NATURAL KILLER CELLS IN HIV-INFECTED SLOW PROGRESSORS

DOCTORAL THESIS

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ABBREVIATIONS

Acquired Immunodeficiency syndrome (AIDS)
Acute Infection Early Disease (AIED)
Adenovirus (Ad)
Antibody dependent cell-mediated viral inhibition (ADCVI) activity
Antibody dependent cellular cytotoxicity (ADCC)
Antigen presenting cells (APCs).
Antiretroviral (ARV)
Antiretroviral therapy (ART)
Arginine (R)
Azidothymidine (AZT)
Broadly neutralizing Abs (bNAbs)
Broadly neutralizing antibodies (bNAbs)
C-C chemokine receptor type 5 (CCR5)
C-X-C chemokine receptor type 4 (CXCR4)
Capsid (CA)
CC-chemokine receptor 7 (CCR7),
$CD4^+$ T-Helper 1 (Th1)
$CD4^+$ T-Helper 2 (Th2)
Cellular exportin -1 (CRM-1)
Centers for Disease Control and Prevention (CDC)
Chemokine Receptor Antagonists (CRA)
Chemokine receptor-7 (CCR7).
Chimpanzee simian immunodeficiency virus (SIVcpz)
Chronic Infection (CI)
Circulating recombinant forms (CRFs)
Cyclophilin A (CypA)
Cyclophillin A (Cyp A)
Cytomegalovirus (CMV)
Cytotoxic T-lymphocytes (CTLs)
Dendritic cell specific ICAM3-grabbing non-integrin (DC-SIGN),
Dendritic cells (DC)
Deoxyribonucleoside triphosphates (dNTPs)
Double stranded RNA (dsRNA)
E4PB4 (E4 nuclear binding protein 4)
Endosomal-sorting complex required for transport (ESCRT)
Envelope (Env)
Epstein Barr Virus (EBV)
Exposed uninfected (EU)
Fusion Inhibitors (FI)
Gag precursor ($Pr55^{Gag}$)
Gastrointestinal (GI)
Genome-wide association study (GWAS)

Glycine (G)
Gut associated Lymphoid Tissue (GALT)
Gut-associated lymphoid tissue (GALT)
Helical heptad repeat 1 (HR1)
Helical heptad repeat 2 (HR2)
Hepatitis C (HCV),
High mobility group (HM)
High mobility group protein (HMG)
Highly Active Antiretroviral Therapy (HAART)
HIV Controller Consortium (HIC)
HIV Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)
HIV Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI)
Homozygotes (hmz)
Host protein of 68 Kilodaltons (HP68)
Human immunodeficiency virus (HIV)
Immunogloblulin-(Ig)-
Immunoreceptor-tyrosine based activation motif (ITAM)-
Immunoreceptor-tyrosine based motif(s) (ITIM)
Inducer of short transcripts (IST)
Influenza (Flu)
Injection drug users (IDU)
Integrase (IN)
Integrase Inhibitors (II)
Killer Immunoglobulin-like Receptor (KIR)
Killer-immunoglobulin-like receptors (KIR)
leukocyte associated inhibitory receptor (LAIR)
leukocyte function associated antigen (LFA-1).
leukocyte Ig-like receptor family (LILR),
Leukocyte region complex (LRC)
Lipopolysaccharide (LPS
Long terminal repeats (LTR)
Lysine (K)
Macrophage inflammatory proteins (MIP)
Major histocompatibitlity complex (MHC)
Matrix (MA)
Mean fluorescence intensity (MFI)
Men who have sex with men (MSM)
Minus strong stop DNA (-) ssDNA)
Monocyte-derived macrophages (MDM)
Monocyte-derived macrophages (MDM)
Myeloid (mDC)
National Institutes of Health (NIH)
Natural cytotoxicity receptors (NCRs)
Natural Killer Cell (NK)
Negative factor (Nef)
Neutralizing antibodies (NAbs)

Neutralizing antibodies (NAbs)
Non-human primates (NHP)
Nuclear localization sequence (NLS)
Nuclear pore complexes (NPC)
Nucleocapsid (NC)
Opportunistic infections (OIs)
PAMP recognition receptors (PRR)
Plasma membrane (PM)
Plasma viral load (pVL)
Plasmacytoid (pDC)
Poly-purine tract (PPT)
Pre-integration complex (PIC)
Primary Infection (PI)
Primer binding site (PBS)
Programmed dealth (PD)-1
Protease (Pro)
Protease Inhibitors (PrI)
Regulated upon Activation, Normal T-cell Expressed and secreted (RANTES)
Regulator of Virion (Rev)
Regulatory T-cells
Rev response element (RRE)
Rev Response Element (RRE)
Reverse Transcriptase (RT)
Reverse transcription complex (RTC)
SH2 containing protein tyrosine phosphatase (SHP)
SH2-containing inositol polyphosphate 5-phosphatase (SHIP)
Simian immunodeficiency virus (SIV)
Slow progressrors (SP)
Sooty mangabey SIV (SIVsm)
Stromal derived factor-1 (SDF-1)
Surface plamon resonance (SPR)[1]
T-cell receptor (TCR)
Toll-like Receptor-9 (TLR9
Transactivation response element (TAR).
Transcriptional Transactivator (Tat)
Transforming growth factor-β (TGF-β)
Tumor necrosis factor-β (TNF-β)
Tumor susceptibility gene 101 (Tsg101)
Tumour necrosis factor alpha related apoptosis inducing lignand (TRAIL)
Type-1 interferons (IFN
Unique recombinant forms (URFs)
US Food and Drug Administration (US-FDA)
Viral infectivity factor (Vif)
Viral protein R (Vpr)
Viral Protein U (Vpu)
Viral replicative capacity (VRC)

Wild-type (WT)
Zidovudine (ZDV)

ABSTRACT

Acquired immunodeficiency syndrome AIDS-related illnesses are a leading cause of infectious disease mortality worldwide. The development of a safe and effective prophylactic HIV vaccine is an imperative global public health priority fraught with significant obstacles because the answers to fundamental immunological questions remain unknown. One such gap in knowledge includes the identification of the elusive correlates of immune protection against HIV.

Untreated HIV infection is characterized by severe dysregulation of the antiviral immune response that begins during the earliest stages of infection. A rare subset of HIV infected individuals demonstrate sustained ability to control HIV replication and/or maintain stable $CD4^+$ cell counts without therapy. Determining the genetic and immunological bases underlying their benign disease course will aid in the development of novel anti-viral strategies and suggest ways the immune system can be manipulated in a vaccination setting to support the development of protective immunity.

Epidemiological studies suggest that licensed NK cells may play a significant role in disease progression by associating the co-carriage of certain KIR/HLA combined genotypes with favorable disease outcomes. The projects described in this thesis provide a functional basis in support of these epidemiological data and contribute to our understanding of how interactions between protective HLA alleles and NK cell receptors may enhance the control of viremia by NK cells.

