune system, Natural Killer (NK) cells are important for tumor and viral control. However, given their potent cytolytic and cytokine secretion potential, their activity needs to be tightly regulated. The activation state of an NK cell is determined partly by the integration of stimulatory and inhibitory signals following interaction of surface NK cell receptors (NCR), including the highly diverse Killer Immunoglobulin-like Receptor (KIR) family, with ligands found on target cells[1]. The polygenic and polymorphic KIR genetic region encodes for receptors with varying protein structures[2], ligand affinities[3-5], cell surface expression levels[6,7], and types (activating or inhibitory) of transmitted intracellular signals[2]. These subtle variations could explain why NK cells from individuals with certain KIR-ligand combinations seem better able to activate NK cell responses in the presence of specific pathogens[8-11]. This was highlighted by a number of epidemiologic studies linking the generic KIR3DS/L1 haplotypes with HIV disease progression or resistance to infection[9,10,12-15].

The KIR3DS/L1 locus has the particularity of encoding for a large polymorphic group of inhibitory alleles (KIR3DL1 – hereafter 3DL1) and a smaller set of activating alleles (KIR3DS1 – hereafter 3DS1), of which 3DS1*013 is the most common across several populations[16]. The polymorphisms of 3DL1 alleles translate into molecules with cell surface levels of expression, which can be high (*h; 3DL1*001, *002, *008, *009, *015, *020), intermediate/low (*l; *005, *006, *007, *028, *053) or null (not expressed at the surface; *004)[5-7,9].

In recent studies, various 3DL1 allotypes in combination with their known, or putative ligands, HLA alleles belonging to the Bw4 group, were shown to be associated with differential HIV disease progression, protection from opportunistic infections and viral load (VL) set point[9-11]. The genotype conferring the highest protection compared with Bw6 homozygotes (hmz), who lack any HLA-B ligand for KIR3DL1 with respect to these outcomes, was a combination referred to as *h/*y+B*57. This genotype is composed of a 3DL1 genotype termed 3DL1*h/*y (i.e. lacking 3DL1*l alleles) with HLA-B*57, an HLA-Bw4 allele with an isoleucine at position 80 (i.e. Bw4-80I). In line with these findings, our group recently reported an association between carrying the *h/*y+B*57 combination with resistance to infection in a population of HIV exposed uninfected (EU) individuals[14]. The *h/*y+B*57 genetic combination is not the only 3DL1/HLA-B pair reported to influence HIV disease progression; 3DL1*004 with HLA-Bw4, 3DL1*l/*x (a KIR3DL1 genotype with at least one *l expressing allele) with HLA-B*27, and 3DL1*h/*y with HLA Bw4-80I were all shown to confer protection in terms of slower progression to AIDS when compared to Bw6 hmz[9]. However, while these data support a role for NK cells in control of HIV that varies depending on carriage of certain alleles encoding NK receptors and their ligands, there are few studies linking the carriage of these KIR/HLA combined genotypes with NK function.

NK cells can be divided into 2 populations based on the intensity of the CD56 marker at the cell surface. $CD56^{dim}$ NK cells are the more cytotoxic subset, while $CD56^{bright}$ cells are poorly cytotoxic and preferentially secrete cytokines when activated [17,18]. However, the cytokine secretion and cytolytic functions are not exclusive to either

cell subset [19-21]. IFN-γ and TNF-α secretion as well as cytolysis have documented anti-viral activity and are functions attributable to both NK and $CD8^+$ T cells[17,22-27]. In HIV infection, where there is evidence that $CD8^+$ T cells play a role in controlling virus, it is not so much the frequency or breadth of epitope recognition by these T cells, but rather the functional quality of their anti-HIV responses that correlates best with VL control[28,29]. This provided the rationale for characterizing the quality of NK cell functional potential by measuring secretion of cytokines (IFN-γ and TNF-α) and CD107a expression, a marker for degranulation[30,31]. In order to determine whether NK cells from individuals bearing KIR/HLA combinations associated with distinct outcomes with respect to HIV infection could be distinguished based on functional potential, we stimulated peripheral blood mononuclear cells (PBMC) with HLA-devoid K562 cells and measured the frequency of NK cells displaying all possible functional patterns. We found that NK cells from HIV uninfected individuals carrying KIR/HLA genotype combinations linked to control of HIV resulted in higher frequencies of polyfunctional NK cells compared to NK cells from Bw6 hmz.

III. Material and Methods

Study population

Fifty-four EUs were included in this study. Thirty-three individuals were KIR3DL1 homozygous, 17 were KIR3DS/L1 heterozygous and 4 were KIR3DS1 homozygous (all of the latter were HLA-Bw6 hmz). These individuals were either enrolled in the St-Luc injection drug user (IDU) cohort[32] or were the seronegative partner in a serodiscordant couple[33]. All subjects are a subset of a previously described study population (in which KIR3DL1*high expression alleles included 3DL1*001, 3DL1* 002, 3DL1*008, 3DL1*009, 3DL1*015, 3DL1*020 and KIR3DL1*low expression alleles included 3DL1*005, 3DL1*007) typed for major histocompatibility complex (MHC) class I alleles, KIR3DL1 generic genotypes and allotypes[14,15]. Figure 1A shows how this study population was grouped according to their KIR/HLA genotypes and, given that some of these groups are not mutually exclusive, the number of study subjects that appear in more than a single group. Informed consent was obtained from all study subjects, and the research conformed to ethical guidelines of all the authors' institutions.

Cells

PBMC were isolated by density gradient centrifugation (Ficoll-Paque, Pharmacia Upsala, Sweden) from whole blood obtained by venipuncture into tubes containing EDTA anticoagulant or by leukapheresis as previously reported[34]. The cells were then cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St-Louis, MO) with 90% fetal bovine serum (FBS, Wisent, St-Bruno, Quebec, Canada).

NK cell activation and staining for phenotype and function

Cryopreserved PBMCs were thawed and resuspended at 10^6 cells/ml in RPMI 1640 (Wisent) containing 10% FBS (Wisent), 2 mM L-glutamine (Wisent), 50 IU/ml of penicillin (Wisent) and 50 μg/ml of streptomycin (Wisent). Brefeldin A (at 5 μg/ml, Sigma-Aldrich), monensin (at 6 μg /ml, Golgi Stop; BD Biosciences, Mississauga, Ontario, Canada) and anti-CD107a-FITC antibody (BD) were added to the cells. One million PBMC were stimulated with 1) MHC class I devoid K562 cells (ATCC, Manassas, VA) at a PBMC-to-target cell ratio of 5:1 or 2) medium alone (negative control) or 3) PMA (1.25 μg/mL); ionomycin (0.25 μg/mL, Sigma-Aldrich) (positive control to ensure the cells being tested were able to respond; all stimulation data shown in the manuscript were from cells that generated positive stimulation results with PMA and ionomycin). Stimulated PBMCs were incubated 6 h at 37^oC in 5% CO_2.

Cells were then stained for viability using the Aqua LIVE/DEAD® fixable dead cell stain kit (Invitrogen, Burlington, Ontario, Canada) following manufacturer's instructions. PBMCs were then stained for cell surface markers with anti-CD56-APC, anti-CD16-Pacific blue (BD), and CD3-ECD, CD158e-PE (aka Z27-PE, Beckman Coulter, Mississauga, Ontario, Canada) for 30min. Samples were washed with PBS containing 1% Fetal Bovine Serum (FBS, Wisent) and 0.1% sodium azide (Sigma-Aldrich), fixed and permeabilized using the Fix & Perm® kit (Invitrogen) and stained for intracellular cytokines using Alexa 700 conjugated anti-Interferon-γ (IFN–γ) and PE-Cy7 conjugated anti-Tumor Necrosis Factor-α (TNF–α), (BD) for 30 min. After washing,

samples were fixed with a solution of 1% paraformaldehyde (PFA, Fisher Scientific, Ottawa, Ontario, Canada) and kept in the dark at 4^{o}C until acquisition.

Flow cytometry analysis

Figure 1B displays the flow cytometry gating strategy used to analyze the functional profile of NK cells stimulated with K562 cells. For all analyses, NK cells were defined as $CD3^-$ and either $CD56^+CD16^+$, $CD56^+CD16^-$, or $CD56^-CD16^+$. Boolean gating was used to determine the tri-functional (CD107a, IFN-γ and TNF-α), bi-functional (any combination of 2 of the measured functions) or mono-functional (a single measurable function) profile of $3DL1^+$ (i.e. $Z27^{hi}$), $3DL1^-$ (i.e. $Z27^{dim}$ and $Z27^-$) or all NK cells ($3DL1^+$ + $3DL1^-$ NK cells). Between 400,000 and 600,000 total events were acquired for each sample on a LSRII flow cytometer (BD). Flow cytometry analysis for NK cell activation following K562 stimulation or for NK cell phenotyping was performed using FlowJo software version 8.7.1 (TreeStar, Ashland, Oregon). The data obtained was corrected for background using unstimulated cells before statistical analysis.

Statistics

Statistical analysis and graphical presentation were performed using GraphPad InStat 3.05 and GraphPad Prism 4.01. Mann-Whitney tests were used for between-group comparisons. Wilcoxon signed-rank test was used to compare the percent of $3DL1^+$ to $3DL1^-$ polyfunctional NK cells within individuals. A p-value of less than 0.05 was considered significant.

IV. Results

Certain KIR3DL1 and HLA-Bw4 genotype combinations have been shown to be associated with time to AIDS and VL set point[9] or have been reported to be associated with a reduced risk of HIV infection in EUs[14]. In order to address whether NK cells from individuals carrying some of these KIR/HLA combinations have differential functional potential, we measured the expression of CD107a and secretion of IFN-γ and TNF-α by NK cells from 54 3DL1 and HLA typed EU subjects in response to stimulation with MHC-devoid K562 cells by 8-color flow cytometry. In these experiments, Bw6 hmz (n=24), who carried no HLA-B alleles that could bind 3DL1 or 3DS1, served as a control population. Responses in NK cells from Bw6 hmz were compared to those from individuals with the following non-mutually exclusive 3DL1+Bw4 combinations previously reported to be associated with either rate of HIV disease progression and/or protection from infection: 1) 3DL1*h/*y carrying at least 1 copy of an HLA-Bw4-B*57 allele (*h/*y+B*57, n=5), 2) at least 1 copy of KIR3DL1*004 and at least 1 copy of a Bw4 allele (*004+Bw4, n=13) and 3) 3DL1*h/*y carrying at least 1 copy of an HLA-Bw4-80I allele (*h/*y+80I, n= 10).

Given that the NK cell response is characterized using three functional markers, 8 different functional permutations are possible and define 8 different NK cell subsets. However, only 7 of these subsets define responsive NK cells. For individuals belonging to each of the four genotype combinations described above, the frequency for each response pattern was assessed and the contribution of each combination to the total NK cell response determined. Figure 2A shows that the contribution of tri-functional NK

cells to the total K562 stimulated response was significantly greater in NK cells from subjects with the *h/*y+B*57 (p=0.0226) and the *004+Bw4 (p=0.0402) combined genotypes compared to NK cells from Bw6 hmz (median, [range]) (5.4% [3.7-6.8], 4.0% [0.2-10.8], 0.9% [0.0-9.1] for NK cells from individuals typing as *h/*y+B*57, *004+Bw4 and Bw6 hmz, respectively, Mann-Whitney test). Figure 2A also shows that NK cells from individuals carrying *h/*y+B*57 (5.0% [3.2-11.7]) had an increased contribution of cytokine-only (IFN-γ and TNF-α) secreting cells compared to Bw6 hmz (1.1% [0.0-14.5], p=0.0086, Mann-Whitney test). Furthermore, subjects typing as *004+Bw4 (23.1% [15.0-31.9]) had an increased contribution of K562 stimulated NK cells secreting IFN-γ and expressing CD107a compared Bw6 hmz (20.0% [5.9-31.3], p=0.0087, Mann-Whitney test). The *h/*y+80I combined genotype has also been reported to be associated with slower HIV disease progression and lower VL. However the contribution of tri-, bi- or mono-functional NK cells to the total K562 stimulated response from individuals with this genotype combination did not differ from that of NK cells from Bw6 hmz. In summary, NK cells from individuals carrying the *h/*y+B*57 and *004+Bw4 genotype combinations associated with slower HIV disease progression, lower VL and/or protection from HIV infection have a greater multifunctional potential upon stimulation with HLA devoid cells than NK cells from individuals who are Bw6 hmz. For further analyses, only results relating to functional profiles for genotypes that were significantly greater than that seen in Bw6 hmz will be shown (i.e. tri-functional for *h/*y+B*57 and *004+Bw4 individuals, $CD107a^{-}IFN\text{-}\gamma^{+}TNF\text{-}\alpha^{+}$ for *h/*y+B*57 individuals and $CD107a^{+}IFN\text{-}\gamma^{+}TNF\text{-}\alpha^{-}$ for *004+Bw4 individuals) although data on all functional profiles were collected and compared.

The HLA-B*57 allele belongs to the Bw4 serotype and has an isoleucine at position 80 (80I). Therefore we questioned whether the increased functional potential of NK cells from *h/*y+B*57 individuals was due to the presence of B*57 or could also be observed by NK cells from individuals with other HLA alleles belonging to either the Bw4 or Bw4-80I allele subsets. To answer this question, the tri-functional response of NK cells from non-B*57 individuals carrying KIR3DL1*h/*y with other Bw4 or Bw4-80I alleles was compared to the response from the *h/*y+B*57 group. The contribution of tri-functional responses by stimulated NK cells from *h/*y+B*57 individuals was greater than that from non-B*57 individuals who were *h/*y+Bw4 (n=11) (0.8% [0.0-2.6], p=0.0022), and was also greater than that from the 5 individuals in this latter group who were *h/*y+80I (0.8% [0.0-2.6], p=0.0079) (Mann Whitney test) (figure 2B). The percent contribution of stimulated tri-functional NK cells in these later 2 study populations was not significantly different from that seen in Bw6 hmz. When a similar analysis was performed for cytokine only responses (IFN-γ and TNF-α, figure 2C), there was a non significant trend towards greater functionality of stimulated NK cells from *h/*y+B57 carriers compared with non-B*57 individuals who were *h/*y+80I (1.2% [0.0-5.8], p=0.0952) and significantly greater functionality compared with NK cells from non-B*57 *h/*y+Bw4 subjects (0.9% [0.0-5.8], p=0.0092). As well, the proportion of stimulated NK cells exhibiting a response characterized by tri-functionality (figure 2B) and secretion of IFN-γ and TNF-α (figure 2C) was greater when these cells came from *h/*y+B*57 carriers compared to when they originated from non-B*57 individuals carrying *h/*y, regardless of their HLA ligand (data not shown). These results suggest that B*57 is unique among Bw4 and Bw4-80I HLA alleles in mediating increased NK

functional potential in combination with the KIR3DL1*h/*y genotype. Since all the B*57 positive EU individuals we studied were also *h/*y, it was not possible for us to verify whether the tri-functional or CD107a$^-$IFN-γ^+TNF-α^+ NK responses were modulated by receptor combinations found in KIR3DL1 genotypes other than *h/*y or by the B*57 allele alone.

We next determined whether the increased contribution of tri-functional and CD107a$^+$IFN-γ^+TNF-α^- NK cells to K562 stimulation seen in *004+Bw4 individuals (figure 2A) depended on carriage of both 3DL1*004 and Bw4 genes. Figure 2D shows that the contribution of tri-functional NK cells to stimulation by HLA devoid cells was greater when NK cells were from individuals carrying *004+Bw4 (n=13, 4.0% [0.2-10.8], than from those carrying 3DL1*004 without Bw4 (*004 only) (n=8, 0.6% [0.0-6.0], p=0.0327) or Bw4 without 3DL1*004 (Bw4 only) (n=20, 1.3% [0.0-5.4], p=0.0342) (Mann-Whitney test). These data suggest that elevated tri-functionality requires expression of both *004 and Bw4. This was not the case for responses characterized by IFN-γ secretion and CD107a expression (figure 2E). While the absence of a difference between the *004+Bw4 (23.1% [15.0-31.9]) and the *004 only group (20.0% [5.9-31.3], p=0.1033) may be attributed to a relatively small sample size, there were no differences observed between the *004+Bw4 and the Bw4 only groups (22.7% [5.7-54.7], p=0.8683, Mann-Whitney test, figure 2E). This suggests 3DL1-receptors other than *004 may play a role in increasing the contribution of this CD107a$^+$IFN-γ^+TNF-α^- NK functional subset to the K562 stimulated response.

The monoclonal antibody Z27 (also known as CD158e) binds both KIR3DL1 and KIR3DS1. However, the fluorescence intensity of antibody binding to the inhibitory versus activating forms of these receptors differs such that $3DL1^+$ NK cell ($Z27^{hi}$) and $3DL1^-$ NK cell ($Z27^{dim}$ or $Z27^-$) are readily distinguishable[35,36]. For individuals carrying at least one copy of a 3DL1 allele and having a measurable tri-functional or responses characterized by the $CD107a^+IFN\text{-}\gamma^+TNF\text{-}\alpha^-$ or $CD107a^-IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ profiles, it was possible to attribute function to either the $3DL1^+$ or $3DL1^-$ NK cell subset for the populations carrying the different KIR/HLA genotype combinations studied. As shown in figure 3A, the percent of $3DL1^+$ NK cells that were tri-functional was greater when they originated from *h/*y+B*57 individuals than from Bw6 hmz (1.8% [0.9-3.9] and 0.2% [0.0-1.6] for *h/*y+ B*57 and Bw6 hmz, respectively, p=0.0026, Mann-Whitney test). The percent of $3DL1^-$ NK cells that were tri-functional was not significantly different between these two study groups (figure 3B), despite a non-significant increase in individuals carrying *h/*y+B*57 (0.5% [0.2-1.2] and 0.2% [0.0-1.6] for *h/*y+B*57 and Bw6 hmz, respectively, p=0.0868; Mann-Whitney test). For responses characterized by the $CD107a^-IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ profile, the percent of both $3DL1^+$ (1.4% [0.5-7.4] and 0.3% [0.0-3.7] for *h/*y+B*57 and Bw6 hmz, respectively, p=0.0185; Mann-Whitney test) and $3DL1^-$ (0.5% [0.2-2.8] and 0.1% [0.0-3.5] for *h/*y+B*57 and Bw6 hmz, respectively, p=0.0291; Mann-Whitney test) NK cells was greater in *h/*y+B*57 carriers than Bw6 hmz (figure 3C and D). Furthermore, within the *h/*y+B*57 group, there was a non-significant trend towards an increased percentage of $CD107a^-IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ $3DL1^+$ (1.4% [0.5-7.4]) versus $3DL1^-$ (0.5% [0.2-2.8]) NK cells (p=0.0625, Wilcoxon signed-rank test, figure 3C and D). In contrast, in Bw6 hmz the contribution of the $3DL1^+$ and

3DL1$^-$ subsets to the CD107a$^-$IFN-γ^+TNF-α^+ NK response stimulated by K562 was similar (Figure 3C and D). In summary the increased functional potential of NK cell from subjects carrying *h/*y+B*57 was most apparent in the KIR3DL1$^+$ NK cells.

We also examined whether tri-functional responses and those characterized by the CD107a$^+$IFN-γ^+TNF-α^- functional profile were differentially observed in 3DL1$^+$ or 3DL1$^-$ NK subsets in *004+Bw4 carriers compared with Bw6 hmz. Given that 3DL1*004 is not expressed at the cell surface[37,38], any effect this molecule would have on mediating NK function would be expected to be observed in the 3DL1$^-$ NK subset. For the 2 relevant functional profiles, i.e. tri-functional (not shown) and CD107a$^+$IFN-γ^+TNF-α^- responses (figure 3E and F), the percent of 3DL1$^+$ NK cells with these functions was not greater in individuals carrying *004+Bw4 versus those who were Bw6 hmz. There was a non-significant trend towards a higher contribution of tri-functional cells in the 3DL1$^-$ NK subset from *004+Bw4 versus Bw6 hmz carriers and a statistically significant greater contribution of NK cells characterized by the CD107a$^+$IFN-γ^+TNF-α^- profile in 3DL1$^-$ NK cells from *004+Bw4 versus Bw6 hmz (2.3% [0.7-8.2] and 1.4% [0.06-4.8], respectively p=0.0143; Mann-Whitney test). While the 3DL1$^-$ NK cell population includes 3DL1*004 expressing cells, it also includes other non-3DL1$^+$ NK cells. This may limit our ability to attribute whether the increase in these functional profiles in stimulated NK cells from *004+Bw4 individuals is the result of the action of 3DL1*004. However, these data suggest that the increase in functionality observed in NK cells from *004+Bw4 individuals is not mediated specifically by cells with measurable surface 3DL1, which would be expected if this effect was due to *004$^+$ NK cells.