In chapter II, I investigate whether T-cell immune activation levels account for the heterogeneity in longitudinal changes in the rates of CD4 counts among HIV-infected elite controllers (EC) with undetectable viral load (VL) and demonstrate that EC with protective HLA or KIR/HLA combined genotypes exhibit elevated immune activation levels which may be indicative of beneficial antiviral immune responses. Chapters III and IV explore novel mechanisms through which licensed NK cells can influence HIV disease progression by demonstrating that KIR/HLA receptor-ligand combinations affect the NK cell functional potential of HIV infected slow progressors (SP).

As mediators of the innate and adaptive immune response, understanding the mechanisms that may underlie the development of protective immunity by NK cells is

key. The work presented in this thesis contributes to our understanding of how protective HLA alleles interact with NK cells to influence HIV pathogenesis and provide insights as to the type of immunity an HIV vaccine should recapitulate.

RESUME

Les maladies liées au syndrome de l'immunodéficience acquise (SIDA) sont principalement responsables de la mortalité par maladies infectieuses dans le monde. La mise au point d'un vaccin préventif sécuritaire et efficace contre le VIH reste un problème urgent et prioritaire détenant des obstacles majeurs pour la santé publique mondiale, car on ignore les réponses aux questions fondamentales sur l'immunologie. Un tel fossé dans la science inclut la détermination des corrélats indéfinissables quant à la protection immunitaire contre le VIH.

Une rare cohorte d'individus infectés par le VIH démontre une capacité continue de limiter la réplication du VIH et/ou de maintenir un taux stable de cellules $CD4^+$ sans traitement. Déterminer les bases génétiques et immunologiques qui sous-tendent la progression de leur maladie bénigne favorisera la création de stratégies antivirales inédites et évoquera des façons dont le système immunitaire peut être manipulé dans un contexte de vaccination dans le but d'appuyer le renforcement d'une immunité protectrice.

Une infection par le VIH non traitée se caractérise par une dérégulation sévère de la réponse immunitaire antivirale qui débute pendant les premières phases de l'infection. Les études épidémiologiques avancent que les cellules NK sont susceptibles de jouer un rôle important dans la progression de la maladie en associant l'expression de certaines combinaisons des génotypes KIR/HLA avec des résultats favorables de la maladie. Les projets décrits dans cette thèse fournissent une base utile en faveur des données épidémiologiques et contribuent à notre compréhension de la manière dont les interactions entre les allèles HLA et les récepteurs des cellules NK sont susceptibles d'améliorer le contrôle de virémie par les cellules NK.

Dans le chapitre II, j'examine si les niveaux d'activation immunitaire des cellules T expliquent l'hétérogénéité dans les changements longitudinaux des taux des lymphocytes CD4 parmi les « contrôleurs élite » (EC) ayant une charge virale indétectable et je démontre que ces derniers, qui possèdent des gènes HLA protecteurs ou une association des génotypes KIR/HLA, montrent des niveaux d'activation immunitaire élevés, ce qui indiqueraient que des réponses immunes antivirales sont bénéfiques. Les

chapitres III et IV fournissent à l'appui de cellules NK la progression lente de la maladie et démontrent que les combinaisons des récepteurs-ligands KIR/HLA influencent le potentiel fonctionnel des patients infectés du VIH à progression lente.

En tant que médiateurs des réponses immunitaires innées et adaptives, comprendre les mécanismes qui sous-tendent la progression de l'immunité protectrice des cellules NK est la clé. Le travail présenté dans cette thèse contribue à notre compréhension de la manière dont les allèles HLA interagissent avec les cellules NK pour influencer la pathogénèse du VIH et donnent un aperçu quant au type d'immunité qu'un vaccin contre le VIH devrait récapituler.

PREFACE

CHAPTER I: Association of T Cell Activation with carriage of protective HLA or KIR/HLA genotypes in HIV-infected Elite Controllers

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PK designed the study, designed and optimized the antibody panel, performed the experiments, analyzed the data and prepared the manuscript. SB aided in study design and critical review of manuscript. CMT, J-PR, RT, PC, M-RB, BL, RK, MO, CK, CLT followed the EC clinically and provided samples for the study. NFB designed the study and prepared manuscript

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CHAPTER II: Receptor-ligand requirements for increased NK cell poly-functional potential in *h/*y+B57 HIV-1 infected Slow progressors

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PK designed the study, performed the experiments, analyzed the data and prepared the manuscript. SB aided in study design, data analysis and critical review of manuscript. Samples were provided by following investigators of the Canadian Cohort of Slow Progressors, CMT, J-PR, RT, PC, M-RB, J-GB, CK, CLT, BL, DL, P-JM, DL, SV. BT, DR, HT, JF, MP, MBK and by SAM and MC. TJS and CB provided study materials. CLT, the Principal Investigator for the Canadian Cohort of HIV Infected Slow Progressors, provided clinical follow up information. NFB designed the study and prepared manuscript

CHAPTER III: Quantitative influence of inhibitory Killer-Immunoglobulin-like Receptors to self HLA-B and HLA-C ligands on NK cell poly-functional potential

Philomena Kamya, Benjamin Tallon, Stephen A. Migueles, Mark Connors, Cecile L. Tremblay, Nicole F. Bernard for the investigators of the Canadian Cohort of HIV Infected Slow Progressors.

Manuscript resubmitted for publication:

PK designed the study, performed the experiments, analyzed the data and prepared the manuscript. BT performed the KIR genotyping and data analysis. Samples were provided by investigators of the Canadian Cohort of HIV Infected Slow Progressors, SAM and MC. CLT, the Principal Investigator for the Canadian Cohort of HIV Infected Slow Progressors, provided clinical follow up information. NFB designed the study and prepared manuscript

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Nothing in the world can take the place of persistence.
Talent alone will not;
Genius alone will not;
Education alone will not;
Persistence and determination and *support* are omnipotent- Adapted from Calvin Coolidge

To my Mother and Father;
I thank you for creating a world of possibility and opportunity.
I thank you for supporting the choices and encouraging the journeys.
I thank you for the comfort and nurture that preserves my sanity, my grace and my fortitude.

To my Supervisor;
I thank you for your unwavering mentorship and guidance.
I thank you for patiently recognizing with intuitive sense; talent.

To my colleagues; George, Yoav, Michel and Salix.
I thank you for your respectful education of one whose learning curve was at best; steep.

To my Sisters and Brothers, Cousins and friends;
I thank you for allowing me to connect with real life in all its uncertainty and complexity.
I thank you for listening and understanding.
Your support fuels my passion.

CHAPTER ONE: INTRODUCTION

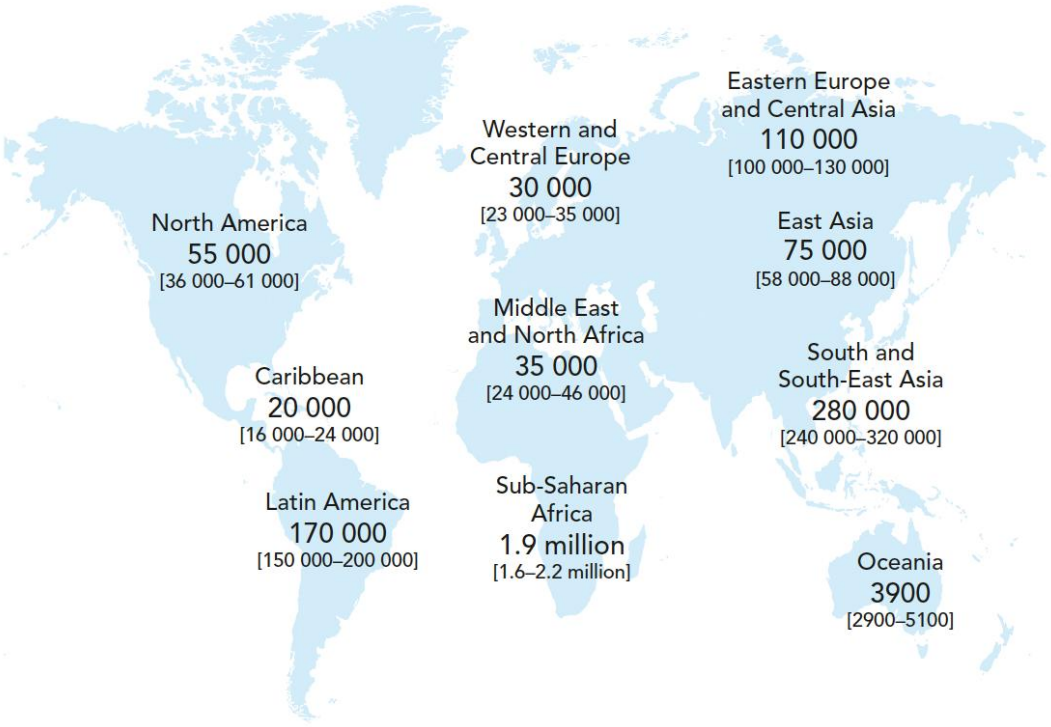
The Global HIV/AIDS pandemic

The first cases of acquired immune deficiency syndrome (AIDS) were recognized in the United States in 1981. By 1985, the human immunodeficiency virus (HIV) had been identified in every region of the world. In this thesis the use of the term HIV will refer to HIV-1. The latest available surveillance data from the Joint United Nations Program on HIV/AIDS (UNAIDS) estimates that 33.2 million (30.6-36.1 million) individuals were living with HIV infection or AIDS as of 2008, an increase from 29.5 million in 2001[1]. However, in this decade, the global prevalence of HIV-1 infection stabilized at 0.8%, and the annual incidence of new HIV infections declined from an estimated 3.0 million in 2001 to 2.7 million in 2007 (2.2-3.2 million).

Despite the dramatic increase in access to HIV antiretroviral (ARV) treatment that decreased the numbers of HIV-related deaths, AIDS is still one of the leading causes mortality among people aged 15-59 years of age and represents an extremely urgent public health challenge. Globally, of the percentage of HIV-infected individuals 50% are women[2]. Adolescents and young adults aged 15-24 years account for 45% of new infections worldwide. The expansion of access to ARV treatment has curtailed a substantial number of mother-child transmissions and led to a striking decline in cases of HIV among infants[3].

[1] www.unaids.org/global2010
[2] www.unaids.org/global/reference book
[3] www.unaids.org/globalreport2010

Figure 1: Estimated number of adults and children newly infected with HIV in 2008 adapted from Joint United Nations program on HIV/AIDS (UNAIDS) 2009 AIDS Epidemic update.



Regional epidemic

Sub-Saharan Africa

Global statistics can mask important regional HIV epidemiological heterogeneity. As seen in Figure 1, Sub-Saharan Africa bears the burden of HIV pandemic with 24.5 million (21.6-27.4 million) infected persons that account for 63% of global infections[4]. The HIV pandemic can be described in two broad patterns. A generalized heterosexual epidemic that afflicts sub-Saharan Africa and the Caribbean and in all other areas of the world an epidemic of marginalized peoples that disproportionally affects men who have sex with men (MSM) and sex workers both female and male. A substantial proportion of heterosexual HIV transmission in sub-Saharan Africa occurs among HIV-discordant stable couples, where women are disproportionately infected [2].

Although sex work is prevalent in most sub-Saharan African countries, data from a recent five-country study reports that sex work accounts for a relatively small proportion of new infections [3]. Mother to child transmission**,** intrauterine, perinatal and through breast milk is still prevalent despite several ARV scale-up programs. Male-male sex is an increasingly recognized mode of transmission [4].

Even within sub-Saharan Africa the epidemic is not a homogenous one from an epidemiological perspective. Whereas infection rates appear to have reached a plateau in several African countries such as Kenya, Zimbabwe and Uganda, in South Africa the

[4] www.unaids.org/globalreport/africa

epidemic is still on the rise. As of 2007, Southern Africa was the most affected region in the global pandemic, with an estimated 43% of all HIV-infected children and 52% of all HIV-infected women residing in this region. [5]. Common genital diseases and helminths endemic to sub-Saharan Africa have been reported to affect HIV acquisition by increasing susceptibility at mucosal surfaces and/or obscuring protective immune responses, thus 'fueling the fire' in a manner that remains unique to the sub-Saharan epidemic.

The concomitant epidemic of HIV and tuberculosis plaguing South Africa have exacerbated each other and further compounded the burden of the HIV/AIDS epidemic in the region. In communities where HIV prevalence exceeds 30% in pregnant women, the annual tuberculosis notifications rates were as high as 1468 per 100,000 in 2004 and have continued to increase. A vast majority of tuberculosis cases are associated with HIV and over 50% of new tuberculosis cases are in patients with HIV co-infection. Even in communities where HIV prevalence is starting to plateau, tuberculosis rates continue to rise because of the increasing levels of population wide immunodeficiency. Not surprisingly, the exceedingly high prevalence of HIV and highest number of reported tuberculosis cases coincide in the province of KwaZulu-Natal [6].

North America

Previous estimates of HIV incidence in North America painted the picture of a stabilizing epidemic due to widely available successful ARV treatment programs. However, recent reports highlight huge disparities in the disease burden along racial,

ethnic and socioeconomic lines. The estimated incidence of HIV per 100,000 in 2006 was 115.7, 43.1, and 19.6 in African American, Hispanic, and white men, respectively and 83.8, 29.4 and 11.5 in African American, Hispanic, and white women, respectively[5]. The HIV epidemic among visible minorities in North America has been referred to as a 'silent epidemic' that is propagated by a number of underlying issues, such as access to adequate health care, socioeconomic factors, under recognition, delayed presentation and other comorbid conditions [7].

Canada has seen significant advances in the health and well-being of people living with HIV/AIDS since the advent of effective nationwide ARV treatment in 1996. One group however appears to be marginalized with respect to these national improvements in treatment for HIV in Canada. Aboriginal women represent close to half of all positive test reports among Aboriginal peoples compared to 19.2% among non-Aboriginal women. Furthermore, recent studies have demonstrated that Aboriginal women die of AIDS sooner than the general population and have an overall lower utilization of ARV programs [8].

There is a wealth of literature revealing the unfortunate association between socioeconomic, political, and historical factors and risk for HIV infection [9]. In Canada, at a population level Aboriginal women experience the highest rates of poverty and violence compared to other women and are at a particular risk for interpersonal violence, which has been clearly implicated in the potential for acquiring HIV infection [10, 11]. Despite these startling statistics with the exception of a handful of programs, Aboriginal woman remain virtually invisible in HIV/AIDS research, policy, and services in Canada [12].