V. Discussion

In this report, we compared the functional profile of NK cells from individuals carrying different allele combinations of KIR3DL1 and HLA-B. NK cells from individuals with 2 genotype combinations, *h/*y+B*57 and *004+Bw4, exhibited an increased contribution of polyfunctional NK cells to the total HLA-devoid K562 stimulated response. These genetic combinations have been previously linked to slower HIV disease progression, decreased VL and/or protection from HIV infection[9,14]. For cells from individuals with the *h/*y+B*57 combined genotype, the increased polyfunctionality was more likely to be seen in NK cells expressing 3DL1. In contrast, this was not the case for cells from individuals with the *004+Bw4 genotype combination. This finding would be consistent with 3DL1*004 alleles being retained within the cell cytoplasm and not expressed at the cell surface[37,38].

HLA B*57 is an HLA allele previously reported to be associated with slow HIV disease progression[9,39-41]. It has been reported that $CD8^+$ T-cell responses restricted by this allele may have unique qualities that influence the efficiency with which they control HIV[42-44]. For example, $CD8^+$ T cell responses restricted by HLA-B*57 contribute more to the total virus specific response during early infection, recognize a greater fraction of potential HIV-restricted epitopes and are associated with a reduction in the level of the response restricted by other alleles carried by the same individual[42-44]. However, given the role of NK cells in controlling viral infections through cytokine secretion and cytolysis, the potency and polyfunctionality of these innate immune cells in HLA-B*57 individuals may provide an additional mechanism favoring the slow disease progression

that has been linked to carrying this allele. Potent NK cell mediated anti-viral responses induced early in HIV infection may directly suppress HIV as well as induce DC maturation via a number of mechanisms, including IFN-γ secretion, favoring a Th1 response that could be beneficial in controlling the infection[45,46].

While epidemiological data suggest a role for the *h/*y+B*57 combined genotype in controlling HIV infection, we could not exclude the possibility that carriage of B*57 was sufficient for increased NK cell polyfunctionality and could not explore the impact of different 3DL1 alleles since all the B*57 individuals studied also carried 3DL1*h/*y. However, in these individuals, NK cell polyfunctionality occurred more frequently in $3DL1^+$ than $3DL1^-$ cells, suggesting that this molecule is probably involved in the modulation of the NK response. On the other hand, since NK cells from non-B*57 subjects with other Bw4-80I or Bw4 alleles did not exhibit increased polyfunctionality following stimulation with K562 cells, nor did NK cells from non-B*57 individuals with the *h/*y genotype alone (see Figure 2 and data not shown), it is clear that B*57 plays a central role in modulating the NK cell functional profile.

It is not known why this HLA allele is superior to other Bw4 or Bw4-80I alleles in favoring the development of polyfunctional NK cells. Amongst Bw4-80I alleles, which are particularly good receptors for 3DL1[47-49], B*5705 and B*5711 (neither of which were present in our population) are the only members of the B*57 family that do not have a valine residue at position 97[50]. Of the other Bw4-80I alleles, only B*5813 has a valine at this position; the remaining Bw4-80I alleles have either arginine, asparagines, threonine, tryptophan or serine at this position[50]. These residues differ markedly from valine either

in terms of polarity or size. While it is known that the amino acid at position 97 is involved in modulating the interaction between HLA-B and 3DL1, its precise role is still unknown[51]. It is possible that the presence of a valine at position 97 influences receptor-ligand interactions, which directly affect the NK licensing. Licensing is an NK cell maturation process in which cells acquire inhibitory KIR receptors for self HLA ligands until tolerance to self is achieved[9,19;52]. Mature NK cell subsets that express potent inhibitory receptors for self HLA may therefore have greater activation potential when the inhibitory signal is interrupted through interactions with HLA-deficient cells such as K562 or HIV infected cells in which Nef downmodulates HLA-A and B cell surface expression[53,54]. The licensing concept provides an explanation for how an inhibitory KIR such as KIR3DL1 may play a role in NK cells function that ultimately influences activation of anti-viral functions.

We are the first to demonstrate that KIR3DL1*004 allele can have an effect on NK cell function. A recent epidemiological study found that, in order to observe an effect on HIV disease progression, both 3DL1*004 and its Bw4 ligand are required[9]. Our data suggest that this combination is also necessary to observe influences on NK cell function. Thus, despite being retained within the cell, this allele may somehow interact with HLA molecules in order to modulate NK cell function and thereby modify HIV disease outcome. One could argue that most 3DL1*004 individuals in our cohort posses a second 3DL1 allele, expressed at the cell surface, which could mediate the increase in polyfunctionality observed. Our results suggest this is unlikely given that the increase in function was not observed in $3DL1^{+}$ NK cells as it was in *h/*y+B*57 individuals (see Figure 3). These findings provide additional evidence that the 3DL1*004 allele is not a

null or inactive allele. Elucidating how intracellular 3DL1*004 is able to modify NK cell function or whether it is linked with a gene that is capable of doing so will be important for improving our understanding of how this allele functions.

Given that the *h/*y+80I combination has also been linked to slower HIV disease progression, we expected that NK cells from individuals with this genotype would also have a greater functional potential when compared to Bw6 hmz. A limited sample size (n=10) could explain why we were not able to detect any functional differences between these groups. It is also possible that the epidemiological effect observed on HIV disease progression in individuals with this genetic combination is dependent on the presence of the virus, as it has been proposed in the case of KIR3DS1 and HLA-Bw4-80I, another combined genotype linked to slower HIV disease progression[10,55,56]. If this is the case, a different experimental system in which induction of the NK cells occurs in the context of HIV infected cell stimulation may be required to observe the effect of the *h/*y+80I genotype on NK cell function[56]. Finally, perhaps the effects of this HLA allele family on NK cell function would be better assessed using functional markers not measured by our experiments, such as perforin or granzyme, recently proposed to be better correlates of immune protection in HIV-specific T cell responses[27].

As shown in figure 2, we observe a high level of variability in the distribution of the contribution of some functional responses in the Bw6 hmz group (Figure 2). As has been done in previous epidemiological studies, our genetic groupings do not take into account that some HLA-A alleles (A*23, A*24, A*25, and A*32) have a Bw4 epitope because the information on the interaction of between these HLA molecules and

KIR3DL1 is limited and somewhat controversial[5,9,49,57,58]. However, when individuals with a Bw4-containing HLA-A molecule are removed from the control population, the variation in response contribution in this group decreases. Future studies clarifying the interaction of Bw4 carrying HLA-A and KIR3DL1 would be necessary in order to better design epidemiological and functional studies. It is important to also consider the possibility that the variation in NK response profile between individuals belonging to the same group may be the result of other KIR/HLA combinations or other NK receptors that may be expressed at the NK cell surface. As alluded to above, induction of NK cell function is likely to differ depending on whether the stimulus is provided by K562 cells or HIV infected cells. In HIV infected cells the Nef gene product downregulates surface expression of HLA-A and –B but does not affect HLA-C and –E levels, which are ligands for some NK cell receptors[53,59,60]. In addition, the presence of HIV viral peptides bound to the HLA molecules could influence their binding to KIR molecules[5]. Thus, while K562 stimulation of NK cells provides insights into their functional potential, there are limitations associated with this method and the next step in understanding the mechanisms underlying the association of certain KIR/HLA combination with favorable HIV outcome would be to investigate the influence of these combined genotypes of NK function using HIV-infected cells as stimuli.

In conclusion, we provide a link between KIR3DL1/HLA-B genotypes that have been associated with slower HIV disease progression and/or resistance to infection and increased NK cell functional potential. Understanding the mechanisms underlying these effects will contribute to our appreciation of the role of the innate immune system in HIV infection and may lead to new HIV vaccine design strategies.

VI. Acknowledgements

We would like to acknowledge Mr. Martin Rioux for coordination of the St. Luc Injecting Drug Use (IDU) cohort, Mr. Sylvain Gimmig and Mme Laurence Lejeune for their assistance in the development of panel of monoclonal antibodies for multiparametric flow cytometry, Mr. Benjamin Tallon, Mr. Saeid Sharafi, Ms. Marianna Kleyman and Ms. Tsoarello Mabanga for technical support. This study received support from the Canadian Institutes for Health Research #MOP-79515 and the Fonds de la Recherche en Santé du Québec (FRSQ). S Boulet was supported by a PhD. scholarship from FRSQ.

VII. Authorship

S.B. designed the study, performed the experiments and data analysis and prepared the manuscript; J.B. and C.M.T. provided clinical samples; N.F.B. designed the study and prepared the manuscript. The authors declared no conflict of interest.

VIII. Figure legends

Figure 1

Study design. KIR3DL1 and HLA-B genetic distribution of our study population **(A)**. 54 individuals were studied, 24 Bw6 homozygotes and 30 individuals subdivided in 4 KIR3DL1 and HLA-Bw4 allelic combination that were not mutually exclusive. Flow cytometry gating strategy **(B)**. The live, lymphocytic singlet population was used to gate on NK cells, which were defined as $CD3^-$ and either $CD56^+CD16^+$, $CD56^+CD16^-$, or $CD56^-CD16^+$. Boolean gating was then used to determine the possible combinations of CD107a, IFN-γ, TNF-α and Z27 expression on NK cells.

Figure 2

NK cells from *h/*y+B*57 and *004+Bw4 individuals have a distinct functional profile. Panel A shows the percent contribution to the total response of NK cells with seven different functional profiles from individuals with four different KIR3DL1 and HLA-B genetic combinations shown by each bar: KIR3DL1*h/*y+HLA-B*57 (*h/*y+B*57, n=5), KIR3DL1*004+HLA-Bw4 (*004+Bw4, n=13), KIR3DL1*h/*y+HLA-Bw4-80I (*h/*y+80I, n=10) and HLA-Bw6 homozygotes (Bw6 hmz, n=24). Below the x-axis, dots refer to the presence of each functional marker (CD107a, IFN-γ and TNF-α) in that profile. The height of each bar represents the median for the group and the height of the error bar the upper range for that group. A "*" over a bar indicates that the contribution of the functional subset for the study population the bar represents was significantly greater than that for Bw6 hmz. Tri-functional $CD107a^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ **(B)** and $CD107a^-$

IFN-γ^+TNF-α^+ **(C)** NK cell contribution to the total response was compared between *h/*y+B*57 individuals and non-B*57 individuals who were *h/*y+80I, *h/*y+Bw4 or Bw6 hmz. Tri-functional CD107a$^+$IFN-γ^+TNF-α^+ **(D)** and CD107a$^+$IFN-γ^+TNF-α^- **(E)** NK cell contribution to the total K562 stimulated response was compared between *004+Bw4 individuals and individuals with *004 only (absence of HLA-Bw4 alleles), Bw4 only (absence of KIR3DL1*004 alleles) or Bw6 hmz. A Mann-Whitney test was used to assess the statistical significance of between group comparisons and a p-value <0.05 was considered significant.

Figure 3

Functional contribution in NK cells from *h/*y+B*57 but not *004+Bw4 individuals is mainly due to KIR3DL1$^+$ cells. The scatter plots show the percent of the total 3DL1$^+$ (panels A, C and E) or 3DL1$^-$ (panels B, D and F) NK cells that are CD107a$^+$IFN-γ^+TNF-α^+ (panels A, B), CD107a$^-$IFN-γ^+TNF-α^+ (panels C, D) and CD107a$^+$IFN-γ^+TNF-α^- (panel E, F) for the study groups with the KIR/HLA combined genotypes shown. A Mann-Whitney test was used to assess the significance of between group comparisons (*h/*y+B*57 versus Bw6 hmz in panels A-D and *004+Bw4 versus Bw6 hmz in panels E-F) and a p-value <0.05 was considered significant and shown with a *. The p-value of 0.0625 in panel C-D is the result of a Wilcoxon signed-rank test comparing the percent of 3DL1$^+$ cells and 3DL1$^-$ cells that are CD107a$^-$IFN-γ^+TNF-α^+ within each *h/*y+B*57 individual. As described in the text, all Bw6 hmz individuals included in these analyses had at least one copy of the 3DL1 gene. This is indicated by the designation 'Bw6 hmz (3DL1$^+$)'.

IX. Tables and Figures

Figure 1A

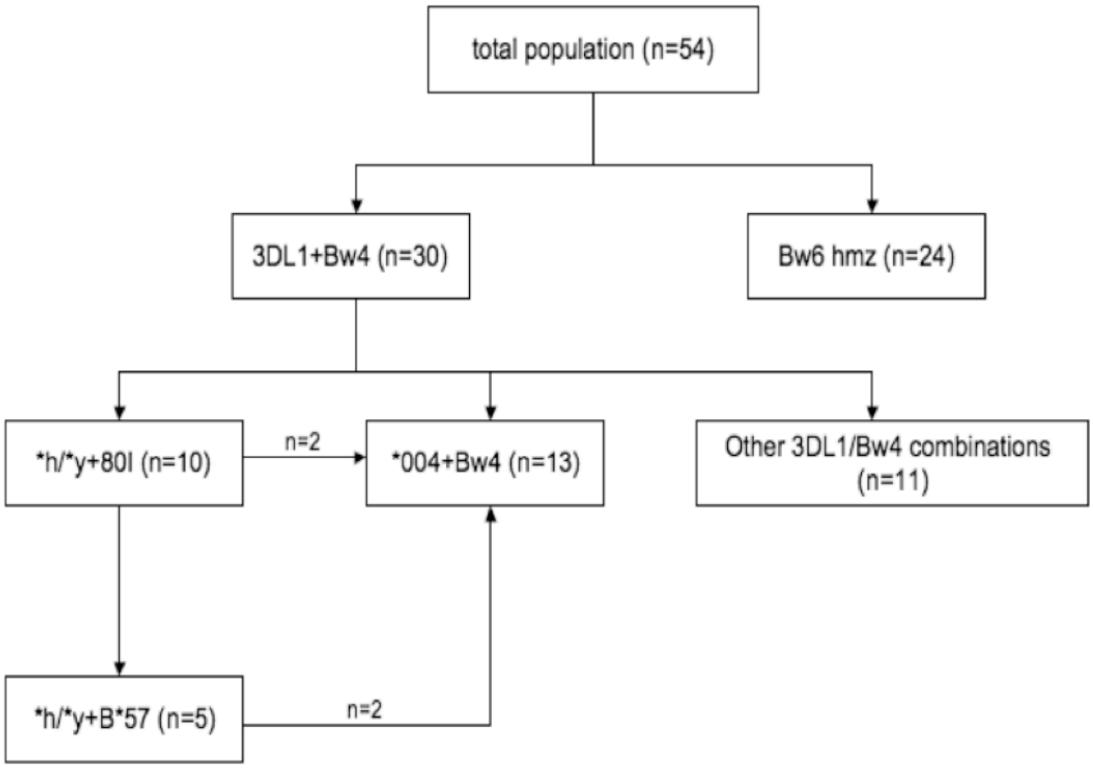


Figure 1B

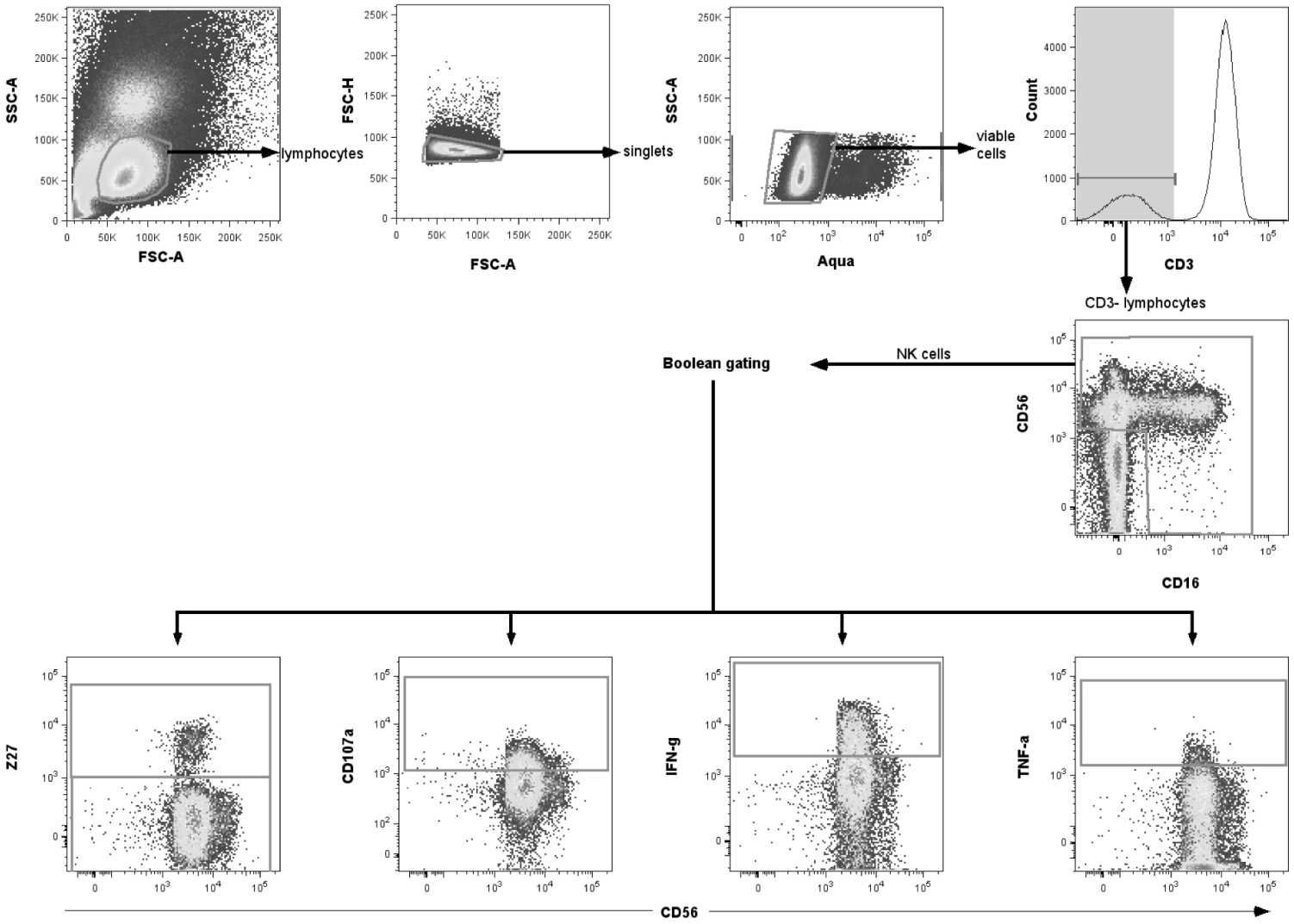

SSC-A
FSC-A
lymphocytes
FSC-H
FSC-A
singlets
SSC-A
Aqua
viable cells
Count
CD3
CD3- lymphocytes
NK cells
Boolean gating
CD56
CD16
Z27
CD107a
IFN-g
TNF-a
CD56