[5] www.cdc.gov/morbwklyrep/2008

Molecular Epidemiology

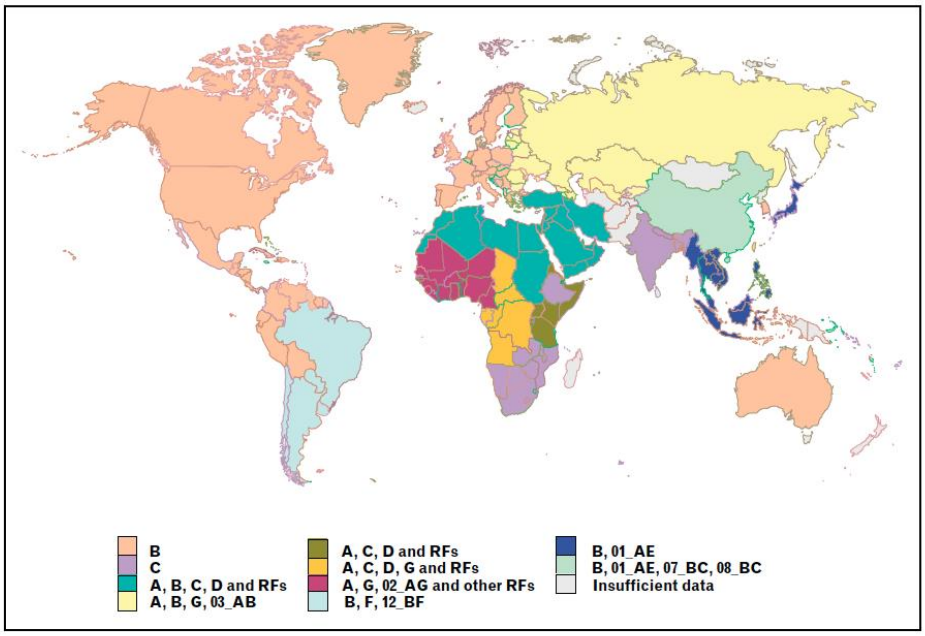
HIV is an extremely genetically diverse virus. There exist three phylogenetically distinct groups of HIV based on genomic sequencing namely: groups M (main), O (outlier), and N (non-M, non-O) [13]. Each group has evolved from independent cross-species events of chimpanzee simian immunodeficiency virus (SIVcpz) to humans. HIV group M has spread worldwide and is the cause of the global AIDS pandemic [14]. Group O infections are uncommon and restricted to individuals living in or epidemiologically linked to Central Africa, specifically Cameroon. Group N infections are very rare and only a few cases have been described in Cameroon [15].

HIV-2 is a distinct primate lentivirus related to HIV-1 that is both less pathogenic and less transmissible. HIV-2 evolved from a cross species transmission of sooty mangabey SIV (SIVsm) and is prevalent in West Africa nations that were former colonies of Portugal such as Guinea-Bissau. Dual infections with HIV-1 and HIV-2 have been described however no recombination events between the two viruses have been reported.

High rates of viral replication coupled with continuous mutation and recombination events have resulted in the rapid genetic diversification of HIV group M viruses. M group strains have diversified into nine distinct subtypes (or clades), over 34 different circulating recombinant forms (CRFs) and an undocumented number of unique recombinant forms (URFs). The initial genetic diversification of HIV group M viruses is

likely to have occurred in Central Africa where the largest genetic diversity and earliest cases of HIV-1 were identified [15] [6].

Figure 2: Regional HIV-1 subtype and circulating recombinant forms (CRF) distribution adapted from Woodman et al.. HIV molecular epidemiology Current Opinion in HIV and AIDS 2009



Modified and updated from Hemelaar *et al.* [6]. 03_AB = CRF03_AB; 12_BF = CRF12_BF; 01_AE = CRF01_AE; 07_BC = CRF07_BC; 08_BC = CRF08_BC; RF = unique recombinant form.

Subtype C, the dominant subtype in Southern Africa, Ethiopia and India causes 50% of the HIV infections worldwide. The predominance of subtype C especially in

[6] www.hiv.lanl.gov

countries with high-prevalence epidemics has led to the speculation that this subtype may have an increased fitness for transmission. Subtype A accounts for 12% of infections worldwide and has a broad geographic distribution. CRF01_AE and CRF02_AG are two additional recombinants that are epidemiologically important in South East Asia and West Africa, respectively. The emergence of these CRFs has raised some concerns as to the selection of viruses with increased fitness, enhanced immune escape or transmissibility. Subtype B is the predominant subtype in the Americas and Western Europe [16] .

The Retrovirus

The Origins of HIV

HIV-1 and HIV-2 cause AIDS. They were introduced to humans during the 20th century and are thus considered relatively new pathogens [14]. The cross-species transmission of lentiviruses from African primates to humans selected for viral adaptations, which subsequently facilitated human-to-human transmission. In several African countries, many species of indigenous nonhuman primates are naturally infected with related lentiviruses yet AIDS is not observed in these hosts [17].

HIV-1 evolved from a strain of SIVcpz that infects a particular subspecies of the chimpanzee (*Pan troglodytes*). HIV-2 originated from SIVsm that infects sooty

mangabeys (*Cerecobus atys*). Notably, among the three SIVcpz ancestors of HIV-1 that have successfully crossed over to humans, only one has given rise to the global AIDS pandemic. [17]

SIVs do not cause AIDS in their natural African hosts. However, similar to humans, several species of Asian macaques (*Macca* spp) develop AIDS when infected with SIVsm. This has provided a powerful experimental system in which specific host as well as viral factors can be controlled and independently studied [18].

It has become increasingly evident that host genetic factors, such as species-specific 'restriction factors,' vary between primate species and ultimately affect the susceptibility of certain primates to develop AIDS. The evolutionary selection and species specificity of lentiviral pathogens implicated by these defense mechanisms indicates that certain natural hosts are relatively resistant to developing AIDS because they have co-evolved with the virus to avoid the immunopathological events that accompany infection and simply act as natural lentivirus reservoirs. Unfortunately, HIV infection in humans has not reached this point as the virus continues to co-evolve and adapt to human populations [17].