Figure 2A

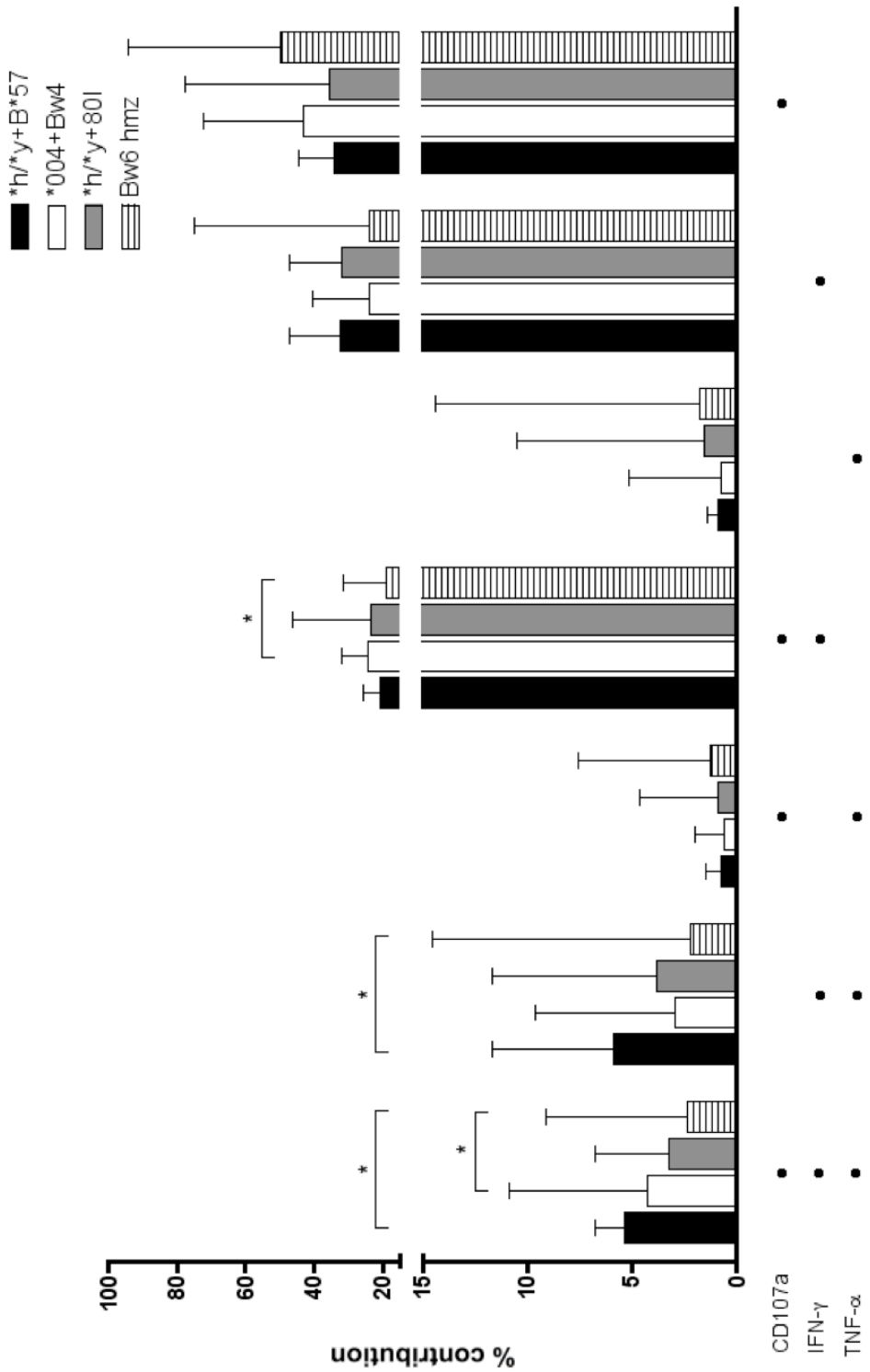

*h/*y+B*57
*004+Bw4
*h/*y+80I
Bw6 hmz
% contribution
100
80
60
40
20
15
10
5
0
CD107a
IFN-γ
TNF-α
*
*
*
*

Figure 2B, C

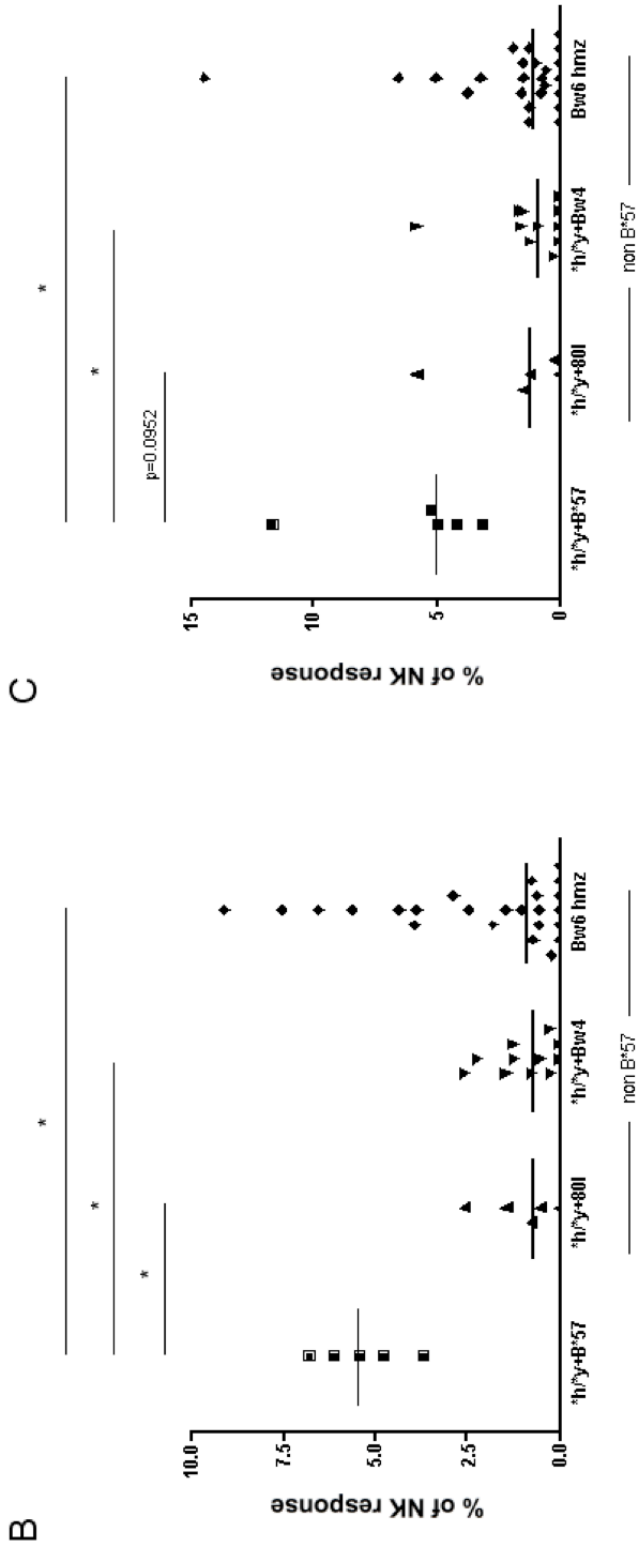
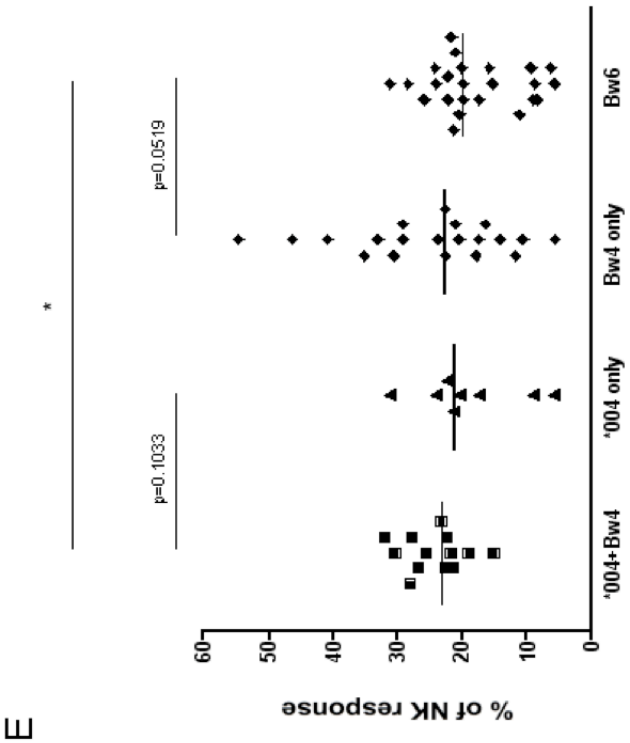

B
% of NK response
10.0
7.5
5.0
2.5
0.0
*h/*y+B*57
*h/*y+80I
*h/*y+Bw4
Bw6 hmz
non B*57
C
% of NK response
15
10
5
0
p=0.0952
*h/*y+B*57
*h/*y+80I
*h/*y+Bw4
Bw6 hmz
non B*57

Figure 2 D, E


E
% of NK response
0
10
20
30
40
50
60
'004+Bw4
'004 only
Bw4 only
Bw6
p=0.1033
p=0.0519
*

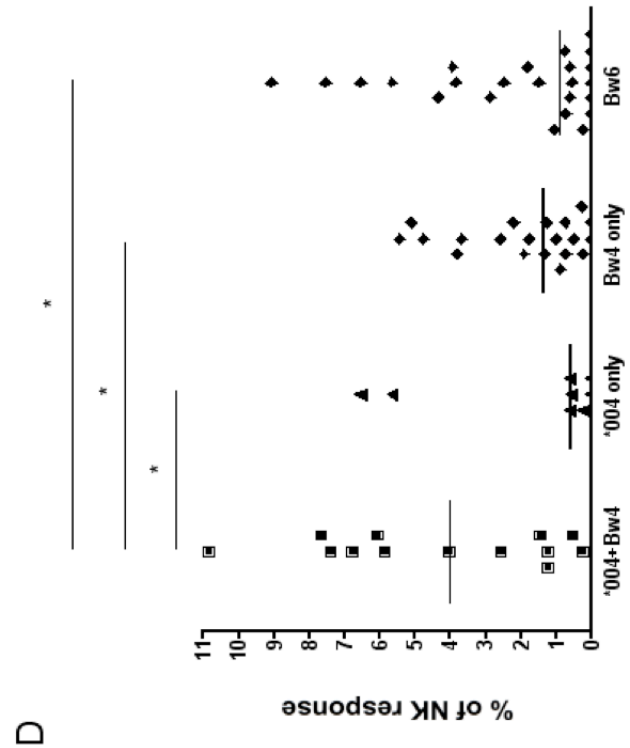

D
% of NK response
0
1
2
3
4
5
6
7
8
9
10
11
'004+Bw4
'004 only
Bw4 only
Bw6
*
*
*

Figure 3A, B

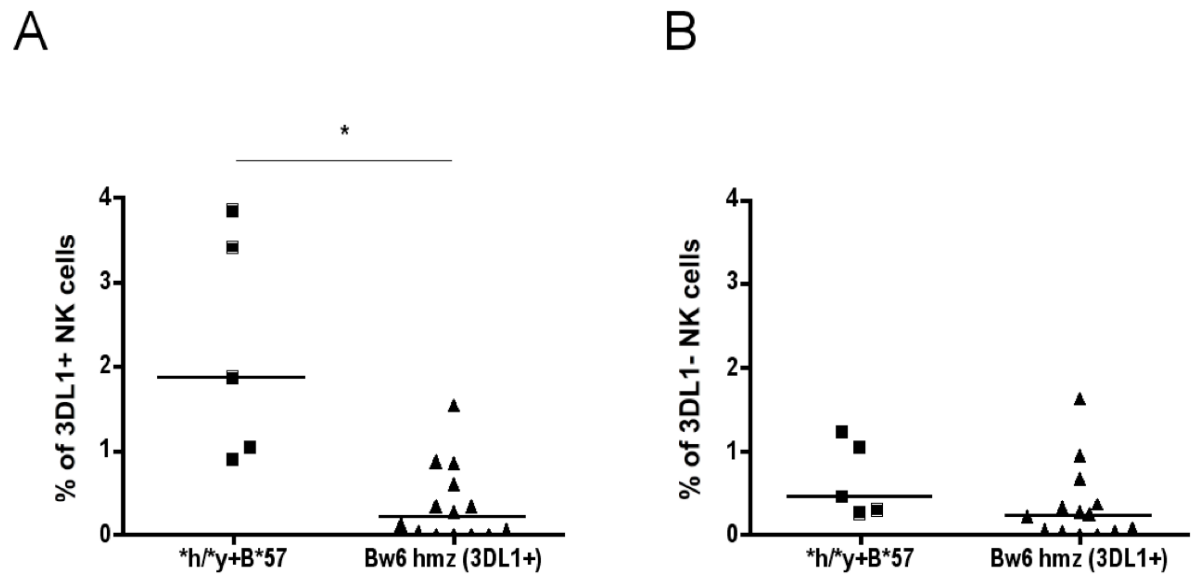
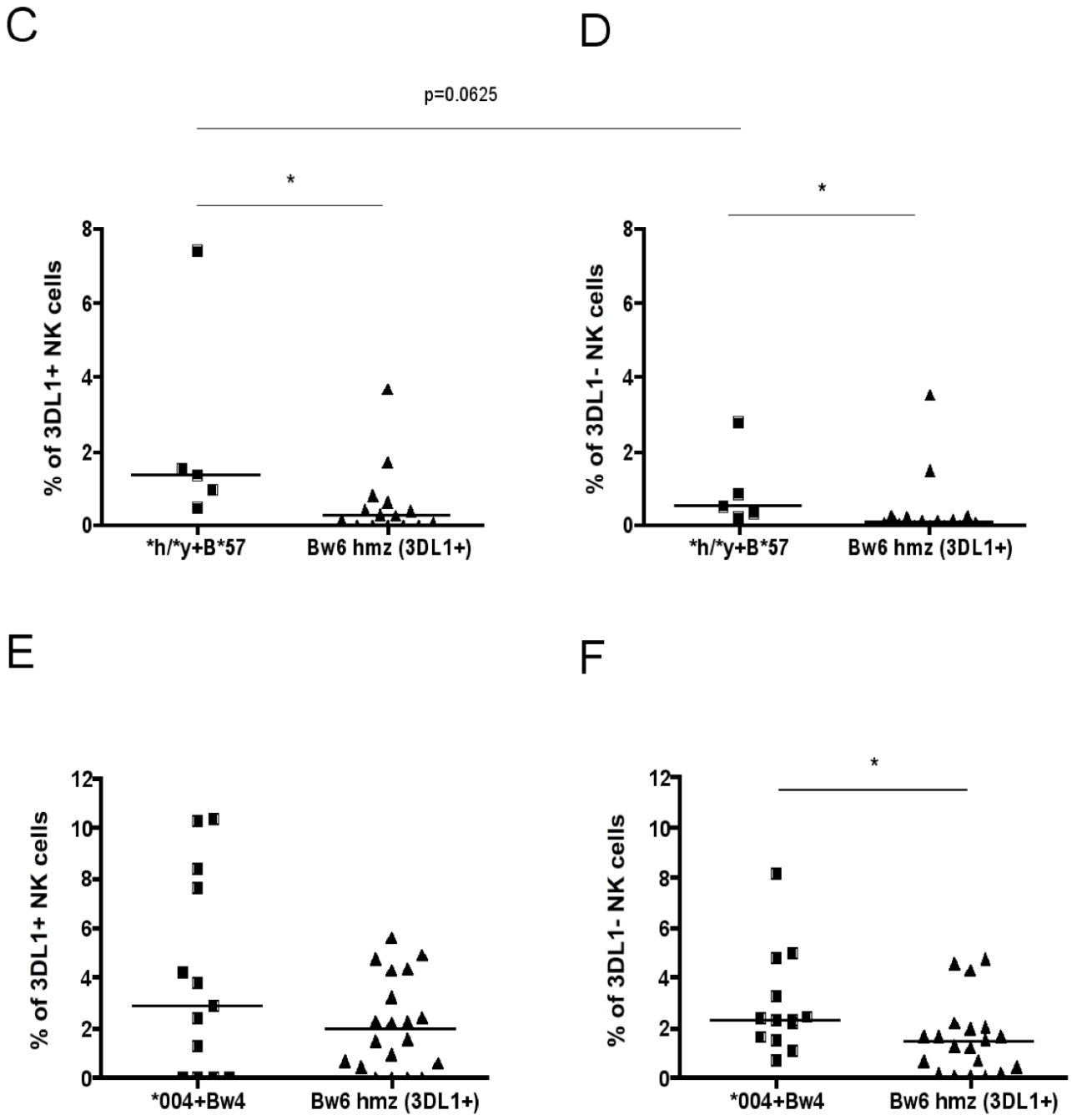


Figure 3, C, D, E, F



X. Reference List

1. Gardiner CM. Killer cell immunoglobulin-like receptors on NK cells: the how, where and why. Int.J.Immunogenet. 2008;35:1-8.

2. Bashirova AA, Martin MP, McVicar DW, Carrington M. The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. Annu.Rev.Genomics Hum.Genet. 2006;7:277-300.

3. Stewart-Jones GB, di GK, Kollnberger S et al. Crystal structures and KIR3DL1 recognition of three immunodominant viral peptides complexed to HLA-B*2705. Eur.J.Immunol. 2005;35:341-351.

4. O'Connor GM, Hart OM, Gardiner CM. Putting the natural killer cell in its place. Immunology 2006;117:1-10.

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

6. Yawata M, Yawata N, Draghi M et al. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J.Exp.Med. 2006;203:633-645.

7. Thomas R, Yamada E, Alter G et al. Novel KIR3DL1 alleles and their expression levels on NK cells: convergent evolution of KIR3DL1 phenotype variation? J.Immunol. 2008;180:6743-6750.

8. Khakoo SI, Thio CL, Martin MP et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 2004;305:872-874.

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

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

11. Qi Y, Martin MP, Gao X et al. KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. PLoS.Pathog. 2006;2:e79.

12. Barbour JD, Sriram U, Caillier SJ et al. Synergy or independence? Deciphering the interaction of HLA Class I and NK cell KIR alleles in early HIV-1 disease progression. PLoS.Pathog. 2007;3:e43.

13. Lopez-Vazquez A, Mina-Blanco A, Martinez-Borra J et al. Interaction between KIR3DL1 and HLA-B*57 supertype alleles influences the progression of HIV-1 infection in a Zambian population. Hum.Immunol. 2005;66:285-289.

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

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

16. Norman PJ, bi-Rached L, Gendzekhadze K et al. Unusual selection on the KIR3DL1/S1 natural killer cell receptor in Africans. Nat.Genet. 2007;39:1092-1099.

17. Farag SS, Caligiuri MA. Human natural killer cell development and biology. Blood Rev. 2006;20:123-137.

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

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

20. Cooper MA, Fehniger TA, Turner SC et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 2001;97:3146-3151.

21. Alter G, Teigen N, Davis BT et al. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. Blood 2005;106:3366-3369.

22. Levy DE, Garcia-Sastre A. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. Cytokine Growth Factor Rev. 2001;12:143-156.

23. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J.Leukoc.Biol. 2004;75:163-189.

24. Wong GH, Goeddel DV. Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. Nature 1986;323:819-822.

25. Mestan J, Digel W, Mittnacht S et al. Antiviral effects of recombinant tumour necrosis factor in vitro. Nature 1986;323:816-819.

26. Lichterfeld M, Yu XG, Waring MT et al. HIV-1-specific cytotoxicity is preferentially mediated by a subset of CD8(+) T cells producing both interferon-gamma and tumor necrosis factor-alpha. Blood 2004;104:487-494.

27. Migueles SA, Osborne CM, Royce C et al. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. Immunity. 2008;29:1009-1021.

28. Addo MM, Yu XG, Rathod A et al. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J.Virol. 2003;77:2081-2092.

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

30. Betts MR, Brenchley JM, Price DA et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J.Immunol.Methods 2003;281:65-78.

31. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J.Immunol.Methods 2004;294:15-22.

32. Boileau C, Bruneau J, Al-Nachawati H, Lamothe F, Vincelette J. A prognostic model for HIV seroconversion among injection drug users as a tool for stratification in clinical trials. J.Acquir.Immune.Defic.Syndr. 2005;39:489-495.

33. Bernard NF, Yannakis CM, Lee JS, Tsoukas CM. Human immunodeficiency virus (HIV)-specific cytotoxic T lymphocyte activity in HIV-exposed seronegative persons. J.Infect.Dis. 1999;179:538-547.

34. Boulassel MR, Spurll G, Rouleau D et al. Changes in immunological and virological parameters in HIV-1 infected subjects following leukapheresis. J.Clin.Apher. 2003;18:55-60.

35. Carr WH, Rosen DB, Arase H et al. Cutting Edge: KIR3DS1, a gene implicated in resistance to progression to AIDS, encodes a DAP12-associated receptor expressed on NK cells that triggers NK cell activation. J.Immunol. 2007;178:647-651.

36. Pascal V, Yamada E, Martin MP et al. Detection of KIR3DS1 on the cell surface of peripheral blood NK cells facilitates identification of a novel null allele and assessment of KIR3DS1 expression during HIV-1 infection. J.Immunol. 2007;179:1625-1633.

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

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

39. Migueles SA, Sabbaghian MS, Shupert WL et al. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc.Natl.Acad.Sci.U.S.A 2000;97:2709-2714.

40. Kaslow RA. Host genes and HIV infection: implications and applications. Emerg.Infect.Dis. 1997;3:401-402.

41. Trachtenberg E, Korber B, Sollars C et al. Advantage of rare HLA supertype in HIV disease progression. Nat.Med. 2003;9:928-935.

42. Altfeld M, Addo MM, Rosenberg ES et al. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS 2003;17:2581-2591.

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

44. Scherer A, Frater J, Oxenius A et al. Quantifiable cytotoxic T lymphocyte responses and HLA-related risk of progression to AIDS. Proc.Natl.Acad.Sci.U.S.A 2004;101:12266-12270.

45. Mocikat R, Braumuller H, Gumy A et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity. 2003;19:561-569.

46. Gerosa F, Gobbi A, Zorzi P et al. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. J.Immunol. 2005;174:727-734.

47. Gumperz JE, Barber LD, Valiante NM et al. Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. J.Immunol. 1997;158:5237-5241.

48. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. J.Immunol. 2005;175:5222-5229.

49. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. J.Exp.Med. 1994;180:1235-1242.

50. Robinson J, Waller MJ, Parham P et al. IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. Nucleic Acids Res. 2003;31:311-314.

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

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

53. Cohen GB, Gandhi RT, Davis DM et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity. 1999;10:661-671.

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

55. Altfeld M, Goulder P. 'Unleashed' natural killers hinder HIV. Nat.Genet. 2007;39:708-710.

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

57. Stern M, Ruggeri L, Capanni M, Mancusi A, Velardi A. Human leukocyte antigens A23, A24, and A32 but not A25 are ligands for KIR3DL1. Blood 2008;112:708-710.

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

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

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

Chapter 5: Summary of Original Scholarship

From Chapter 2:

1. We were the first to demonstrate that the homozygous KIR3DS1 genotype is overrepresented in the EU population when compared to individuals in the primary stage of HIV infection.

2. We demonstrated that, unlike its effect on HIV disease progression, resistance to infection conferred by the KIR3DS1 allele is not likely to depend on the presence of HLA-Bw4-80I. This HLA allele still has not been shown to physically interact with KIR3DS1.

3. Our study remains the largest genetic association study analyzing the effect of KIR3DS/L1 on resistance to HIV infection in EU (n=80).

4. Our study is the only genetic association study analyzing the effect of KIR3DS/L1 on resistance to HIV infection performed in a largely Caucasian population.

From Chapter 3:

1. We were the first to demonstrate that the KIR3DL1*h/*y+HLA-B*57 combined genotype is overrepresented in the EU population when compared to individuals in the primary stage of HIV infection.

2. We were the first to link a KIR genotype associated with slower HIV disease progression to resistance from infection.

3. We were the first to comprehensively compare the distribution of KIR3DL1 allotypes in the EU population.

4. We were the first to suggest that HLA-B*57 alone may have an effect on resistance from infection. Although this remains inconclusive for the moment, this observation warrants further investigation.

From Chapter 4:

1. We were the first to show the increased functional profile of NK cell isolated from individuals with genotypes linked to slower HIV disease progession and/or resistance from infection (i.e. KIR3DL1*h/*y+HLA-B*57 and KIR3DL1*004+HLA-Bw4).
2. We were the first to apply the concept of cell polyfunctionality to NK cells. Subsequently, we were the first to suggest that increased polyfunctional profile is is favorable in NK cells as well as T-cell responses.
3. We were the first to suggest that, despite the fact that KIR3DL1*004 is not expressed at the cell surface, it may positively affect the NK cell functional profile.
4. We were the first to suggest a role for the amino acid 97 in HLA-B*57 in increasing NK cell functional potential.

Chapter 6: Discussion

There are two main conclusions that may be drawn from the data presented in this thesis. Firstly, the KIR3DS/L1 locus, which had been previously shown to affect HIV disease progression, is linked by population association studies to the decreased infection susceptibility observed in EUs. More specifically, EUs were more likely to be KIR3DS1 homozygotes or carry KIR3DL1*h/*y + HLA-B*57 than individuals in HIV primary infection. Secondly, KIR3DL1 genotypes associated with favorable HIV clinical outcomes, particularly KIR3DL1*h/*y + HLA-B*57, were shown to have increased NK cell functional potential. Increased NK cell function may lead to HIV control.

I. POPULATION ASSOCIATION STUDIES

The rationale behind the research presented in this thesis is that, given the epidemiological link observed between the rate of HIV disease progression and the KIR3DS/L1 locus and the functional importance of NK cells in the early stages of an infection, perhaps this NK cell receptor locus may also play a role in the reduced susceptibility to HIV infection observed in EUs. It is clear that rate of HIV disease progression and protection from infection are two very different clinical outcomes, and that what may apply to one does not necessarily apply to the other. While several markers of decreased susceptibility, such as the HLA-A A2/6802 supertype are not found in individuals that progress more slowly to AIDS, the effect of a locus on both HIV disease progression and resistance from infection has been previously observed in the case of CCR5 and CCL3L1[330,335,344,351,387].

The association between a phenotype and a genotype can be established by comparing the gene content of a population of individuals with the phenotype (EUs) to a

population without the phenotype (infected individuals)[388]. This type of association study has become a useful tool in population genetics. The statistical association between a locus and a phenotype can arise in three ways[389]. Firstly, there can be a direct association, where the gene is causally related to the phenotype. Ideally, results from association studies fall into this category.

Secondly, the locus itself may not be causal, but it is sufficiently close to a causal locus to be in linkage disequilibrium with it. This is termed indirect association. For the moment, it is impossible to determine whether the data presented in chapters 2 and 3 are the result of an indirect association, considering that the KIR3DS/L1 is located in a genetic region that contains several genes involved in the immune response. The issue is additionally complex because KIR3DS1 is specific to KIR B haplotypes.[390] Therefore, the associations described in this thesis could result from the inheritance of haplotypes or specific genes within the haplotypes rather than the KIR3DS/L1 locus. However, because in vitro experiments have demonstrated an effect on HIV viral replication mediated by NK cells expressing KIR3DS1 (see section IIIB), this locus should be considered as the prime candidate for mediating decreased susceptibility to infection in EUs[310]. Further studies evaluating the capacity of NK cells expressing specific KIR molecules to inhibit HIV replication should be able to demonstrate whether the effect is mediated specifically by the KIR3DS/L1 locus.

Finally, the association may be due to confounding factors and therefore be of little scientific interest. Such false positive findings can be the result of population stratification, where individuals with or without the outcome are of different genetic background[388]. This confounder was minimized in chapters 2 and 3 by recruiting the EU

and control population within a single geographical location and by insuring that both study groups would be ethnically similar. Alternatively, false positive associations between genotype and phenotype may be the result of multiple testing[388]. Because association studies are statistical in nature, there is an inherent rate of type I errors (α); the probability of making one false-positive inference. As, genetic association studies often tend to test more than one hypothesis (i.e. look for more than a single association), the number of possible false positive results increases proportionally with the number of statistical tests carried out. This means that, on average, if 100 independent statistical tests were carried, about five false positives would appear, linking a gene and a trait even if they are unrelated. To account for this error rate, a correction may be required. An often-used method is the Bonferroni correction, where the threshold for significance of a p-value is increased proportionally to the number of analyses performed[391]. Therefore, using the Bonferroni correction, if 100 tests are performed, then only p-values below 0.0005 would be considered significant. However, this type of correction results in increased rates of type II errors, i.e. false negatives[392]. This means that associations that are not spurious would be missed, an outcome that is also undesirable. Therefore, the best way to diminish type I errors is to increase sample size. EU studies typically have relatively small sample sizes because of the rarity of the phenotype. The size of the study population presented in chapters 2 and 3 compares favorably with another published study associating KIR with the EU phenotype (n=21) but is still much smaller than epidemiological studies linking KIR3DS/L to HIV disease progression (n > 1000)[150,151,386]. Efforts to increase the size of the EU cohort in Montreal are ongoing, but

the current recruitment rate is too low to expect significant changes in the data interpretation in the near future.

It is more likely that reproducibility of the results in other EU cohorts will confirm/refute the validity of the genetic associations presented in this thesis. This has been the case for the CCR5Δ32 mutation and the increased presence of the HLA A2/6802 supertype in EUs[330,345,346,393]. Unfortunately, the frequency of the KIR3DS/L1 genes varies greatly amongst populations. Several EU cohorts are located in Africa, because the HIV pandemic is concentrated in this region. However, the frequency of KIR3DS1 in individuals of African descent is low (0.05) compared to North American Caucasian populations (0.22)[150]. This explains the weaker association between the combined genotype KIR3DS1/HLA-Bw4 80I and HIV disease progression in African Americans[150]. The lower frequency of KIR3DS1 in individuals of African descent may also explain why Jennes et al did not observe an increased proportion of KIR3DS1 homozygous individuals in their Ivorian EU cohort (0/21)[386]. Therefore, any attempt to reproduce our findings in other EU cohorts should first be performed in cohorts of European origin.

II. THE MONTREAL EU COHORTS

The EU cohort described in this thesis is composed of two distinct populations; individuals recruited in the St-Luc cohort IDU (n=64/80, 80%) and seronegative sexual partners of HIV infected patients (n=16/80, 20%). There is no organized recruitment of potential EUs within the homosexual community in Montreal. Therefore, the majority of EUs included for analysis in this thesis were parenterally exposed to HIV. This contrasts with individuals recruited in the Quebec PI (primary infection) cohort, approximately

60% of which report sexual exposure as the suspected cause of HIV acquisition. This stratification difference between the control (PI) and test (EU) population may introduce a bias in the genetic analyses. To avoid this bias, comparisons in the distribution of the KIR3DS1 homozygous and KIR3DL1*h/*y+HLA-B*57 genotypes could be performed on PI and EU individuals reporting a single risk factor of HIV exposure. For example, utilizing a similar analytic strategy to that performed in chapters 2 and 3, amongst parenterally exposed EUs, 7/64 (10.9%) were KIR3DS1 homozygous and 5/37 (13.5%) were KIR3DL1*h/*y+HLA-B*57. In parentally exposed PI individuals, 5/103 (4.9%) were KIR3DS1 homozygous and 1/47 (2.1%) were KIR3DL1*h/*y+HLA-B*57. The decreased sample size due to the exclusion of mucosally exposed individuals affects the statistical power of these comparisons. However, proportional differences are still observed between EUs and PIs for these genotypes. Increasing the sample size of parenterally and mucosally exposed individuals will be necessary in order to determine whether KIR3DS/L1 loci associated with decreased HIV susceptibility function in either or both contexts.

One of the great strengths of data obtained from the Pumwani cohort of HIV resistant women results from the geographical location of the cohort. Prevalence in Nairobi is such that the exposure levels of commercial sex workers to HIV are elevated and favor the selection of individuals with reduced susceptibility to infection. EUs in Montreal were defined as individuals with at least five exposures, sexual or intravenous, to a known HIV seropositive source. This arbitrary number ensured a balance between identifying individuals with sufficient high-risk behavior and a reasonable sample size. In the case of seronegative sexual partners of seropositive individuals, unprotected sexual

activity was documented several times over the course of minimum eight months and up to several years. For IDUs of the St-Luc cohort, in which behavioral data was collected by trained professionals during one-on-one interviews, an exposure was defined by needle sharing with a known HIV^+ individual. While five such sharing events were required for inclusion in the resistant cohort, the average number of known HIV exposures for the IDU EUs included in the studies presented in this thesis was 60. In addition, IDUs in this cohort were also shown to share needles with individuals of unknown serostatus. Recruiting individuals through needle exchange programs, prisons or detoxification centers, the SurvUDI network was able to estimate levels of HIV infections in IDUs in Montreal, Canada[394]. Between 1995 and 2007, 4686 individuals were recruited in the network. Approximately 17.6% of these were shown to be HIV^+, a quarter of which were unaware of their serostatus. Therefore, in Montreal, sharing needles with individuals of unknown serostatus carries great risk of HIV infection. Finally, increased prostitution and unprotected sex amongst IDUs, results in additional risks of HIV exposure in this population[21]. For these reasons, despite the fact that the study presented in this thesis was performed in Canada, which has a relatively low HIV prevalence, selection criteria for inclusion in the EU cohort allowed for a defined group of individuals that were highly exposed to HIV, but remained uninfected. In the 80 individuals included in the EU group, over 4000 exposures to HIV were documented. These numbers do not include exposures to individuals of unknown HIV serostatus. It also does not account for the fact that IDUs may underreport their drug related habits and therefore may have had more exposures than documented[395-397]. Therefore, the probability that all these individuals remained uninfected based on 'chance' alone is

virtually nil. This provides a rationale for exploring biological explanations for the reduced susceptibility to HIV infection observed in some individuals in Montreal, Canada.

III. OF POLYFUNCTIONALITY

Evaluation of NK cell responses has traditionally relied on two methodologies. Cytolytic capacity of NK cells was measured by their co-incubation with radiolabeled targets. On the other hand, the secretion of each chemokine or cytokines in response to various stimuli was measured separately either by flow cytometry or ELISA. The separation of these methods was the result of two technical impediments: the lack of alternative cytotoxicity assays and the limited number of markers that could be used in a single flow-based experiment. Recently, measurement of CD107 translocation to the cell surface by flow cytometry was proven an adequate surrogate marker for cytotoxic potential of T- and NK cells[263,264]. In addition, the latest generations of flow cytometers are designed to detect up to eighteen different fluorochrome-associated antibodies, a dramatic increase from the only four combinations available in older instruments. These methodological advances allow for the simultaneous detection of NK cell specific cytokine/chemokine secretion and cytotoxic potential in a single experiment.

Using these new technical developments, Betts et al. compared the functional profile of $CD8^+$ T cells isolated from individuals with a nonprogressive course of HIV infection to those from individuals with a chronic progressive course by simultaneously measuring their IFN-γ, IL-2, MIP-1β, CD107 and TNF-α secretion in response to HIV peptide stimulation[398]. They showed that nonprogressors had a greater proportion of T-

cells concurrently secreting 5 or 4 functions. The benefits of T-cells responses capable of several simultaneous functions is not exclusive to HIV and applies in the context of *Leishmania major* infection[399]. Mice subjected to a live vaccine that induced a greater proportion of *Leishmania*-specific T-cells capable of concurrent secretion of IFN-γ, IL-2 and TNF-α had a decreased parasitic load and lesion size upon infectious challenge when compared to mice subjected to a vaccine that induced mostly T-cells capable of secreting only one of these functions. These experiments demonstrate the importance of 'polyfunctional' T-cells in infection.

To our knowledge, we are the first to attempt to comprehensively characterize NK cells by simultaneously measuring three known anti-viral functions. Whether polyfunctional NK cells are also biologically important has not been demonstrated. This could possibly be done by comparing the functional profile of NK cells from nonprogressive individuals to those from progressors. In chapter 4, we show that polyfunctional NK cells are increased in individuals with KIR3DS/L1 and HLA-B combined genotypes associated with slower HIV disease progression, decreased viral load and/or decreased susceptibility to infection. This suggests a possible biological role for polyfunctional NK cells, but needs to be confirmed.

We have defined our NK cell response through the use of three functional markers: CD107a, IFN-γ and TNF-α. These are well known NK cell anti-viral functions. However, several other NK cell functions could have been included in our panel. MIP-1α competitively inhibits HIV entry into cells and is secreted by NK cells. While CD107 is usually accepted as a surrogate marker for cytotoxicity, recent characterization of T-cell responses have shown that a significant proportion of T-cells that are positive for CD107

translocation to the cell surface upon stimulation do not secrete perforin and may therefore not be cytotoxic[400]. Including perforin as a functional marker in the experiments presented in chapter 4 could allow for a more accurate measure of cytotoxicity. The absence of a functional marker in a panel can result in inconclusive data. This is exemplified by a study in which the frequency of HIV-specific cells secreting IFN-γ and IL-2 correlated positively with CD4 count in HIV infected individuals, whereas IFN-γ secretion alone did not[401]. An incomplete functional panel could partly explain why NK cells isolated from individuals with genetic combinations that were shown to be associated with slower HIV disease progression, such as HLA-B Bw4-80I with KIR3DL1*h/*y, did not appear to have superior functional potential, as characterized by IFN-γ, CD107a, and TNF-α detection, compared to NK cells isolated from HLA-Bw6 homozygous individuals.

IV. NK CELL STIMULATION

In section IIIA of chapter 1, the controversy surrounding the increased or decreased functional capacity of NK cells in HIV infected individuals was partly explained by the use of different NK cell stimuli in different studies. The K562 cell line, used as a stimulus in chapter 4, was isolated in 1970 from a 53-year-old female suffering from chronic myelogenous leukemia[402]. It does not express HLA molecules and fails to inhibit NK cells by interacting with HLA-binding ITIM-containing receptors. Stimulation with K562 targets therefore mimics the outcome of an interaction with most tumors or virally infected cells, which often have decreased cell-surface expression of HLA molecules. While methodologically convenient and conceptually useful, the interpretation

of experiments using K562 as a stimulus for NK cells must consider certain potential pitfalls.

Firstly, K562 stimulation of NK cells is not receptor specific. While KIR3DL1 signaling should be affected by the lack of HLA-B on K562 cells, several other inhibitory NK cell receptors do not find their ligand on the leukemia cell line. This is the case for KIR2DL1, KIR2DL2 and KIR2DL3, which bind HLA-C molecules, and KIR3DL2, which binds HLA-A*3 and HLA-A*11[225]. Therefore the NK cell activation induced by K562 cells results from compounded signals, or rather the lack thereof, transmitted through several NK cell receptors. To circumvent this issue, Yu et al. resorted to flow-based cell identification[251]. Using monoclonal antibodies against several KIR molecules, they were able to exclude NK cells expressing KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, ILT-2 and NKG2A and positively select for KIR3DL1 or KIR3DS1 expressing NK cells. This strategy allowed Yu et al. to more specifically study the effect of KIR3DL1 or KIR3DS1 expression on NK cell function in response to stimulation with MHC-devoid targets at the exclusion of the effect of most other MHC class I receptors. However, antibodies against all KIRs are not yet available. In addition, several of the currently available antibodies are cross-reactive to two or three KIR molecules. For example, the Z27 clone binds both KIR3DS1 and KIR3DL1 while the CH-L clone binds KIR2DL2, KIR2DL3 and KIR2DS2[251,403]. Therefore, selection of NK cells based on KIR expression is, for the moment, technically difficult and incomplete. Alternatively, K562 cells transfected with HLA-A, -C, -E and -G, but not HLA-B could be used to stimulate NK cells. This would provide NK cell stimulation specifically as a result of decreased HLA-B expression. The caveat of this approach would be that the

HLA genotype of the transfected clones should be matched to every individual tested to mimic pre-existing KIR-HLA interactions *in vivo*.