The viral genome

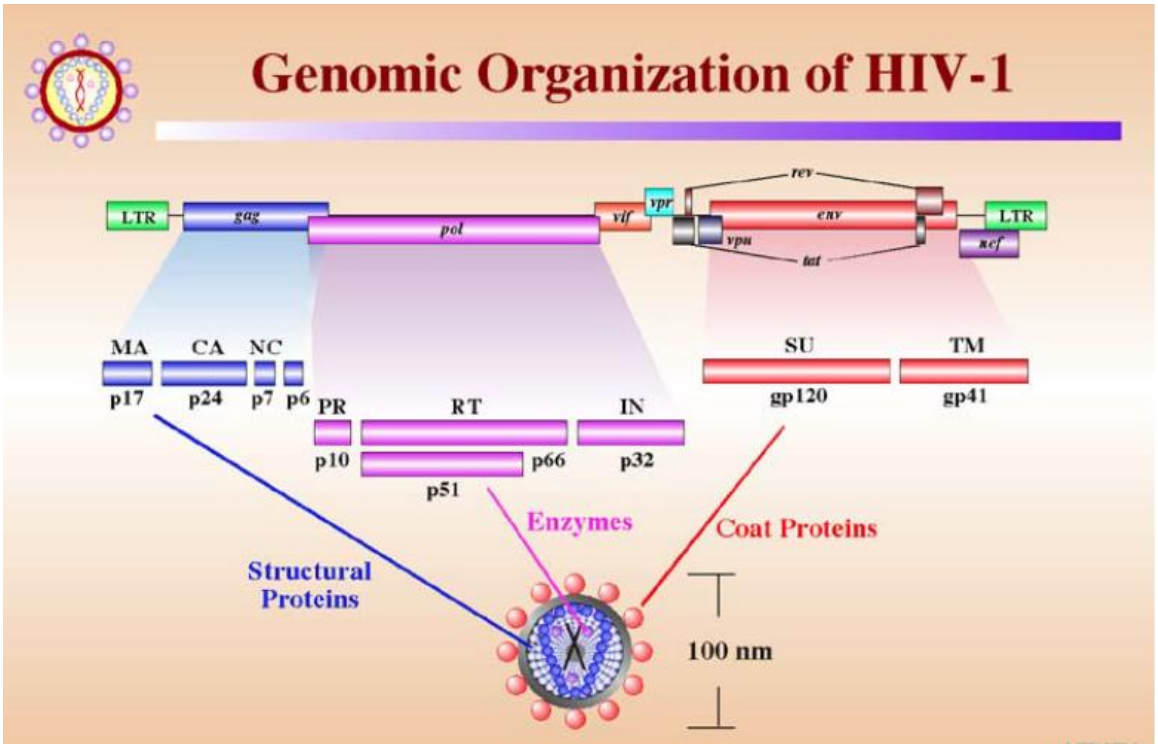
The integrated form of HIV or the provirus is ~9.8 kilobases in length and flanked at both ends by long terminal repeats (LTR). The HIV-1 genome encodes nine gene products that are divided into three classes;

I. The major structural proteins: Gag, Pol, and Env

II. The regulatory proteins: Tat and Rev

III. The accessory proteins: Vpr, Vpu for HIV-1 and Vpx for HIV-2, Vif and Nef

Figure 3: Genomic organization of HIV-1 genome adapted from www.stanford.edu/group.retro



The Viral proteins and their functions

Structural proteins

Gag

The *gag* gene encodes a 55-Kilodalton (KD) Gag precursor protein ($Pr55^{Gag}$) that is expressed from the unspliced viral mRNA. During translation the N-terminus of the precursor is myristoylated triggering its association with the plasma membrane and promoting viral assembly and budding. [19] During the virion particle maturation phase of the viral life-cycle, $Pr55^{Gag}$ is cleaved into four smaller proteins; MA (matrix p17), CA (capsid, p24), NC (nucleocapsid, p9) and p6 [20]. MA molecules remain firmly attached to the inner surface of the virion lipid bilayer thereby stabilizing the particle, and play an integral role in facilitating nuclear transport after reverse transcription [21].

The CA protein forms the conical core of viral particle and interacts with cyclophilin A (CypA). The NC region of Gag recognizes the packaging signal of HIV, which consists of four stem loop structures at the 5'end of the viral RNA and upon binding to this signal mediates the incorporation of heterologous RNA into the HIV genome [22]. The p6 polypetide region mediates interactions between $Pr55^{Gag}$ and Vpr and sequesters Vpr for incorporation into assembling virions [23].

Pol

A ribosomal frame shifting event during which ribosomes shift to the *pol* reading frame without interrupting translation, generates the Gag-Pol fusion protein from which

the viral Protease (Pro), Integrase (IN), RNase H, and Reverse Transcriptase (RT) are translated. During the maturation phase of the viral life cycle, Pro cleaves the Pol polypeptide away from Gag and subsequently digests Pol into Pro (p10), RT (p50), RNase H (p15) and IN (p13).

Protease (Pro)

Pro is an aspartyl protease that acts as a dimer. Pro activity is required for cleavage of the Gag and Gag-Pol polyprotein precursors during virion assembly. This molecule is an important target for ARV drugs [24-26].

Reverse transcriptase (RT)

The RNA-dependent and DNA-dependent RT encoded by the *pol* gene is responsible for the reverse transcription of viral RNA into double stranded DNA. The crystal structure of RT has been determined and used to generate RT inhibitors [27].

Integrase (IN)

The IN protein mediates the insertion of the HIV proviral DNA into the host cell genome. IN has three distinct functions: exonuclease, endonuclease and DNA ligation activity. Remarkably, no exogenous source of energy is required for IN function.

Envelope (Env)

The 160KD Env (gp160) is expressed from a singly spliced mRNA. As newly synthesized Env polypeptides migrate through the Golgi complex they are heavily

glycosylated, a key requirement for viral infectivity. A cellular protease cleaves gp160 into gp41 (the transmembrane domain of Env) and gp120.

Regulatory proteins

Transcriptional Transactivator (Tat)

Tat plays an essential role in the transcriptional activation of integrated proviral DNA by binding to the transactivation response element (TAR). In conjunction with cellular proteins, the binding of Tat to TAR activates transcription from the HIV LTR. Tat exists as either a 72 or 101 aa polypeptide expressed by early fully spliced mRNAs or late incompletely spliced HIV mRNAs, respectively. Aside from activating viral DNA transcription, Tat is documented to activate the expression of other cellular genes including tumor necrosis factor-β (TNF-β) and transforming growth factor-β (TGF-β) [28-30].

Regulator of Virion (Rev)

Rev is a 13-KD sequence-specific RNA binding protein produced from fully spliced mRNAs. Rev induces the transition from the early to the late phase of HIV gene expression. Rev is encoded by two exons and accumulates in the nuclei and nucleoli of infected cells. It binds to a secondary RNA structure called the Rev response element (RRE) and facilitates the export of unspliced and incompletely spliced viral RNAs from the nucleus to the cytoplasm. High concentrations of Rev expression can lead to extensive amounts of intron containing unspliced viral RNA and because Rev is produced

from fully spliced mRNAs, a negative feedback loop is created whereby a decrease in the amount of RNA available for splicing in turn reduces the levels of Rev expression and stops the export of unspliced viral RNA from the nucleus to the cytoplasm [31].

Accessory proteins

The HIV genome encodes four additional genes referred to as accessory genes that are not strictly required for viral replication, but play fundamental roles in viral infectivity and virulence *in vivo*.

Negative factor (Nef)

Nef is a 27 KD myristoylated protein that is encoded by a single exon and is one of the primary viral proteins to accumulate to detectable levels following HIV infection. Nef functions post-translationally to decrease the cell-surface expression of CD4 by increasing the rate of CD4 endocytosis and lysosomal degradation [32]. Nef also down regulates the surface expression of major histocompatibitlity complex (MHC) class I A and B alleles impeding efficient immunosurveillance by cytotoxic T-lymphocytes (CTLs) [33, 34]. Nef perturbs T-cell activation by negatively impacting the induction of the transcription factor NF-κB and IL-2 expression.

Viral protein R (Vpr)

Vpr is incorporated into viral particles via specific interactions with the p6 region of the $Pr55^{Gag}$ in the proteolytically processed protein. Vpr facilitates the nuclear localization of the pre-integration complex (PIC) [35].

Viral Protein U (Vpu)

Vpu is a 16KD polypeptide that is an integral membrane phosphoprotein primarily localized in the internal membranes of the cell. Vpu plays the role of releasing HIV from the surface of an infected cell [36].

Viral infectivity factor (Vif)

HIV is largely immune to the intrinsic antiviral effects of host cell restriction factors belonging to the APOBEC3G family because it encodes the 23KD Vif polypeptide. Vif is an accessory protein that prevents the encapsidation of the APOBEC3 subfamily of proteins into budding virus particles by inducing their degradation through ubiquitin-dependent and independent mechanisms [37].

The viral life cycle

The life cycle of retroviruses is arbitrarily divided into two phases: the early phase refers to the steps from binding to the integration of the viral DNA into the cell genome, and the late phase begins with the expression of viral genes through to the release and maturation of progeny virions.

Early phase of HIV replicative cycle

Binding and entry

For HIV to enter a target cell, the oligomeric Env trimer spike must bind to cell surface CD4 molecules. Primary conformational changes are induced upon the binding of Env to CD4 including the exposure of the co-receptor binding site [38-41]. Env then binds to either the C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) co-receptor on target cells and initiates more conformational changes that trigger the dissociation of gp120 from gp41 [42]. The final outcome of this reaction is the formation of a six-helix bundled gp41 ectodomain core structure comprising of three helical heptad repeat 1 (HR1) domains in the center and three helical heptad repeat 2 (HR2) domains packed on the outside in an antiparallel fashion, referred to as the fusogenic conformation of gp41 [43-47]**.** The formation of this structure leads to the formation of a prehairpin structure that allows for viral and target cell membranes to fuse and the viral core to enter into the target cell [48].

Uncoating

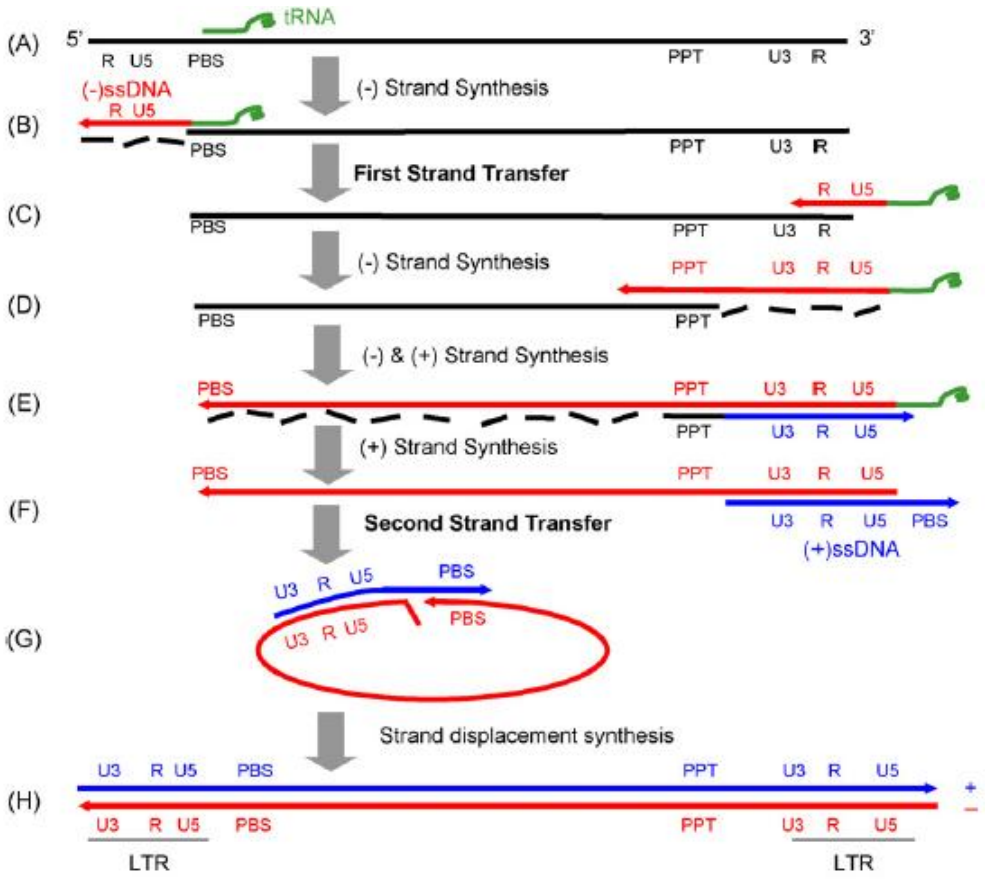
HIV uncoating is defined as the deposition of viral capsid/core within the cytoplasm of infected cells before entry of the viral genome into the nucleus. It is an obligatory step in the HIV replication cycle and accompanies the transition between reverse transcription complexes (RTC), in which reverse transcription occurs and pre-integration complexes (PICs), which are competent to integrate into the host genome. The uncoating process enables HIV and other lentiviruses to replicate efficiently in metabolically active non-dividing cells due to the active nuclear import of their genome across the nuclear membrane of interphasic nuclei by way of a nuclear pore [49, 50]

Reverse transcription

The HIV genome undergoes reverse transcription to convert its single-positive diploid RNA genome into double-stranded DNA. HIV RT can use both DNA and RNA as a template for DNA synthesis and exhibits both DNA polymerase and RNase H activities required to complete the reverse transcription process. The DNA polymerase activity of HIV incorporates deoxyribonucleoside triphosphates (dNTPs) that form 3'-5' phosphodiester bonds with the 3'hydroxyl terminus of the primer resulting in the release of pyrophosphate, the elongation of the primer and generation of nascent DNA. The RNaseH activity of RT utilizes exonuclease, endonuclease and DNA ligation activities to carry out essential functions during the reverse transcription process [51].

Characteristic features of HIV RT include relatively poor processivity, the lack of proofreading capability and inability to excise mis-paired nucleotides [52, 53]. HIV RT does not remain attached to the primer terminus for a large number of successive nucleotide additions and therefore is strongly impaired at secondary RNA structures that are common in the HIV RNA genome. Additionally, HIV RT has an approximate 100-fold lower fidelity than other cellular DNA polymerases that possess the proofreading 3'-exonuclease activity and is 10-100 fold more error prone. Collectively, the lower fidelity coupled with the abundance of recombination events during HIV reverse transcription translates into the very high mutation rate of HIV RT [54].

Figure 4: Steps of the reverse transcription process adapted from Vandana et al. Strand transfer events during HIV-1 reverse transcription Virus research 2008.