K562 stimulation of NK cells also differs in several points from the events that occur upon the encounter of an HIV-infected cell. As previously discussed, HLA-A and -B are downregulated in HIV infection, but some residual expression is maintained[306]. In addition, other HLA molecules such as HLA-E and HLA-C are maintained at the cell surface[306]. Finally, infected cells should present HLA molecules loaded with HIV antigens in the peptide-binding groove at the cell surface, which affect KIR-HLA interactions[232]. These factors all combine to make the HIV-infected cells stimulus considerably different from the K562 NK stimulus. To understand the effect the KIR3DS/L1 locus has on HIV disease progression, viral load and resistance from infection, it may be more appropriate to measure NK cell function following stimulation with autologous $CD4^+$ T cells infected with HIV. Alter et al. studied the inhibition of viral replication capacity of NK cells isolated from individuals with the combined KIR3DS1 and HLA-B Bw4-80I genotype by co-incubating their NK cells with HIV infected $CD4^+$ T cells[310]. The HIV virion production, as measured by ELISA assay for the p24 protein, was compared between $CD4^+$ T cells alone or co-incubated with NK cells. Because their measure of NK cell potency was limited to inhibition of viral replication, Alter et al. were not able to determine which function(s) (i.e. cytotoxicity, cytokine secretion, chemokine secretion) was (were) responsible for the superior viremic control demonstrated by NK cells isolated from individuals with the combined genotype. Perhaps modifications to the assay used by Alter et al., to include flow cytometry-based

characterization of NK cell function, would clarify whether tri-functionality is indeed linked to HIV control.

Although not shown in chapter 4, analysis of NK cell function in response to K562 stimulation was also performed for individuals that are KIR3DS1 homozygous. This genotype was linked to the EU phenotype in chapter 2 and an increased NK cell functional profile was hypothesized. However, NK cells from KIR3DS1 homozygote individuals were not found to be functionally different than those from HLA-Bw6 homozygote controls. Preliminary data suggests that NK cells from KIR3DS1 homozygotes may be more potent inhibitors of HIV replication *in vitro* (see Appendix I). Because it has been suggested that the ligand for KIR3DS1 is an HIV-peptide-bound HLA-B Bw4-80I molecule, this observation further supports the importance of studying NK cell function in the context of HIV-specific stimulation[319].

V. ANIMAL MODELS

EUs provide a model in which immune responses contributing to the control of HIV may be uncovered. However, for practical and ethical considerations, there are limitations in the type of experiments than can be carried out in human populations. In order to better understand the role that the KIR3DS/L1 locus may have in HIV resistance, animal models would be useful.

Animal EU models have been developed in Rhesus macaques[404-406]. These usually involve SIV low-dose mucosal inoculations. Using the Rhesus macaque model, virus-specific T-cell responses were detected in exposed seronegative animals, duplicating observations made in human EU cohorts[327,328,340,357,363,405,406]. KIR and MHC molecules

are found in macaques. However, genetic information concerning KIR haplotypes is currently incomplete. Additional knowledge will be gained from the completion of macaque genome projects as well as characterization of haplotypes from several animals[407]. Recently, Bostik et al. sequenced KIR3DL, which has 74-77% amino acid identity to human KIR3DL1, in 38 SIV-infected Rhesus macaques[408,409]. They defined 14 different KIR3DL alleles, which differed in cell surface expression levels. Therefore, the polymorphic nature of KIR3DL is also found in macaques. Interestingly, certain KIR3DL polymorphisms in Rhesus macaques were associated with increased or decreased levels of SIV plasma viral loads[408]. This suggests that Rhesus macaques may provide an animal model in which the mechanism linking the KIR3DS/L1 locus to decreased HIV susceptibility in humans could be studied. In such a context, the number of viral exposures, viral and host genetics could be more easily controlled and host cell samples would be more readily accessible. Perhaps it would be possible to evaluate whether certain KIR3DL alleles are associated with decreased susceptibility to SIV infection and whether these affect NK cell polyfunctionality.

Mice with genetic defects have often been used for immunological studies. The NOG mouse, which has the NOD (non-obese diabetic), SCID (severe combined immunodeficiency) and γ_c (common gamma chain, found in several cytokine receptors) mutations, lacks functional T-, B- and NK cells[410]. It therefore tolerates engraftment of human tissue, such as peripheral blood mononuclear cells, which are susceptibile to subsequent HIV infection. Humanized mice can therefore be used to evaluate the effect of compounds or infused cells on HIV replication. In these animals, measuring the effects

of transfused NK cells expressing specific KIR3DS/L1 alleles on viral load could further provide evidence as to the role of KIR3DS/L1 in HIV protection.

VI. NK CELL MEMORY?

Innate and adaptive immunity are largely distinguished on the basis that cells belonging to the latter are capable of immune memory, in addition to their evolving receptors. Immunological memory is characterized by the rapid expansion of antigen-specific cells following a second encounter, allowing for long-term protection against a pathogen[280]. NK cells are normally considered innate immune cells because the receptors controlling their immunological response are genetically inherited. However, a recent study by Sun et al. demonstrates that NK cells may have some features of memory[411].

In mice, the MCMV protein m157 is a known ligand for Ly49H, an activating NK cell receptor with DAP-12 dependent intracellular signaling[412]. In addition, $Ly49H^+$ NK cells are protective against MCMV infection[413,414]. In DAP-12 deficient mice, Sun et al. showed that the population of $Ly49H^+$ NK cells transferred from DAP-12 competent mice specifically expanded in response to MCMV infection. This 'virus experienced' NK cell population was maintained for an extended period of time and showed increased expression of Ly49H receptor and capacity to produce IFN-γ when compared to 'virus naïve' $Ly49H^+$ NK cells isolated from the same DAP-12 competent mouse. Finally, adoptive transfer of 'virus experienced' $Ly49H^+$ NK cells into a second DAP-12 deficient mouse was tenfold more protective against MCMV-induced death than transfer of the 'virus naïve' counterpart. Antigen-specific expansion, rapid and stronger recall responses and increased protection are all features normally characteristic of a memory response.

NK cell 'memory' may also exist in humans. If the interaction between KIR3DS/L1 and their ligands is strongly modulated by the presence of HIV peptides in the HLA peptide-binding groove, then perhaps NK cell 'memory' could provide a mechanistic explanation for the decreased susceptibility to infection observed in individuals with specific KIR3DS/L1 alleles. A primary exposure to HIV following high-risk behavior could induce expansion and stimulation of NK cells bearing protective KIR alleles. Upon subsequent exposures, the protective NK cell population would already be 'primed' and provide additional protection against HIV infection. To test this hypothesis, it will be important to (1) find the ligand for KIR3DS1, (2) determine the effect of HIV peptides on the interaction of different KIR3DS/L1 alleles with their ligands, (3) determine the level of activity of KIR3DS/L1^{+} NK cells in EUs compared to unexposed individuals, (4) utilize animal models of infection such as Rhesus macaques or humanized mice to confirm the increased protective effect from 'virus experienced' NK cells.

VII. THERAPEUTIC OPPORTUNITIES

Section III of chapter 1 describes the relationship between HIV and NK cells. HIV infection alters the phenotype of NK cells, rendering them suboptimal for HIV control by affecting different aspects of the cell's function (summarized in figure 26)[271]. On the other hand there is strong epidemiological and *in vitro* evidence demonstrating that NK cells can kill infected cells, inhibit viral replication and influence the rate of disease progression[150,151,191,287,305]. Therefore, if NK cell function can be therapeutically restored

in HIV infected individuals, it may be possible to achieve some measurable clinical benefits.

Several therapeutics strategies, shown in figure 27, that harness the potential of NK cells have been developed[415]. These include the adoptive transfer of alloreactive NK cells, the alteration of NK cell receptor signaling, administration of NK cell

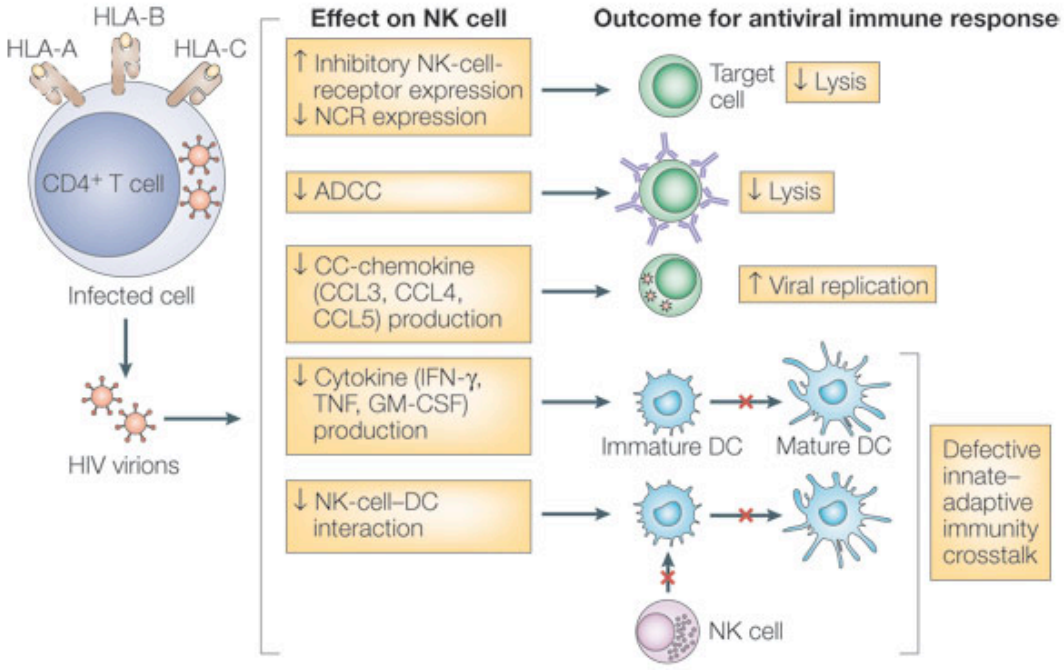


Figure 26[271]**:** Effects of HIV infection on NK cell function. (Reproduced by permission from Macmillan Publishers Ltd: Nature Reviews Immunolgy, Fauci et al, 5(11): 835-43, 2005).

suppressors or activators and modulation of NK/DC crosstalk. In a clinical setting, most of these strategies have been developed for cancer therapy. For example, in 2005, Miller et al. demonstrated that a successful engraftment of NK cells isolated from a mismatched KIR donor (i.e. the graft recipient lacked MHC molecules capable of acting as ligands for recipient KIR) increased remission rates in patients with acute myeloid leukemia who had poor prognosis[189]. In these patients, the graft versus leukemia effect was strong because

NK cell function could not be inhibited through KIR signaling. Another example of NK cell based therapy is the monoclonal antibody rituximab. Specific for CD20, a molecule found on B-cells, rituximab is used for the treatment of non-Hodgkin lymphomas and functions by mediating NK cell and monocyte ADCC against the tumor.

Therapeutic strategies based on modulating NK cell function in infectious settings are only still at an early stage of development. *In vitro* experiments have shown that addition of IL-21 to NK cells isolated from HIV infected individuals increased NK cell perforin secretion[416]. Also, Gupta et al. developed a fusion protein that is capable of inducing NK cell mediated ADCC by binding both HIV gp120 and CD16[417]. Similarly, direct KIR3DS/L1 stimulation in HIV-infected individuals with the goal of inducing NK cell functional profiles observed in EUs may lead to better control of viremia. These strategies provide interesting HIV treatment options that warrant further investigation. It is likely that such an approach would not cure HIV infection but may provide a novel treatment option. The most attractive NK cell targeted therapeutic strategy in the case of HIV infection is probably the modulation of NK-DC crosstalk. It is thought that by modulating the interaction between these cells, perhaps by adding the right kind of adjuvants in a HIV-vaccine, the qualitative function of both these cells would be improved. Given the known impact of DC and NK cells on the development of adaptive immune responses (see section IID, chapter 1), the modulation of these cells' function could theoretically result in qualitatively better vaccine-induced HIV-specific T cell responses. Research on the manipulation of NK-DC crosstalk is in its infancy. For example, vaccination of melanoma patients with tumor-derived heat-shock protein gp96 has successfully resulted in both increased NK and T-cell responses, suggesting

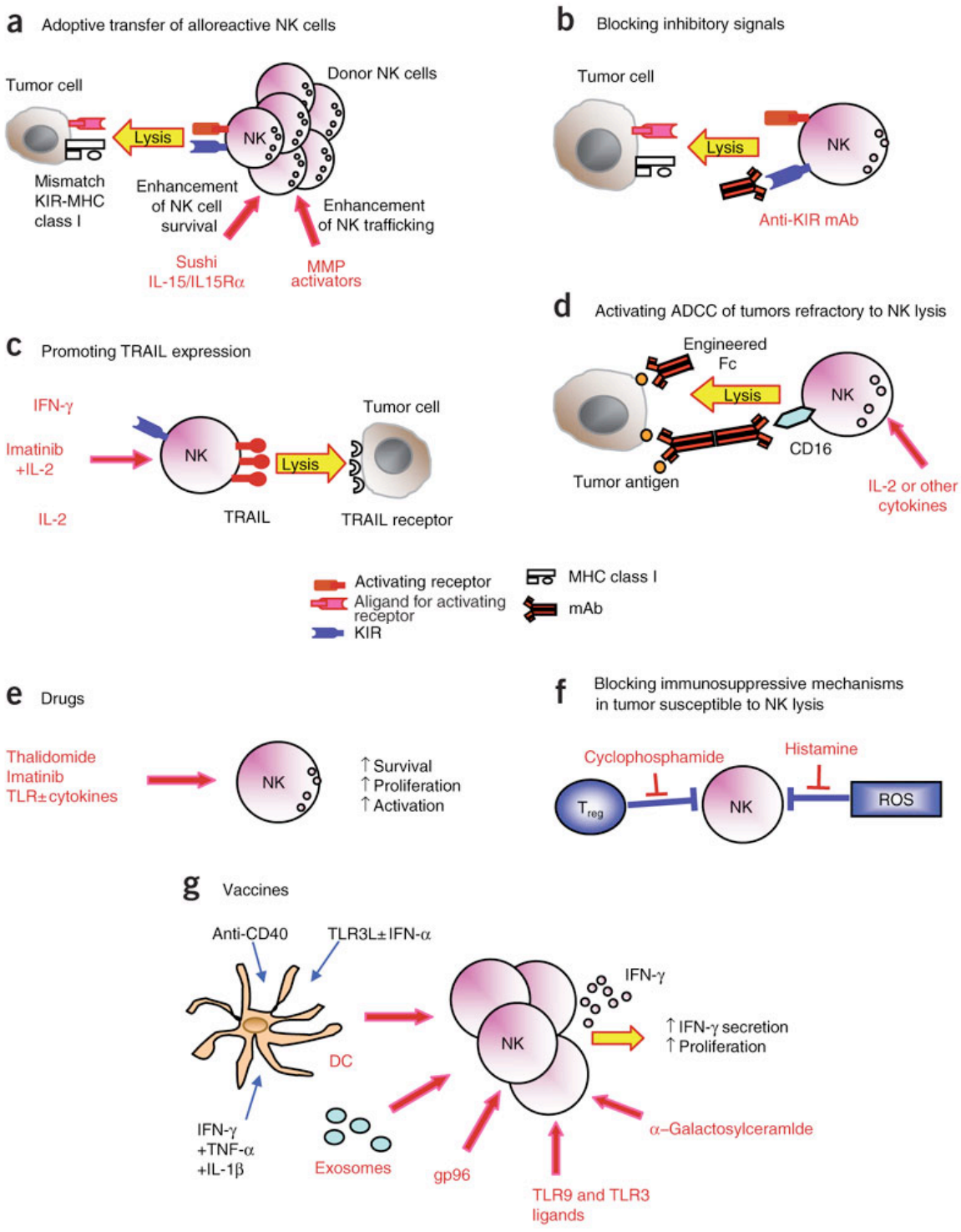


Figure 27: Therapeutic strategies harnessing NK cell function. Red-arrows depict NK cell stimulation. Blue-arrows depict DC stimulation. Substances that act on NK cells are depicted in red. ROS; reactive oxygen species. (Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology, Terme et al. 9(5): 486-94, 2008.

a possible link between NK cell activity and adaptive immune response following vaccination[418]. However, whether the NK cell responses were involved in shaping the anti-tumor T-cell response was not directly demonstrated. Also, NK cell mediated lysis of antigen-loaded targets increases cross-presentation of antigen in DCs and robustly induces antigen-specific adaptive immune responses[255]. The biological mechanism linking KIR3DS/L1 to slower HIV-disease progression, diminished plasma viral loads and decreased susceptibility to infection is poorly understood. Once it is clarified, however, whether or not adjuvant-directed stimulation of KIR3DS/L1 would lead to improved HIV-specific adaptive immunity may become an active area of investigation in the field of HIV vaccines. The study of NK cells in EU could provide a better understanding of their effect on adaptive immunity. HIV-specific adaptive immune responses have been repeatedly observed in EUs[326,355,356,360,365]. Additionally, there is increasing evidence for a role of NK cells in decreased susceptibility in EUs[152,385,386,419,420]. The possibility that there could be a link between KIR genotype, NK cell function, DC maturation and development of adaptive immune responses in EUs is an interesting hypothesis. Characterizing the functional profile of NK cells isolated from KIR genotyped EUs that develop HIV-specific adaptive immune responses may reveal a possible association between NK cells and adaptive immunity in EUs. Alternatively, *in vitro* co-incubation of activated NK cells with autologous DC can be used to measure the DC maturation-inducing (increased cell surface expression of CD80 or CD83; induction of TNF-α or IFN-γ secretion) capacity of NK cells[267]. Subsequently, the NK cell-exposed DCs can be co-incubated with autologous T-cells to measure their effect on these cells[421]. While indirect, if NK cells isolated from KIR3DS1 homozygotes or

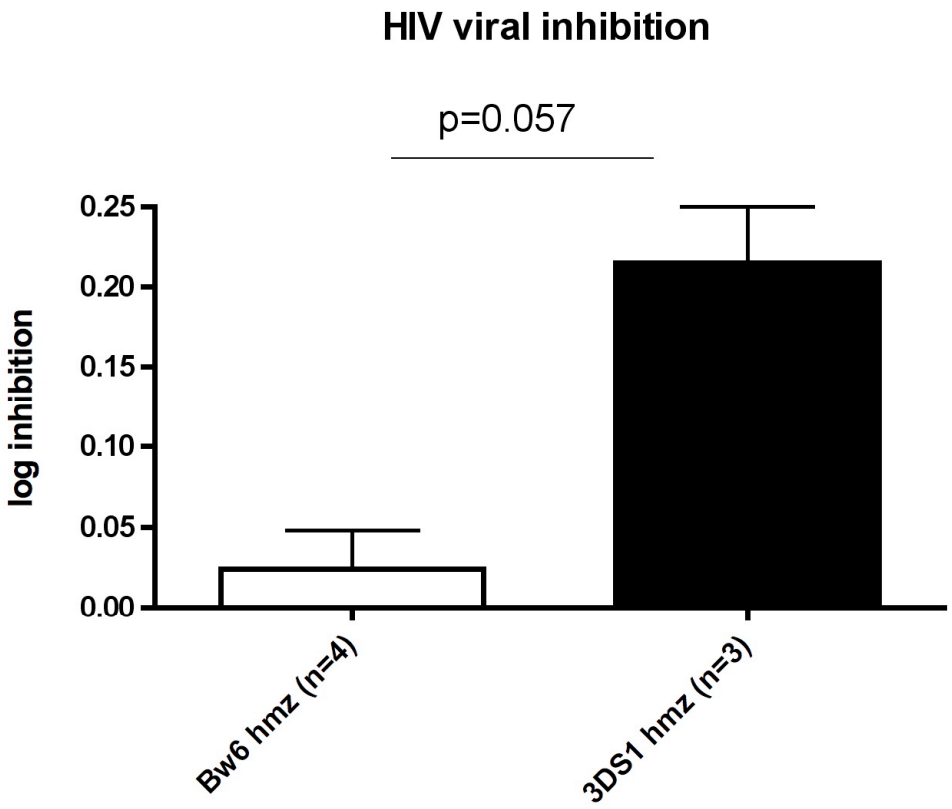
KIR3DL1*h/*y+B*57 individuals induce greater DC maturation and, in turn, greater T-cell activation, it would provide evidence for a link between NK cells and adaptive immunity in EUs.