Steps of the reverse transcription process

a) Minus strand synthesis: Reverse transcription is initiated from the 3'end of a $tRNA^{lys,3}$ partially annealed to a primer binding site (PBS). The primer extends through the rest of the RNA genome, generating a minus strand strong-stop DNA (-) ssDNA.

b) First strand transfer: The 5'-end of the genomic RNA is degraded by the RNase H activity of the RT and (-) ssDNA is translocated to the 3'-end of the genomic RNA.

c) Minus strand synthesis: A sequence of nucleotides referred to as the 'R' at the 3'-end of the (-) ssDNA binds to the a complimentary 'R' region of the genomic RNA allowing for (-) ssDNA synthesis to continue forming a hybrid between (-) ssDNA and the RNA genome.

d) Minus and plus strand synthesis: RNase H activity of RT degrades the RNA strand of the hybrid.

e) Plus DNA strand synthesis: The plus strand of the DNA template is initiated from a region referred to as the poly-purine tract (PPT) within the genomic RNA.

f) Preparation for second strand transfer: Upon the completion of the plus strand of the DNA template, a portion of the primer $tRNA^{lys,3}$ a DNA copy of the PBS is created.

g) The complimentary copies of the PBS sequences at the 3'-ends of the newly generated plus strand DNA and the nascent minus strand DNA form base pairs.

h) Second strand transfer and strand displacement synthesis: A second strand transfer event occurs allowing for minus and plus strand DNA synthesis to continue with each strand using each other as a template until the double stranded DNA is fully completed creating long terminal repeats (LTR) at either end.

The final product of HIV reverse transcription is linear double stranded DNA with a central DNA Flap [55]. Once the central DNA Flap is formed the viral complex

becomes a pre-integration complex (PIC), competent for import into the nucleus and integration within the host chromatin.

Other accessory factors are involved in the reverse transcription process. These include the viral NC of HIV, which enhances the annealing of the $tRNA^{lys3}$ primer, facilitates polymerization at regions of template secondary structures and accelerates strand transfers [56]. Importantly, NC influences the mutation rate of HIV by enhancing the incorporation of mutations during minus strand elongation and facilitating the annealing of mutated minus strand DNA during transfers [56]. Other proteins implicated in reverse transcription include, IN, Tat, Vpr, Nef and Vif and the cellular protein cyclophillin A (Cyp A).

Nuclear Entry

In the PIC, the ~9.7kb HIV-1 genome is compacted into a 56nm diameter body with the aid of viral and/or cellular proteins that render the PIC karyophilic for passage through the nuclear pore. The components of the PIC have been difficult to identify. However, it is known that the PIC is devoid of detectable CA proteins but contains IN, MA, NC, Vpr and several other cellular factors such as the high mobility group (HMG) protein HMG 1 (Y) [57]. These cellular proteins may assist in tethering the PIC to chromatin, determining integration site selection and expediting the integration process itself [58-60].

Nuclear pore complexes (NPC) are large supramolecular protein structures that span the nuclear membrane and protrude into both the cytoplasm and nucleoplasm. Four different viral components contribute to the nuclear import of the HIV PIC. IN harbors a

non-classical nuclear localization sequence (NLS). MA is kayophilic and contains a classical basic NLS [61]. The part Vpr plays in HIV PIC nuclear import is controversial since Vpr-deficient viruses still retain the capacity to infect non-dividing cells efficiently [62, 63]. Lastly, the central DNA flap structure present in the viral DNA of lentiviruses after the completion of the reverse transcription process supports the nuclear import of the PIC [64].

Integration

The integration of reverse-transcribed viral DNA into a host genome is an obligatory step in the HIV life cycle that is mediated by the 32kDa viral protein IN. IN possesses three structural domains, consisting of a N-terminal domain that contains a zinc binding motif, a catalytic core domain and a less-conserved C-terminal domain [65]. Retroviral integration occurs in two well-characterized catalytic steps referred to as 3' processing and strand transfer. The 3' processing step, which takes place in the cytoplasm within the PIC nucleoprotein complex, entails the removal of a pGT dinucleotide at each 3' end of the viral LTR, adjacent to a highly conserved CA dinucleotide. The processed CA-3'-OH viral DNA ends are simultaneously ligated to the 5'-O-phosphate ends of the target DNA. The integration process is completed by cleavage of unpaired dinucleotides from the 5'-ends of the viral DNA and repair of the single stranded gaps created between the viral and target DNA generating a fully integrated provirus [65, 66]**.**

Late phase of HIV replicative cycle

Transcription from provirus

The regulation of HIV gene expression involves the complex interplay between chromatin-associated proviral DNA, cellular transcription factors and viral Tat. Following integration, the HIV promoter is under the control of the local chromatin environment, which governs the basal transcriptional activity [67]. Multiple cellular transcription factors bind a nucleosome-free region of the LTR core promoter and enhancer. Sequences near the RNA initiation site contain regulatory elements such as the putative inducer of short transcripts (IST), the initiator and the TAR element, which interacts with Tat to enhance Tat-mediated trans-activation [68-70]. LTR promoter activation is primarily driven by Tat. Tat can reverse transcriptional blocks imposed by chromatin and interacts with cellular cofactors and TAR, to increase transcriptional levels of integrated viral DNA [71-74].

Successful transcription leads to the generation of approximately 30 different viral transcripts from the provirus. All these viral transcripts are derived from a single full-length transcript that is subsequently spliced. The spliced viral RNA can be grouped into three classes; the multiply spliced mRNA encoding early regulatory proteins such as Tat, Nef and Rev, the singly spliced mRNA that encodes Vpu, Vpr, Vif and Env and the full-length mRNA encoding the Gag-Pol polyprotein [75, 76].

The transcription and translation of the viral genome occurs in two separate stages of gene expression. First, a Rev-independent phase of regulatory gene expression leads to the translation of Tat, Rev Vif, Vpr, Vpu and Nef proteins. When the concentration of Rev exceeds a certain threshold, the nuclear export of unspliced (*gag*-

pol-env) and singly spliced (*env*) transcripts occurs. These encode the structural proteins CA, MA and NC, the enzymes PR, RT and IN and the Env glycoproteins (gp120, gp41).

Both singly-spliced and un-spliced RNAs are intron-containing transcripts that carry a secondary structure, the Rev Response Element (RRE) within the 3' end of the intron region. Specific interactions between Rev and RRE permit nuclear export of incompletely spliced viral transcripts. Rev binds directly to RRE and multimerizes, which stabilizes the formation of a complex between REV, cellular exportin-1 (CRM-1) and the GTPase Ran. This complex targets the mRNA complex to the nuclear pore complex for export. The shuttling of Rev between the cytoplasm and nucleus and its interaction with RRE are fundamentally important for the regulation of HIV gene expression. A low Rev level would restrict viral gene expression and may send viral replication into a latent state [76].

Virion assembly, packaging and release

Virus assembly in T-cells occurs at specific sites knows as microdomains or lipid rafts in the plasma membrane (PM), whereas assembly can occur in endosomal vacuoles in other cells [77-79]. The $Pr55^{Gag}$ plays a central role in assembly and is sufficient for viral assembly and the production of non-infectious virus particles in the absence of other viral proteins [80]. The $Pr55^{Gag}$ contains three functional domains that are intimately involved in viral assembly; the membrane-binding (M) domain, the Gag-Gag interaction domain (I) and the late domain (L). The assembly process can be artificially divided into the following stages:

1) Gag dimerization and multimerization.
2) Binding of Gag complexes to genomic viral RNA.
3) Transport of Gag/RNA complexes, Gag/Pol, Gag/p55 and Env to the site of assembly.
4) Assembly of nascent virions.
5) Budding and maturation of infectious viral particles.