VIII. CONCLUSIONS

In this thesis, we demonstrate that certain KIR3DS/L1 genotypes are overrepresented in the EU population we screened. In addition, we show that one of these genotypes, HLA-B*57+KIR3DL1*h/*y, is linked with increased NK cell function. Therefore, the evidence presented in this thesis further supports the role of NK cells, and particularly of the KIR3DS/L1 locus, in the decreased susceptibility to HIV infection observed in EUs. As cells that are directly involved in viral suppression and that interact with both the adaptive and innate arm of the immune system, NK cell offer several therapeutic opportunities against HIV. Because B- and T-cell responses are thought to be crucial to the design of successful vaccines, scientific efforts aimed at describing the immune response to HIV have been largely focused on investigating the role these cells have in fighting the virus. The failure of recent HIV vaccine trials has underlined the lack understanding of the correlates of immune protection against HIV and suggests that, perhaps, a better understanding of the anti-HIV immune response provided by other cells of the immune system is required to successfully curb the HIV pandemic.

Appendices

Appendix I: Supplemental data



Effect of KIR3DS1 homozygosity on HIV inhibition. HIV-infected CD4 + T cells were co-incubated or not with autologous NK cells for 15 days. On the 15th day, HIV replication was evaluated by measuring p24 in the culture supernatant by ELISA. Viral inhibition was calculated by measuring the decrease in p24 production in NK cell co-cultures when compared to cultures with infected CD4 T cells only. The capacity to inhibit HIV replication was compared between three KIR3DS1 homozygous (3DS1 hmz) individuals and four Bw6 homozygous (Bw6 hmz) controls. Although preliminary, statistical comparison (Mann Whitney test) indicates a strong non-statistically significant trend towards the increased capacity of NK cells from KIR3DS1 homozygous individuals to inhibit HIV viral replication *in vitro.*

References

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

2. Masur H, Michelis MA, Greene JB, et al. An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. N Engl J Med. 1981;305:1431-1438.

3. Pneumocystis pneumonia--Los Angeles. MMWR Morb Mortal Wkly Rep. 1981;30:250-252.

4. Update on acquired immune deficiency syndrome (AIDS)--United States. MMWR Morb Mortal Wkly Rep. 1982;31:507-508, 513-504.

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

6. Gallo RC, Sarin PS, Gelmann EP, et al. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science. 1983;220:865-867.

7. Prusiner SB. Historical essay. Discovering the cause of AIDS. Science. 2002;298:1726.

8. Ottmann M, Innocenti P, Thenadey M, Micoud M, Pelloquin F, Seigneurin JM. The polymerase chain reaction for the detection of HIV-1 genomic RNA in plasma from infected individuals. J Virol Methods. 1991;31:273-283.

9. Piatak M, Jr., Saag MS, Yang LC, et al. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science. 1993;259:1749-1754.

10. Asjo B, Morfeldt-Manson L, Albert J, et al. Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. Lancet. 1986;2:660-662.

11. Weiss SH, Goedert JJ, Gartner S, et al. Risk of human immunodeficiency virus (HIV-1) infection among laboratory workers. Science. 1988;239:68-71.

12. Siliciano R. Acquired Immune Deficiency Syndrome (AIDS). Encyclopedia of life science. London: Nature Publishing Group; 2001.

13. UNAIDS. Declaration of commitment on HIV/AIDS: five years later. Report of the Secretary-General. Vol. 2009; 2006.

14. UNAIDS. 2008 Report on the global AIDS epidemic. Vol. 2008; 2008.

15. Cohen MS, Hellmann N, Levy JA, DeCock K, Lange J. The spread, treatment, and prevention of HIV-1: evolution of a global pandemic. J Clin Invest. 2008;118:1244-1254.

16. Touloumi G, Hatzakis A. Natural history of HIV-1 infection. Clin Dermatol. 2000;18:389-399.

17. Epidemiology of HIV/AIDS--United States, 1981-2005. MMWR Morb Mortal Wkly Rep. 2006;55:589-592.

18. Simon V, Ho DD, Abdool Karim Q. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet. 2006;368:489-504.

19. Quinn TC. HIV epidemiology and the effects of antiviral therapy on long-term consequences. AIDS. 2008;22 Suppl 3:S7-12.

20. Kallings LO. The first postmodern pandemic: 25 years of HIV/ AIDS. J Intern Med. 2008;263:218-243.

21. HIV/AIDS Epi Updates. National HIV Prevalence and Incidence Estimates for 2005: Public Health Agency of Canada; 2007.

22. Frohlich KL, Ross N, Richmond C. Health disparities in Canada today: some evidence and a theoretical framework. Health Policy. 2006;79:132-143.

23. Holmes EC. On the origin and evolution of the human immunodeficiency virus (HIV). Biol Rev Camb Philos Soc. 2001;76:239-254.

24. Wainberg MA. HIV-1 subtype distribution and the problem of drug resistance. AIDS. 2004;18 Suppl 3:S63-68.

25. Heeney JL, Dalgleish AG, Weiss RA. Origins of HIV and the evolution of resistance to AIDS. Science. 2006;313:462-466.

26. Daniel MD, Letvin NL, King NW, et al. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. Science. 1985;228:1201-1204.

27. Letvin NL, Daniel MD, Sehgal PK, et al. Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. Science. 1985;230:71-73.

28. Schneider J, Hunsmann G. Simian lentiviruses--the SIV group. AIDS. 1988;2:1-9.

29. Hahn BH, Shaw GM, De Cock KM, Sharp PM. AIDS as a zoonosis: scientific and public health implications. Science. 2000;287:607-614.

30. Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature. 1989;339:389-392.

31. Gao F, Bailes E, Robertson DL, et al. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature. 1999;397:436-441.

32. Bailes E, Gao F, Bibollet-Ruche F, et al. Hybrid origin of SIV in chimpanzees. Science. 2003;300:1713.

33. Hirsch VM, Dapolito G, Goeken R, Campbell BJ. Phylogeny and natural history of the primate lentiviruses, SIV and HIV. Curr Opin Genet Dev. 1995;5:798-806.

34. Worobey M, Santiago ML, Keele BF, et al. Origin of AIDS: contaminated polio vaccine theory refuted. Nature. 2004;428:820.

35. Worobey M, Gemmel M, Teuwen DE, et al. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. Nature. 2008;455:661-664.

36. Peterlin BM, Trono D. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat Rev Immunol. 2003;3:97-107.

37. White DO, Fenner, F.J. Medical Virology (ed 4th Edition): Academic Press; 1994.

38. Wang WK, Chen MY, Chuang CY, Jeang KT, Huang LM. Molecular biology of human immunodeficiency virus type 1. J Microbiol Immunol Infect. 2000;33:131-140.

39. Freed EO. HIV-1 replication. Somat Cell Mol Genet. 2001;26:13-33.

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

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

42. Este JA, Telenti A. HIV entry inhibitors. Lancet. 2007;370:81-88.

43. Bjorndal A, Deng H, Jansson M, et al. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J Virol. 1997;71:7478-7487.

44. Burleigh L, Lozach PY, Schiffer C, et al. Infection of dendritic cells (DCs), not DC-SIGN-mediated internalization of human immunodeficiency virus, is required for long-term transfer of virus to T cells. J Virol. 2006;80:2949-2957.

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

46. Livingstone WJ, Moore M, Innes D, Bell JE, Simmonds P. Frequent infection of peripheral blood CD8-positive T-lymphocytes with HIV-1. Edinburgh Heterosexual Transmission Study Group. Lancet. 1996;348:649-654.

47. Kwon DS, Gregorio G, Bitton N, Hendrickson WA, Littman DR. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. Immunity. 2002;16:135-144.

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

49. Hu WS, Bowman EH, Delviks KA, Pathak VK. Homologous recombination occurs in a distinct retroviral subpopulation and exhibits high negative interference. J Virol. 1997;71:6028-6036.

50. Bukrinsky MI, Sharova N, Dempsey MP, et al. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci U S A. 1992;89:6580-6584.

51. Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell. 2000;101:173-185.

52. Gallay P, Hope T, Chin D, Trono D. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc Natl Acad Sci U S A. 1997;94:9825-9830.

53. Bukrinsky MI, Haggerty S, Dempsey MP, et al. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature. 1993;365:666-669.

54. Sherman MP, de Noronha CM, Heusch MI, Greene S, Greene WC. Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr. J Virol. 2001;75:1522-1532.

55. Chen H, Engelman A. The barrier-to-autointegration protein is a host factor for HIV type 1 integration. Proc Natl Acad Sci U S A. 1998;95:15270-15274.

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

57. Taube R, Fujinaga K, Wimmer J, Barboric M, Peterlin BM. Tat transactivation: a model for the regulation of eukaryotic transcriptional elongation. Virology. 1999;264:245-253.

58. Kao SY, Calman AF, Luciw PA, Peterlin BM. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature. 1987;330:489-493.

59. Wei P, Garber ME, Fang SM, Fischer WH, Jones KA. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell. 1998;92:451-462.

60. Price DH. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol Cell Biol. 2000;20:2629-2634.

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

62. Gottlinger HG, Dorfman T, Sodroski JG, Haseltine WA. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc Natl Acad Sci U S A. 1991;88:3195-3199.

63. Berkowitz R, Fisher J, Goff SP. RNA packaging. Curr Top Microbiol Immunol. 1996;214:177-218.

64. Bukrinskaya AG. HIV-1 assembly and maturation. Arch Virol. 2004;149:1067-1082.

65. Quinn TC, Wawer MJ, Sewankambo N, et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. N Engl J Med. 2000;342:921-929.

66. Chan DJ. Pathophysiology of HIV-1 in semen: current evidence for compartmentalisation and penetration by antiretroviral drugs. Curr HIV Res. 2005;3:207-222.

67. Thea DM, Steketee RW, Pliner V, et al. The effect of maternal viral load on the risk of perinatal transmission of HIV-1. New York City Perinatal HIV Transmission Collaborative Study Group. AIDS. 1997;11:437-444.

68. Lehman DA, Farquhar C. Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. Rev Med Virol. 2007;17:381-403.

69. Musey L, Hughes J, Schacker T, Shea T, Corey L, McElrath MJ. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. N Engl J Med. 1997;337:1267-1274.

70. Galvin SR, Cohen MS. The role of sexually transmitted diseases in HIV transmission. Nat Rev Microbiol. 2004;2:33-42.

71. Pilcher CD, Tien HC, Eron JJ, Jr., et al. Brief but efficient: acute HIV infection and the sexual transmission of HIV. J Infect Dis. 2004;189:1785-1792.

72. Royce RA, Sena A, Cates W, Jr., Cohen MS. Sexual transmission of HIV. N Engl J Med. 1997;336:1072-1078.

73. Gray RH, Wawer MJ, Brookmeyer R, et al. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. Lancet. 2001;357:1149-1153.

74. Vittinghoff E, Douglas J, Judson F, McKirnan D, MacQueen K, Buchbinder SP. Per-contact risk of human immunodeficiency virus transmission between male sexual partners. Am J Epidemiol. 1999;150:306-311.

75. de Vincenzi I. A longitudinal study of human immunodeficiency virus transmission by heterosexual partners. European Study Group on Heterosexual Transmission of HIV. N Engl J Med. 1994;331:341-346.

76. Monzon OT, Capellan JM. Female-to-female transmission of HIV. Lancet. 1987;2:40-41.

77. Rothenberg RB, Scarlett M, del Rio C, Reznik D, O'Daniels C. Oral transmission of HIV. AIDS. 1998;12:2095-2105.

78. Weber J, Desai K, Darbyshire J. The development of vaginal microbicides for the prevention of HIV transmission. PLoS Med. 2005;2:e142.

79. Cutler B, Justman J. Vaginal microbicides and the prevention of HIV transmission. Lancet Infect Dis. 2008;8:685-697.

80. Quinn TC. Circumcision and HIV transmission. Curr Opin Infect Dis. 2007;20:33-38.

81. Williams BG, Lloyd-Smith JO, Gouws E, et al. The potential impact of male circumcision on HIV in Sub-Saharan Africa. PLoS Med. 2006;3:e262.

82. Sawires SR, Dworkin SL, Fiamma A, Peacock D, Szekeres G, Coates TJ. Male circumcision and HIV/AIDS: challenges and opportunities. Lancet. 2007;369:708-713.

83. Nduati R, John G, Mbori-Ngacha D, et al. Effect of breastfeeding and formula feeding on transmission of HIV-1: a randomized clinical trial. JAMA. 2000;283:1167-1174.

84. Thior I, Lockman S, Smeaton LM, et al. Breastfeeding plus infant zidovudine prophylaxis for 6 months vs formula feeding plus infant zidovudine for 1 month to reduce mother-to-child HIV transmission in Botswana: a randomized trial: the Mashi Study. JAMA. 2006;296:794-805.

85. Coovadia H, Kindra G. Breastfeeding to prevent HIV transmission in infants: balancing pros and cons. Curr Opin Infect Dis. 2008;21:11-15.

86. Guay LA, Musoke P, Fleming T, et al. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. Lancet. 1999;354:795-802.

87. Mathers BM, Degenhardt L, Phillips B, et al. Global epidemiology of injecting drug use and HIV among people who inject drugs: a systematic review. Lancet. 2008;372:1733-1745.

88. Kaplan EH, Heimer R. HIV prevalence among intravenous drug users: model-based estimates from New Haven's legal needle exchange. J Acquir Immune Defic Syndr. 1992;5:163-169.

89. Des Jarlais DC. Structural interventions to reduce HIV transmission among injecting drug users. AIDS. 2000;14 Suppl 1:S41-46.

90. Valdiserri RO, Ogden LL, McCray E. Accomplishments in HIV prevention science: implications for stemming the epidemic. Nat Med. 2003;9:881-886.

91. Ensuring Blood Safety in Africa: World Health Organization (WHO), Geneva; 2001.

92. Donegan E, Stuart M, Niland JC, et al. Infection with human immunodeficiency virus type 1 (HIV-1) among recipients of antibody-positive blood donations. Ann Intern Med. 1990;113:733-739.

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

94. Cohen DE, Walker BD. Human immunodeficiency virus pathogenesis and prospects for immune control in patients with established infection. Clin Infect Dis. 2001;32:1756-1768.

95. Stevenson M. HIV-1 pathogenesis. Nat Med. 2003;9:853-860.

96. Addo MM, Draenert R, Rathod A, et al. Fully differentiated HIV-1 specific CD8+ T effector cells are more frequently detectable in controlled than in progressive HIV-1 infection. PLoS ONE. 2007;2:e321.

97. Iweala OI. HIV diagnostic tests: an overview. Contraception. 2004;70:141-147.

98. Forster SM. Diagnosing HIV infection. Clin Med. 2003;3:203-205.

99. Kessler HA, Blaauw B, Spear J, Paul DA, Falk LA, Landay A. Diagnosis of human immunodeficiency virus infection in seronegative homosexuals presenting with an acute viral syndrome. JAMA. 1987;258:1196-1199.

100. Busch MP, Lee LL, Satten GA, et al. Time course of detection of viral and serologic markers preceding human immunodeficiency virus type 1 seroconversion: implications for screening of blood and tissue donors. Transfusion. 1995;35:91-97.

101. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol. 1994;68:6103-6110.

102. Koup RA, Safrit JT, Cao Y, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol. 1994;68:4650-4655.

103. Mellors JW, Munoz A, Giorgi JV, et al. Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. Ann Intern Med. 1997;126:946-954.

104. Giorgi JV, Hultin LE, McKeating JA, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. J Infect Dis. 1999;179:859-870.

105. Liu Z, Hultin LE, Cumberland WG, et al. Elevated relative fluorescence intensity of CD38 antigen expression on CD8+ T cells is a marker of poor prognosis in HIV infection: results of 6 years of follow-up. Cytometry. 1996;26:1-7.

106. Mowat AM, Viney JL. The anatomical basis of intestinal immunity. Immunol Rev. 1997;156:145-166.

107. Li Q, Duan L, Estes JD, et al. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. Nature. 2005;434:1148-1152.

108. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature. 2005;434:1093-1097.

109. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006;12:1365-1371.

110. Pandrea IV, Gautam R, Ribeiro RM, et al. Acute loss of intestinal CD4+ T cells is not predictive of simian immunodeficiency virus virulence. J Immunol. 2007;179:3035-3046.

111. Douek DC, Roederer M, Koup RA. Emerging Concepts in the Immunopathogenesis of AIDS. Annu Rev Med. 2008.

112. Phillips AN. CD4 lymphocyte depletion prior to the development of AIDS. AIDS. 1992;6:735-736.

113. Goedert JJ, Kessler CM, Aledort LM, et al. A prospective study of human immunodeficiency virus type 1 infection and the development of AIDS in subjects with hemophilia. N Engl J Med. 1989;321:1141-1148.

114. From the Centers for Disease Control and Prevention. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. JAMA. 1993;269:729-730.

115. Gail MH, Tan WY, Pee D, Goedert JJ. Survival after AIDS diagnosis in a cohort of hemophilia patients. Multicenter Hemophilia Cohort Study. J Acquir Immune Defic Syndr Hum Retrovirol. 1997;15:363-369.

116. Ezzell C. AZT given the green light for clinical treatment of AIDS. Nature. 1987;326:430.

117. Sluis-Cremer N, Arion D, Parniak MA. Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). Cell Mol Life Sci. 2000;57:1408-1422.

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

119. Ren J, Stammers DK. Structural basis for drug resistance mechanisms for non-nucleoside inhibitors of HIV reverse transcriptase. Virus Res. 2008;134:157-170.

120. FDA. Drugs Used in the Treatment of HIV Infection. Vol. 2009; 2009.

121. Lataillade M, Kozal MJ. The hunt for HIV-1 integrase inhibitors. AIDS Patient Care STDS. 2006;20:489-501.

122. Crum NF, Riffenburgh RH, Wegner S, et al. Comparisons of causes of death and mortality rates among HIV-infected persons: analysis of the pre-, early, and late HAART (highly active antiretroviral therapy) eras. J Acquir Immune Defic Syndr. 2006;41:194-200.

123. Murphy EL, Collier AC, Kalish LA, et al. Highly active antiretroviral therapy decreases mortality and morbidity in patients with advanced HIV disease. Ann Intern Med. 2001;135:17-26.

124. Kaufmann D, Pantaleo G, Sudre P, Telenti A. CD4-cell count in HIV-1-infected individuals remaining viraemic with highly active antiretroviral therapy (HAART). Swiss HIV Cohort Study. Lancet. 1998;351:723-724.

125. Hirsch M, Steigbigel R, Staszewski S, et al. A randomized, controlled trial of indinavir, zidovudine, and lamivudine in adults with advanced human immunodeficiency virus type 1 infection and prior antiretroviral therapy. J Infect Dis. 1999;180:659-665.

126. Montessori V, Press N, Harris M, Akagi L, Montaner JS. Adverse effects of antiretroviral therapy for HIV infection. CMAJ. 2004;170:229-238.

127. Chariot P, Drogou I, de Lacroix-Szmania I, et al. Zidovudine-induced mitochondrial disorder with massive liver steatosis, myopathy, lactic acidosis, and mitochondrial DNA depletion. J Hepatol. 1999;30:156-160.

128. Nunez M. Hepatotoxicity of antiretrovirals: incidence, mechanisms and management. J Hepatol. 2006;44:S132-139.

129. Bonfanti P, Capetti A, Rizzardini G. HIV disease treatment in the era of HAART. Biomed Pharmacother. 1999;53:93-105.

130. Mannheimer S, Friedland G, Matts J, Child C, Chesney M. The consistency of adherence to antiretroviral therapy predicts biologic outcomes for human immunodeficiency virus-infected persons in clinical trials. Clin Infect Dis. 2002;34:1115-1121.

131. Paterson DL, Swindells S, Mohr J, et al. Adherence to protease inhibitor therapy and outcomes in patients with HIV infection. Ann Intern Med. 2000;133:21-30.

132. Bangsberg DR, Moss AR, Deeks SG. Paradoxes of adherence and drug resistance to HIV antiretroviral therapy. J Antimicrob Chemother. 2004;53:696-699.

133. Garcia-Lerma JG, Heneine W. Resistance of human immunodeficiency virus type 1 to reverse transcriptase and protease inhibitors: genotypic and phenotypic testing. J Clin Virol. 2001;21:197-212.

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

135. Fisher K, Feuer, C., Finley, B., Harrison, P., Vuthoori, S., Warren, M. Abstract TUAE0101: Building a comprehensive response: funding for HIV vaccines, microbicides and other new prevention tools: 2000 to 2007. XVII International AIDS Conference. Mexico City; 2008.

136. Gaschen B, Taylor J, Yusim K, et al. Diversity considerations in HIV-1 vaccine selection. Science. 2002;296:2354-2360.

137. Walker BD, Burton DR. Toward an AIDS vaccine. Science. 2008;320:760-764.

138. Barouch DH. Challenges in the development of an HIV-1 vaccine. Nature. 2008;455:613-619.

139. Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis. 2005;191:654-665.

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

141. McElrath MJ, De Rosa SC, Moodie Z, et al. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. Lancet. 2008;372:1894-1905.

142. Perreau M, Pantaleo G, Kremer EJ. Activation of a dendritic cell-T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells. J Exp Med. 2008;205:2717-2725.

143. Pantaleo G. HIV-1 T-cell vaccines: evaluating the next step. Lancet Infect Dis. 2008;8:82-83.

144. Miedema F. A brief history of HIV vaccine research: stepping back to the drawing board? AIDS. 2008;22:1699-1703.

145. Medzhitov R, Littman D. HIV immunology needs a new direction. Nature. 2008;455:591.

146. Levine AJ. Why do we not yet have a human immunodeficiency virus vaccine? J Virol. 2008;82:11998-12000.

147. Andoniou CE, Coudert JD, Degli-Esposti MA. Killers and beyond: NK-cell-mediated control of immune responses. Eur J Immunol. 2008;38:2938-2942.

148. Belz G, Mount A, Masson F. Dendritic cells in viral infections. Handb Exp Pharmacol. 2009:51-77.

149. McCoy CE, O'Neill LA. The role of toll-like receptors in macrophages. Front Biosci. 2008;13:62-70.

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

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

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

153. Hellstrom IE, Hellstrom KE, Pierce GE, Bill AH. Demonstration of cell-bound and humoral immunity against neuroblastoma cells. Proc Natl Acad Sci U S A. 1968;60:1231-1238.

154. Hellstrom I, Hellstrom KE, Sjogren HO, Warner GA. Demonstration of cell-mediated immunity to human neoplasms of various histological types. Int J Cancer. 1971;7:1-16.

155. Fossati G, Colnaghi MI, Porta GD, Cascinelli N, Veronesi U. Cellular and humoral immunity against human malignant melanoma. Int J Cancer. 1971;8:344-350.

156. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer. 1975;16:230-239.

157. Cudkowicz G, Bennett M. Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F 1 hybrid mice. J Exp Med. 1971;134:1513-1528.

158. Lanier LL, Phillips JH, Hackett J, Jr., Tutt M, Kumar V. Natural killer cells: definition of a cell type rather than a function. J Immunol. 1986;137:2735-2739.

159. Herberman RB. Natural killer cells. Annu Rev Med. 1986;37:347-352.

160. Alter G, Altfeld M. NK cell function in HIV-1 infection. Curr Mol Med. 2006;6:621-629.

161. Farag SS, Caligiuri MA. Human natural killer cell development and biology. Blood Rev. 2006;20:123-137.

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

163. Alter G, Teigen N, Davis BT, et al. Sequential deregulation of NK cell subset distribution and function starting in acute HIV-1 infection. Blood. 2005;106:3366-3369.

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

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

166. Orange JS, Ballas ZK. Natural killer cells in human health and disease. Clin Immunol. 2006;118:1-10.

167. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med. 1989;320:1731-1735.

168. Wendland T, Herren S, Yawalkar N, Cerny A, Pichler WJ. Strong alpha beta and gamma delta TCR response in a patient with disseminated Mycobacterium avium infection and lack of NK cells and monocytopenia. Immunol Lett. 2000;72:75-82.

169. Bernard F, Picard C, Cormier-Daire V, et al. A novel developmental and immunodeficiency syndrome associated with intrauterine growth retardation and a lack of natural killer cells. Pediatrics. 2004;113:136-141.

170. Etzioni A, Eidenschenk C, Katz R, Beck R, Casanova JL, Pollack S. Fatal varicella associated with selective natural killer cell deficiency. J Pediatr. 2005;146:423-425.

171. Notarangelo LD, Mazzolari E. Natural killer cell deficiencies and severe varicella infection. J Pediatr. 2006;148:563-564; author reply 564.

172. Eidenschenk C, Dunne J, Jouanguy E, et al. A novel primary immunodeficiency with specific natural-killer cell deficiency maps to the centromeric region of chromosome 8. Am J Hum Genet. 2006;78:721-727.

173. Ballas ZK, Turner JM, Turner DA, Goetzman EA, Kemp JD. A patient with simultaneous absence of "classical" natural killer cells (CD3-, CD16+, and NKH1+) and expansion of CD3+, CD4-, CD8-, NKH1+ subset. J Allergy Clin Immunol. 1990;85:453-459.

174. Akiba H, Motoki Y, Satoh M, Iwatsuki K, Kaneko F. Recalcitrant trichophytic granuloma associated with NK-cell deficiency in a SLE patient treated with corticosteroid. Eur J Dermatol. 2001;11:58-62.

175. Fleisher G, Starr S, Koven N, Kamiya H, Douglas SD, Henle W. A non-x-linked syndrome with susceptibility to severe Epstein-Barr virus infections. J Pediatr. 1982;100:727-730.

176. Komiyama A, Kawai H, Yabuhara A, et al. Natural killer cell immunodeficiency in siblings: defective killing in the absence of natural killer cytotoxic factor activity in natural killer and lymphokine-activated killer cytotoxicities. Pediatrics. 1990;85:323-330.

177. Gazit R, Garty BZ, Monselise Y, et al. Expression of KIR2DL1 on the entire NK cell population: a possible novel immunodeficiency syndrome. Blood. 2004;103:1965-1966.

178. Komiyama A, Kawai H, Yamada S, et al. A killing defect of natural killer cells with the absence of natural killer cytotoxic factors in a child with Hodgkin's disease. Blood. 1987;69:1686-1690.

179. Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. Proc Natl Acad Sci U S A. 2000;97:2731-2736.

180. Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol. 2001;13:459-463.

181. Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer. 2002;2:850-861.

182. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene. 2008;27:5932-5943.

183. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410:1107-1111.

184. Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest. 2007;117:1137-1146.

185. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet. 2000;356:1795-1799.

186. Coca S, Perez-Piqueras J, Martinez D, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer. 1997;79:2320-2328.

187. Ishigami S, Natsugoe S, Tokuda K, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer. 2000;88:577-583.

188. Villegas FR, Coca S, Villarrubia VG, et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer. 2002;35:23-28.

189. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105:3051-3057.

190. Huard B, Fruh K. A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells. Eur J Immunol. 2000;30:509-515.

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

192. Brooks CR, Elliott T, Parham P, Khakoo SI. The inhibitory receptor NKG2A determines lysis of vaccinia virus-infected autologous targets by NK cells. J Immunol. 2006;176:1141-1147.

193. Vankayalapati R, Garg A, Porgador A, et al. Role of NK cell-activating receptors and their ligands in the lysis of mononuclear phagocytes infected with an intracellular bacterium. J Immunol. 2005;175:4611-4617.

194. Orange JS, Wang B, Terhorst C, Biron CA. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. J Exp Med. 1995;182:1045-1056.

195. Liu ZX, Govindarajan S, Okamoto S, Dennert G. NK cells cause liver injury and facilitate the induction of T cell-mediated immunity to a viral liver infection. J Immunol. 2000;164:6480-6486.

196. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science. 2004;305:872-874.

197. Croy BA, Ashkar AA, Minhas K, Greenwood JD. Can murine uterine natural killer cells give insights into the pathogenesis of preeclampsia? J Soc Gynecol Investig. 2000;7:12-20.

198. Guimond MJ, Wang B, Croy BA. Engraftment of bone marrow from severe combined immunodeficient (SCID) mice reverses the reproductive deficits in natural killer cell-deficient tg epsilon 26 mice. J Exp Med. 1998;187:217-223.

199. Ashkar AA, Croy BA. Interferon-gamma contributes to the normalcy of murine pregnancy. Biol Reprod. 1999;61:493-502.

200. Guimond M, Wang B, Croy BA. Immune competence involving the natural killer cell lineage promotes placental growth. Placenta. 1999;20:441-450.

201. Santoni A, Zingoni A, Cerboni C, Gismondi A. Natural killer (NK) cells from killers to regulators: distinct features between peripheral blood and decidual NK cells. Am J Reprod Immunol. 2007;58:280-288.

202. Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol. 2002;2:656-663.

203. Le Bouteiller P, Piccinni MP. Human NK cells in pregnant uterus: why there? Am J Reprod Immunol. 2008;59:401-406.

204. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11:237-244.

205. Trinchieri G, Santoli D. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. J Exp Med. 1978;147:1314-1333.

206. Becker S, Kiessling R, Lee N, Klein G. Modulation of sensitivity to natural killer cell lysis after in vitro explantation of a mouse lymphoma. J Natl Cancer Inst. 1978;61:1495-1498.

207. Welsh RM, Karre K, Hansson M, Kunkel LA, Kiessling RW. Interferon-mediated protection of normal and tumor target cells against lysis by mouse natural killer cells. J Immunol. 1981;126:219-225.

208. Gidlund M, Orn A, Pattengale PK, Jansson M, Wigzell H, Nilsson K. Natural killer cells kill tumour cells at a given stage of differentiation. Nature. 1981;292:848-850.

209. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986;319:675-678.

210. Ljunggren HG, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J Exp Med. 1985;162:1745-1759.

211. Kumar V, McNerney ME. A new self: MHC-class-I-independent natural-killer-cell self-tolerance. Nat Rev Immunol. 2005;5:363-374.

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

213. Dimasi N, Biassoni R. Structural and functional aspects of the Ly49 natural killer cell receptors. Immunol Cell Biol. 2005;83:1-8.

214. Natarajan K, Dimasi N, Wang J, Mariuzza RA, Margulies DH. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. Annu Rev Immunol. 2002;20:853-885.

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

216. Vales-Gomez M, Reyburn HT, Erskine RA, Lopez-Botet M, Strominger JL. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. EMBO J. 1999;18:4250-4260.

217. Michaelsson J, Teixeira de Matos C, Achour A, Lanier LL, Karre K, Soderstrom K. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. J Exp Med. 2002;196:1403-1414.

218. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. Nat Rev Immunol. 2003;3:304-316.

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

220. Cosman D, Fanger N, Borges L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity. 1997;7:273-282.

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

222. Lepin EJ, Bastin JM, Allan DS, et al. Functional characterization of HLA-F and binding of HLA-F tetramers to ILT2 and ILT4 receptors. Eur J Immunol. 2000;30:3552-3561.

223. Navarro F, Llano M, Bellon T, Colonna M, Geraghty DE, Lopez-Botet M. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. Eur J Immunol. 1999;29:277-283.

224. Colonna M, Navarro F, Bellon T, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med. 1997;186:1809-1818.

225. Bashirova AA, Martin MP, McVicar DW, Carrington M. The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. Annu Rev Genomics Hum Genet. 2006;7:277-300.

226. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu Rev Immunol. 2002;20:217-251.

227. Kikuchi-Maki A, Catina TL, Campbell KS. Cutting edge: KIR2DL4 transduces signals into human NK cells through association with the Fc receptor gamma protein. J Immunol. 2005;174:3859-3863.

228. Uhrberg M, Parham P, Wernet P. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. Immunogenetics. 2002;54:221-229.

229. Martin AM, Freitas EM, Witt CS, Christiansen FT. The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. Immunogenetics. 2000;51:268-280.

230. Wilson MJ, Torkar M, Haude A, et al. Plasticity in the organization and sequences of human KIR/ILT gene families. Proc Natl Acad Sci U S A. 2000;97:4778-4783.

231. EMBL-EBI. IPD - KIR database. Vol. 2009.

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

233. Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J Exp Med. 2006;203:633-645.

234. Thomas R, Yamada E, Alter G, et al. Novel KIR3DL1 alleles and their expression levels on NK cells: convergent evolution of KIR3DL1 phenotype variation? J Immunol. 2008;180:6743-6750.

235. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. J Exp Med. 1994;180:1235-1242.

236. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. Blood. 2005;105:4416-4423.

237. Dorfman JR, Raulet DH. Acquisition of Ly49 receptor expression by developing natural killer cells. J Exp Med. 1998;187:609-618.

238. Biassoni R. Human natural killer receptors, co-receptors, and their ligands. Curr Protoc Immunol. 2009;Chapter 14:Unit 14 10.

239. Li H, Pascal V, Martin MP, Carrington M, Anderson SK. Genetic control of variegated KIR gene expression: polymorphisms of the bi-directional KIR3DL1 promoter are associated with distinct frequencies of gene expression. PLoS Genet. 2008;4:e1000254.

240. Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity. 1997;7:739-751.

241. Draghi M, Pashine A, Sanjanwala B, et al. NKp46 and NKG2D recognition of infected dendritic cells is necessary for NK cell activation in the human response to influenza infection. J Immunol. 2007;178:2688-2698.

242. Santourlidis S, Trompeter HI, Weinhold S, et al. Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. J Immunol. 2002;169:4253-4261.

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

244. Pascal V, Stulberg MJ, Anderson SK. Regulation of class I major histocompatibility complex receptor expression in natural killer cells: one promoter is not enough! Immunol Rev. 2006;214:9-21.

245. Davies GE, Locke SM, Wright PW, et al. Identification of bidirectional promoters in the human KIR genes. Genes Immun. 2007;8:245-253.

246. Kawasaki H, Taira K. Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature. 2004;431:211-217.

247. Shilling HG, Young N, Guethlein LA, et al. Genetic control of human NK cell repertoire. J Immunol. 2002;169:239-247.

248. Raulet DH, Held W, Correa I, Dorfman JR, Wu MF, Corral L. Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. Immunol Rev. 1997;155:41-52.

249. Williams NS, Kubota A, Bennett M, Kumar V, Takei F. Clonal analysis of NK cell development from bone marrow progenitors in vitro: orderly acquisition of receptor gene expression. Eur J Immunol. 2000;30:2074-2082.

250. Raulet DH, Vance RE. Self-tolerance of natural killer cells. Nat Rev Immunol. 2006;6:520-531.

251. Yu J, Heller G, Chewning J, Kim S, Yokoyama WM, Hsu KC. Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. J Immunol. 2007;179:5977-5989.

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

253. Raulet DH. Missing self recognition and self tolerance of natural killer (NK) cells. Semin Immunol. 2006;18:145-150.

254. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.

255. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008;9:503-510.

256. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol. 2006;6:940-952.

257. Orange JS. Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol. 2008;8:713-725.

258. Smyth MJ, Cretney E, Kelly JM, et al. Activation of NK cell cytotoxicity. Mol Immunol. 2005;42:501-510.

259. Smyth MJ, Trapani JA. Granzymes: exogenous proteinases that induce target cell apoptosis. Immunol Today. 1995;16:202-206.

260. Kagi D, Ledermann B, Burki K, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature. 1994;369:31-37.

261. Krensky AM, Clayberger C. Granulysin: a novel host defense molecule. Am J Transplant. 2005;5:1789-1792.

262. Peters PJ, Borst J, Oorschot V, et al. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med. 1991;173:1099-1109.

263. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003;281:65-78.

264. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods. 2004;294:15-22.

265. Robertson MJ, Soiffer RJ, Wolf SF, et al. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. J Exp Med. 1992;175:779-788.

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

267. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med. 2002;195:327-333.

268. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. J Exp Med. 2002;195:335-341.

269. Andrews DM, Scalzo AA, Yokoyama WM, Smyth MJ, Degli-Esposti MA. Functional interactions between dendritic cells and NK cells during viral infection. Nat Immunol. 2003;4:175-181.

270. Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat Rev Immunol. 2005;5:112-124.

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

272. Martin-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol. 2004;5:1260-1265.

273. Gao N, Jennings P, Yuan D. Requirements for the natural killer cell-mediated induction of IgG1 and IgG2a expression in B lymphocytes. Int Immunol. 2008;20:645-657.

274. Takeda K, Dennert G. The development of autoimmunity in C57BL/6 lpr mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. J Exp Med. 1993;177:155-164.

275. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol. 1999;17:189-220.

276. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004;75:163-189.

277. Levy DE, Garcia-Sastre A. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. Cytokine Growth Factor Rev. 2001;12:143-156.

278. Havell EA. Evidence that tumor necrosis factor has an important role in antibacterial resistance. J Immunol. 1989;143:2894-2899.

279. Pfeffer K, Matsuyama T, Kundig TM, et al. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell. 1993;73:457-467.

280. Janeway CT, P; Walport, M; Capra, JD. Immunobiology: The Immune System in Health and Disease (ed 4th). New York: Elsevier Science/Garland Publishing; 1999.

281. Alter G, Teigen N, Ahern R, et al. Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. J Infect Dis. 2007;195:1452-1460.

282. Alter G, Altfeld M. NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. J Intern Med. 2009;265:29-42.

283. Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science. 1999;283:857-860.

284. Allen TM, Altfeld M, Geer SC, et al. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. J Virol. 2005;79:13239-13249.

285. Allen TM, O'Connor DH, Jing P, et al. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature. 2000;407:386-390.

286. Lifson JD, Nowak MA, Goldstein S, et al. The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. J Virol. 1997;71:9508-9514.

287. Kottilil S, Shin K, Planta M, et al. Expression of chemokine and inhibitory receptors on natural killer cells: effect of immune activation and HIV viremia. J Infect Dis. 2004;189:1193-1198.

288. Azzoni L, Papasavvas E, Chehimi J, et al. Sustained impairment of IFN-gamma secretion in suppressed HIV-infected patients despite mature NK cell recovery: evidence for a defective reconstitution of innate immunity. J Immunol. 2002;168:5764-5770.

289. Parato KG, Kumar A, Badley AD, et al. Normalization of natural killer cell function and phenotype with effective anti-HIV therapy and the role of IL-10. AIDS. 2002;16:1251-1256.

290. Mavilio D, Lombardo G, Benjamin J, et al. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. Proc Natl Acad Sci U S A. 2005;102:2886-2891.

291. Sirianni MC, Ensoli F, Alario C, et al. Distribution of the natural killer-related receptor for HLA-C during highly active antiretroviral therapy for human immunodeficiency virus infection. Hum Immunol. 2001;62:1328-1334.

292. Ahmad R, Sindhu ST, Tran P, et al. Modulation of expression of the MHC class I-binding natural killer cell receptors, and NK activity in relation to viral load in HIV-infected/AIDS patients. J Med Virol. 2001;65:431-440.

293. Mela CM, Burton CT, Imami N, et al. Switch from inhibitory to activating NKG2 receptor expression in HIV-1 infection: lack of reversion with highly active antiretroviral therapy. AIDS. 2005;19:1761-1769.

294. De Maria A, Fogli M, Costa P, et al. The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural

cytotoxicity receptors (NKp46, NKp30 and NKp44). Eur J Immunol. 2003;33:2410-2418.

295. Baron GC, Klimas NG, Fischl MA, Fletcher MA. Decreased natural cell-mediated cytotoxicity per effector cell in acquired immunodeficiency syndrome. Diagn Immunol. 1985;3:197-204.

296. Poli G, Introna M, Zanaboni F, et al. Natural killer cells in intravenous drug abusers with lymphadenopathy syndrome. Clin Exp Immunol. 1985;62:128-135.

297. Alter G, Malenfant JM, Delabre RM, et al. Increased natural killer cell activity in viremic HIV-1 infection. J Immunol. 2004;173:5305-5311.

298. Vitale M, Caruso A, Licenziati S, et al. Differential production of IFN-gamma, analyzed at the single-cell level, by specific subsets of human NK and T cells from healthy and HIV(+) subjects. Cytometry. 2000;39:189-194.

299. Ironson G, Balbin E, Solomon G, et al. Relative preservation of natural killer cell cytotoxicity and number in healthy AIDS patients with low CD4 cell counts. AIDS. 2001;15:2065-2073.

300. Ahmad A, Morisset R, Thomas R, Menezes J. Evidence for a defect of antibody-dependent cellular cytotoxic (ADCC) effector function and anti-HIV gp120/41-specific ADCC-mediating antibody titres in HIV-infected individuals. J Acquir Immune Defic Syndr. 1994;7:428-437.

301. Sirianni MC, Soddu S, Malorni W, Arancia G, Aiuti F. Mechanism of defective natural killer cell activity in patients with AIDS is associated with defective distribution of tubulin. J Immunol. 1988;140:2565-2568.

302. Bonavida B, Katz J, Gottlieb M. Mechanism of defective NK cell activity in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. I. Defective trigger on NK cells for NKCF production by target cells, and partial restoration by IL 2. J Immunol. 1986;137:1157-1163.

303. Sirianni MC, De Sanctis G, Macchi B, et al. Natural killer activity from normal peripheral blood lymphocytes against a human T lymphotropic retrovirus type III (HTLV-III)-infected cell line. Diagn Clin Immunol. 1988;5:297-303.

304. Bandyopadhyay S, Ziegner U, Campbell DE, Miller DS, Hoxie JA, Starr SE. Natural killer cell-mediated lysis of T cell lines chronically infected with HIV-1. Clin Exp Immunol. 1990;79:430-435.

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

306. Cohen GB, Gandhi RT, Davis DM, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity. 1999;10:661-671.

307. Kottilil S. Natural killer cells in HIV-1 infection: role of NK cell-mediated non-cytolytic mechanisms in pathogenesis of HIV-1 infection. Indian J Exp Biol. 2003;41:1219-1225.

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

309. Fehniger TA, Herbein G, Yu H, et al. Natural killer cells from HIV-1+ patients produce C-C chemokines and inhibit HIV-1 infection. J Immunol. 1998;161:6433-6438.

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

311. Qi Y, Martin MP, Gao X, et al. KIR/HLA pleiotropism: protection against both HIV and opportunistic infections. PLoS Pathog. 2006;2:e79.

312. Barbour JD, Sriram U, Caillier SJ, Levy JA, Hecht FM, Oksenberg JR. Synergy or independence? Deciphering the interaction of HLA Class I and NK cell KIR alleles in early HIV-1 disease progression. PLoS Pathog. 2007;3:e43.

313. Lopez-Vazquez A, Mina-Blanco A, Martinez-Borra J, et al. Interaction between KIR3DL1 and HLA-B*57 supertype alleles influences the progression of HIV-1 infection in a Zambian population. Hum Immunol. 2005;66:285-289.

314. Biassoni R, Cantoni C, Falco M, et al. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. J Exp Med. 1996;183:645-650.

315. Muller CA, Engler-Blum G, Gekeler V, Steiert I, Weiss E, Schmidt H. Genetic and serological heterogeneity of the supertypic HLA-B locus specificities Bw4 and Bw6. Immunogenetics. 1989;30:200-207.

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

317. Stern M, Ruggeri L, Capanni M, Mancusi A, Velardi A. Human leukocyte antigens A23, A24, and A32 but not A25 are ligands for KIR3DL1. Blood. 2008;112:708-710.

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

319. Gillespie GM, Bashirova A, Dong T, McVicar DW, Rowland-Jones SL, Carrington M. Lack of KIR3DS1 binding to MHC class I Bw4 tetramers in complex with CD8+ T cell epitopes. AIDS Res Hum Retroviruses. 2007;23:451-455.

320. Norman PJ, Abi-Rached L, Gendzekhadze K, et al. Unusual selection on the KIR3DL1/S1 natural killer cell receptor in Africans. Nat Genet. 2007;39:1092-1099.

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

322. Altfeld M, Goulder P. 'Unleashed' natural killers hinder HIV. Nat Genet. 2007;39:708-710.

323. Lifson AR, Buchbinder SP, Sheppard HW, et al. Long-term human immunodeficiency virus infection in asymptomatic homosexual and bisexual men with normal CD4+ lymphocyte counts: immunologic and virologic characteristics. J Infect Dis. 1991;163:959-965.

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

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

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

327. Clerici M, Sison AV, Berzofsky JA, et al. Cellular immune factors associated with mother-to-infant transmission of HIV. AIDS. 1993;7:1427-1433.

328. Fowke KR, Nagelkerke NJ, Kimani J, et al. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. Lancet. 1996;348:1347-1351.

329. Paxton WA, Martin SR, Tse D, et al. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. Nat Med. 1996;2:412-417.

330. Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996;86:367-377.

331. O'Brien TR, Winkler C, Dean M, et al. HIV-1 infection in a man homozygous for CCR5 delta 32. Lancet. 1997;349:1219.

332. Biti R, Ffrench R, Young J, Bennetts B, Stewart G, Liang T. HIV-1 infection in an individual homozygous for the CCR5 deletion allele. Nat Med. 1997;3:252-253.

333. Heiken H, Becker S, Bastisch I, Schmidt RE. HIV-1 infection in a heterosexual man homozygous for CCR-5 delta32. AIDS. 1999;13:529-530.

334. Michael NL, Nelson JA, KewalRamani VN, et al. Exclusive and persistent use of the entry coreceptor CXCR4 by human immunodeficiency virus type 1 from a subject homozygous for CCR5 delta32. J Virol. 1998;72:6040-6047.

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

336. Martinson JJ, Chapman NH, Rees DC, Liu YT, Clegg JB. Global distribution of the CCR5 gene 32-basepair deletion. Nat Genet. 1997;16:100-103.

337. Stranford SA, Skurnick J, Louria D, et al. Lack of infection in HIV-exposed individuals is associated with a strong CD8(+) cell noncytotoxic anti-HIV response. Proc Natl Acad Sci U S A. 1999;96:1030-1035.

338. Rugeles MT, Solano F, Diaz FJ, Bedoya VI, Patino PJ. Molecular characterization of the CCR 5 gene in seronegative individuals exposed to human immunodeficiency virus (HIV). J Clin Virol. 2002;23:161-169.

339. Lockett SF, Alonso A, Wyld R, et al. Effect of chemokine receptor mutations on heterosexual human immunodeficiency virus transmission. J Infect Dis. 1999;180:614-621.

340. Bernard NF, Yannakis CM, Lee JS, Tsoukas CM. Human immunodeficiency virus (HIV)-specific cytotoxic T lymphocyte activity in HIV-exposed seronegative persons. J Infect Dis. 1999;179:538-547.

341. Mazzoli S, Trabattoni D, Lo Caputo S, et al. HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals. Nat Med. 1997;3:1250-1257.

342. Hutter G, Nowak D, Mossner M, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N Engl J Med. 2009;360:692-698.

343. Sidney J, Peters B, Frahm N, Brander C, Sette A. HLA class I supertypes: a revised and updated classification. BMC Immunol. 2008;9:1.

344. MacDonald KS, Fowke KR, Kimani J, et al. Influence of HLA supertypes on susceptibility and resistance to human immunodeficiency virus type 1 infection. J Infect Dis. 2000;181:1581-1589.

345. MacDonald KS, Embree JE, Nagelkerke NJ, et al. The HLA A2/6802 supertype is associated with reduced risk of perinatal human immunodeficiency virus type 1 transmission. J Infect Dis. 2001;183:503-506.

346. Liu C, Carrington M, Kaslow RA, et al. Association of polymorphisms in human leukocyte antigen class I and transporter associated with antigen processing genes with resistance to human immunodeficiency virus type 1 infection. J Infect Dis. 2003;187:1404-1410.

347. Rohowsky-Kochan C, Skurnick J, Molinaro D, Louria D. HLA antigens associated with susceptibility/resistance to HIV-1 infection. Hum Immunol. 1998;59:802-815.

348. Lockett SF, Robertson JR, Brettle RP, Yap PL, Middleton D, Leigh Brown AJ. Mismatched human leukocyte antigen alleles protect against heterosexual HIV transmission. J Acquir Immune Defic Syndr. 2001;27:277-280.

349. Sriwanthana B, Hodge T, Mastro TD, et al. HIV-specific cytotoxic T lymphocytes, HLA-A11, and chemokine-related factors may act synergistically to determine HIV resistance in CCR5 delta32-negative female sex workers in Chiang Rai, northern Thailand. AIDS Res Hum Retroviruses. 2001;17:719-734.

350. Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. Cytokine Growth Factor Rev. 2002;13:455-481.

351. Gonzalez E, Kulkarni H, Bolivar H, et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science. 2005;307:1434-1440.

352. Kuhn L, Schramm DB, Donninger S, et al. African infants' CCL3 gene copies influence perinatal HIV transmission in the absence of maternal nevirapine. AIDS. 2007;21:1753-1761.

353. Urban TJ, Weintrob AC, Fellay J, et al. CCL3L1 and HIV/AIDS susceptibility. Nat Med. 2009;15:1110-1112.

354. Miyazawa M, Lopalco L, Mazzotta F, Lo Caputo S, Veas F, Clerici M. The 'immunologic advantage' of HIV-exposed seronegative individuals. AIDS. 2009;23:161-175.

355. Rowland-Jones SL, Dong T, Fowke KR, et al. Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. J Clin Invest. 1998;102:1758-1765.

356. Alimonti JB, Kimani J, Matu L, et al. Characterization of CD8 T-cell responses in HIV-1-exposed seronegative commercial sex workers from Nairobi, Kenya. Immunol Cell Biol. 2006;84:482-485.

357. Makedonas G, Bruneau J, Lin H, Sekaly RP, Lamothe F, Bernard NF. HIV-specific CD8 T-cell activity in uninfected injection drug users is associated with maintenance of seronegativity. AIDS. 2002;16:1595-1602.

358. Truong LX, Luong TT, Scott-Algara D, et al. CD4 cell and CD8 cell-mediated resistance to HIV-1 infection in exposed uninfected intravascular drug users in Vietnam. AIDS. 2003;17:1425-1434.

359. Cheynier R, Langlade-Demoyen P, Marescot MR, et al. Cytotoxic T lymphocyte responses in the peripheral blood of children born to human immunodeficiency virus-1-infected mothers. Eur J Immunol. 1992;22:2211-2217.

360. Rowland-Jones SL, Nixon DF, Aldhous MC, et al. HIV-specific cytotoxic T-cell activity in an HIV-exposed but uninfected infant. Lancet. 1993;341:860-861.

361. Goh WC, Markee J, Akridge RE, et al. Protection against human immunodeficiency virus type 1 infection in persons with repeated exposure: evidence for T cell immunity in the absence of inherited CCR5 coreceptor defects. J Infect Dis. 1999;179:548-557.

362. Kebba A, Kaleebu P, Rowland S, et al. Distinct patterns of peripheral HIV-1-specific interferon- gamma responses in exposed HIV-1-seronegative individuals. J Infect Dis. 2004;189:1705-1713.

363. Kaul R, Dong T, Plummer FA, et al. CD8(+) lymphocytes respond to different HIV epitopes in seronegative and infected subjects. J Clin Invest. 2001;107:1303-1310.

364. Kaul R, Rowland-Jones SL, Kimani J, et al. Late seroconversion in HIV-resistant Nairobi prostitutes despite pre-existing HIV-specific CD8+ responses. J Clin Invest. 2001;107:341-349.

365. Kaul R, Trabattoni D, Bwayo JJ, et al. HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers. AIDS. 1999;13:23-29.

366. Fowke KR, Kaul R, Rosenthal KL, et al. HIV-1-specific cellular immune responses among HIV-1-resistant sex workers. Immunol Cell Biol. 2000;78:586-595.

367. Skurnick JH, Palumbo P, DeVico A, et al. Correlates of nontransmission in US women at high risk of human immunodeficiency virus type 1 infection through sexual exposure. J Infect Dis. 2002;185:428-438.

368. Beretta A, Furci L, Burastero S, et al. HIV-1-specific immunity in persistently seronegative individuals at high risk for HIV infection. Immunol Lett. 1996;51:39-43.

369. Jennes W, Vuylsteke B, Borget MY, et al. HIV-specific T helper responses and frequency of exposure among HIV-exposed seronegative female sex workers in Abidjan, Cote d'Ivoire. J Infect Dis. 2004;189:602-610.

370. Furci L, Scarlatti G, Burastero S, et al. Antigen-driven C-C chemokine-mediated HIV-1 suppression by CD4(+) T cells from exposed uninfected individuals expressing the wild-type CCR-5 allele. J Exp Med. 1997;186:455-460.

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

372. Lopalco L, Barassi C, Pastori C, et al. CCR5-reactive antibodies in seronegative partners of HIV-seropositive individuals down-modulate surface CCR5 in vivo and neutralize the infectivity of R5 strains of HIV-1 In vitro. J Immunol. 2000;164:3426-3433.

373. Lopalco L, Pastori C, Cosma A, et al. Anti-cell antibodies in exposed seronegative individuals with HIV type 1-neutralizing activity. AIDS Res Hum Retroviruses. 2000;16:109-115.

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

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

376. Devito C, Hinkula J, Kaul R, et al. Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects. J Acquir Immune Defic Syndr. 2002;30:413-420.

377. Devito C, Hinkula J, Kaul R, et al. Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate. AIDS. 2000;14:1917-1920.

378. Mazzoli S, Lopalco L, Salvi A, et al. Human immunodeficiency virus (HIV)-specific IgA and HIV neutralizing activity in the serum of exposed seronegative partners of HIV-seropositive persons. J Infect Dis. 1999;180:871-875.

379. Buchacz K, Parekh BS, Padian NS, et al. HIV-specific IgG in cervicovaginal secretions of exposed HIV-uninfected female sexual partners of HIV-infected men. AIDS Res Hum Retroviruses. 2001;17:1689-1693.

380. Dorrell L, Hessell AJ, Wang M, et al. Absence of specific mucosal antibody responses in HIV-exposed uninfected sex workers from the Gambia. AIDS. 2000;14:1117-1122.

381. Chiu YL, Greene WC. Multifaceted antiviral actions of APOBEC3 cytidine deaminases. Trends Immunol. 2006;27:291-297.

382. Newman EN, Holmes RK, Craig HM, et al. Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. Curr Biol. 2005;15:166-170.

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

384. Misse D, Yssel H, Trabattoni D, et al. IL-22 participates in an innate anti-HIV-1 host-resistance network through acute-phase protein induction. J Immunol. 2007;178:407-415.

385. Ravet S, Scott-Algara D, Bonnet E, et al. Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIV-exposed uninfected individuals. Blood. 2007;109:4296-4305.

386. Jennes W, Verheyden S, Demanet C, et al. Cutting edge: resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in the absence of their HLA ligands. J Immunol. 2006;177:6588-6592.

387. Trachtenberg E, Korber B, Sollars C, et al. Advantage of rare HLA supertype in HIV disease progression. Nat Med. 2003;9:928-935.

388. Guo GA, D.E. How is a statistical link established between a human outcome and a genetic variant? Sociological Methods & Research. 2008;37:201-226.

389. Balding DJB, M. Cannings, C. Handbook of Statistical Genetics. Vol. 2 (ed Third edition). Chichester: Jjohn Wiley & Sons, Ltd. ; 2007.

390. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005;5:201-214.

391. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. BMJ. 1995;310:170.

392. Perneger TV. What's wrong with Bonferroni adjustments. BMJ. 1998;316:1236-1238.

393. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature. 1996;382:722-725.

394. Parent RA, M. Morissette, C. Roy, E. Leclerc, P. Surveillance des maladies infectieuses chez les utilisateurs de drogue par injection. Épidémiologie du VIH de 1995 à 2006. Épidémiologie de 2003 à 2006. Montréal: Institut National de Santé Publique du Québec; 2008.

395. Morral AR, McCaffrey D, Iguchi MY. Hardcore drug users claim to be occasional users: drug use frequency underreporting. Drug Alcohol Depend. 2000;57:193-202.

396. Solbergsdottir E, Bjornsson G, Gudmundsson LS, Tyrfingsson T, Kristinsson J. Validity of self-reports and drug use among young people seeking treatment for substance abuse or dependence. J Addict Dis. 2004;23:29-38.

397. Fendrich M, Johnson TP, Sudman S, Wislar JS, Spiehler V. Validity of drug use reporting in a high-risk community sample: a comparison of cocaine and heroin survey reports with hair tests. Am J Epidemiol. 1999;149:955-962.

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

399. Darrah PA, Patel DT, De Luca PM, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med. 2007;13:843-850.

400. Makedonas G, Banerjee PP, Pandey R, et al. Rapid up-regulation and granule-independent transport of perforin to the immunological synapse define a novel mechanism of antigen-specific CD8+ T cell cytotoxic activity. J Immunol. 2009;182:5560-5569.

401. Peretz Y, Ndongala ML, Boulet S, et al. Functional T cell subsets contribute differentially to HIV peptide-specific responses within infected individuals: correlation of these functional T cell subsets with markers of disease progression. Clin Immunol. 2007;124:57-68.

402. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood. 1975;45:321-334.

403. Pascal V, Yamada E, Martin MP, et al. Detection of KIR3DS1 on the cell surface of peripheral blood NK cells facilitates identification of a novel null allele and assessment of KIR3DS1 expression during HIV-1 infection. J Immunol. 2007;179:1625-1633.

404. Letvin NL, Rao SS, Dang V, et al. No evidence for consistent virus-specific immunity in simian immunodeficiency virus-exposed, uninfected rhesus monkeys. J Virol. 2007;81:12368-12374.

405. Shacklett BL, Ling B, Veazey RS, et al. Boosting of SIV-specific T cell responses in rhesus macaques that resist repeated intravaginal challenge with SIVmac251. AIDS Res Hum Retroviruses. 2002;18:1081-1088.

406. Wilson LA, Murphey-Corb M, Martin LN, Harrison RM, Ratterree MS, Bohm RP. Identification of SIV env-specific CTL in the jejunal mucosa in vaginally exposed, seronegative rhesus macaques (Macaca mulatta). J Med Primatol. 2000;29:173-181.

407. Bimber B, O'Connor DH. KIRigami: the case for studying NK cell receptors in SIV+ macaques. Immunol Res. 2008;40:235-243.

408. Bostik P, Kobkitjaroen J, Tang W, et al. Decreased NK cell frequency and function is associated with increased risk of KIR3DL allele polymorphism in simian immunodeficiency virus-infected rhesus macaques with high viral loads. J Immunol. 2009;182:3638-3649.

409. Hershberger KL, Shyam R, Miura A, Letvin NL. Diversity of the killer cell Ig-like receptors of rhesus monkeys. J Immunol. 2001;166:4380-4390.

410. Nomura TW, T. Habu, S. Humanized Mice. Vol. 324. Berlin: Springer 2008.

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

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

413. Brown MG, Dokun AO, Heusel JW, et al. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. Science. 2001;292:934-937.

414. Daniels KA, Devora G, Lai WC, O'Donnell CL, Bennett M, Welsh RM. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. J Exp Med. 2001;194:29-44.

415. Terme M, Ullrich E, Delahaye NF, Chaput N, Zitvogel L. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. Nat Immunol. 2008;9:486-494.

416. Strbo N, de Armas L, Liu H, Kolber MA, Lichtenheld M, Pahwa S. IL-21 augments natural killer effector functions in chronically HIV-infected individuals. AIDS. 2008;22:1551-1560.

417. Gupta N, Arthos J, Khazanie P, et al. Targeted lysis of HIV-infected cells by natural killer cells armed and triggered by a recombinant immunoglobulin fusion protein: implications for immunotherapy. Virology. 2005;332:491-497.

418. Pilla L, Patuzzo R, Rivoltini L, et al. A phase II trial of vaccination with autologous, tumor-derived heat-shock protein peptide complexes Gp96, in combination with GM-CSF and interferon-alpha in metastatic melanoma patients. Cancer Immunol Immunother. 2006;55:958-968.

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

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

421. Fanales-Belasio E, Moretti S, Fiorelli V, et al. HIV-1 Tat addresses dendritic cells to induce a predominant Th1-type adaptive immune response that appears prevalent in the asymptomatic stage of infection. J Immunol. 2009;182:2888-2897.