The process is initiated by the interaction of Gag NC with the viral RNA as a scaffold. The complex promotes subsequent Gag-Gag association through dimerization of CA domains [81, 82]. Although Gag multimerization occurs at the PM, recent studies suggest that multimerization commences within intracellular membranes such as multivesicular bodies [83]. Gag dimerization and multimerization enhance membrane binding and the association with lipid rafts, which may serve as concentration platforms for Gag. Thereafter, the intrinsic sphere-forming properties of the Gag protein drive the assembly of the nascent virion particle. The HIV accessory proteins Vpr and Vif are packaged into virions. Vif plays a crucial role in viral core stability and importantly prevents the restriction enzyme APOBEC 3G from being incorporated into the virion [84]. Important advances have been made in the identification of cellular factors that are appropriated by the virus for its replication, assembly and virus infectivity. These include, cyclophilin A, host protein of 68 Kilodaltons (HP68) and tumor susceptibility gene 101 (Tsg101) all of which participate in the late events of the viral life cycle [85, 86].

The Gag domain interacts with cellular proteins to efficiently release virions from the surface of the target cells. Virus particles are initially released as immature particles and subsequently undergo a maturation step, which leads to the condensation of the inner core, formation of a core shell and conversion of the virus particle into an infectious virion. The precise structural principles and underlying mechanisms governing particle maturation and budding still remain one of the most mysterious steps in the HIV viral life cycle [87]. The prevailing model of virion maturation is that the $Pr55^{Gag}$ is cleaved by the virus-encoded Pro to produce mature Gag proteins [88, 89]

Viral latency

Current ARV regimes are incapable of completely eradicating HIV from infected individuals because of the establishment of viral reservoirs. Resting HIV infected cells can persist as reservoirs for years even in the presence of highly active antiretroviral therapy (HAART) [90, 91]. The reservoir that appears to be the foremost barrier to eradication is latently infected resting memory $CD4^+$ T cells that can carry an integrated provirus that is transcriptionally silent. Resting memory $CD4^+$ T cells are well suited to maintaining hidden copies of the virus because of their extremely long half-life combined with the tight control of HIV expression. Characterizing the molecular determinants that permit proviral DNA silencing has become an area of intense investigation.

HIV exploits different strategies to persist within infected individuals. In $CD4^+$ T lymphocytes, the replicative state of the virus is dependent upon the stage of the cell cycle of host cell. Whereas HIV entry into activated $CD4^+$ T lymphocytes leads to productive infection, viral replication blocks such as host cell restriction enzymes delay

the production of progeny. These have been speculated to instigate the silencing of proviral DNA, or promote the generation of pre-integration latent virus [92]. Other reservoirs for HIV infection include, dendritic cells (DC) and macrophages. In monocyte-derived macrophages, virions can persist and retain infectivity for up to several weeks within late endosomes, thus providing an additional mechanism for viral persistence [93]. The long-term viral persistence of HIV hidden in DCs and macrophages is yet to be proven.

Clinical course

Modes of HIV transmission and risk factors

Heterosexual intercourse accounts for approximately 70% of all sexual transmissions of HIV worldwide. In certain European countries and the United States, acquisition of the virus through homosexual contact accounts for close to 50% of new infections. [94]**.** HIV has been isolated from blood, seminal fluid, pre-ejaculate, vaginal secretions, cerebrospinal fluid, saliva, tears and breast milk of infected persons. However, no cases of HIV infections have been documented to arise from saliva or tears [95].

Nonsexual HIV transmission can occur through transfusion with contaminated blood products, injection drug use, occupational exposure or accidental needlesticks. In the absence of ARV therapy, mother-to-child transmission occurred in approximately 25% of births to HIV-infected mothers [96]. Fortunately, certain regimens of ARV therapy can reduce the rate of perinatal transmission by 50% [96, 97]. However,

interventions aimed at preventing mother-child-transmission remain futile if mothers are not provided with safe alternatives to breast-feeding as approximately one third of cases of mother-to-child transmission result from breast-feeding [98].

The Biology of HIV-1 transmission

The transmission of HIV infection is directly correlated with the levels of virus circulating in the source subject [99]. As the rate of transmission is proportionally related to the plasma viral load (VL), it has been surmised that the viral peak attained during PI may be responsible for the increased rate of infectiousness of acutely infected subjects [100].

Evidence from nonhuman primate studies further support this notion as virions isolated from rhesus macaques during acute infection were shown to be more infectious than those from animals in the chronic phase of infection [101]. Interestingly, results from Goodreau et al. highlight the importance of the acute infection period for sustaining the HIV epidemic within a Zimbabwean cohort and propose that the virons from the early infection phase may contain certain biological properties that are significant for HIV transmission[7]. Additionally, enhanced coating of viruses by host-generated antibodies (Ab) during the chronic phase of infection has been suggested to hinder infectivity. Therefore, it appears as though acute phase viruses have unique properties that are advantageous for transmission events and these properties are lost as the infection progresses.

[7] Goodreau S et al… Estimating the fraction of new HIV-1 infections from acute-stage cases in Zimbabwe through network modeling. Presented at Acute Infection meeting. Boston MA. 2009

Genotypic and phenotypic evidence further bolster the hypothesis that during transmission there is a preferential active selection of a minority of variants that diverge away from the infecting founder population as the disease progresses. Key observations include the almost exclusive acquisition of CCR5 as opposed to CXCR4 using variants by newly infected subjects [102, 103].

Newly acquired variants also have shorter or less glycosylated envelope glycoproteins than those in the transmitting partner or among chronically infected subjects [104-106]. However, the identification of viral phenotypic properties that confer an advantage during transmission in support of a biological model of active selection during transmission has thus far remained elusive.

Several lines of evidence indicate that HIV transmission is associated with a population bottleneck [102, 104, 107-110]. Recent seminal work from Keele and colleagues employing technologies capable of the precise identification of the transmitted founder virus population found that over 70% of the transmission events involved in productive clinical infection occurred by a single genetic variant [108]. As encouraging as these data were, it has also become apparent that this transmission block can be overcome, as evidenced by cases describing the transmission of multiple variants [107, 108, 111, 112]. Infection with genetically divergent viral lineages has been associated with a more severe disease course [105].

The development of effective strategies to reduce HIV transmission is an overwhelming challenge further complicated by the fact that acutely infected subjects carrying the most infectious virus are often unaware of their HIV status. This early infection period presents a unique opportunity for intervention given the limited genetic

variation among circulating strains. By understanding the biological mechanisms underlying the selective bottleneck during transmission, it may be possible to design novel interventions that enhance the obstacles to acquisition before swarms of variants gain a foothold.

Primary infection (PI)

PI is defined as the period from initial infection with HIV to the development of an antibody response detectable by standard diagnostic tests. Approximately 87% of individuals who acquire HIV experience a transient flu-like "seroconversion" or acute retroviral syndrome associated with a spike in viremia that can exceed 10^7 viral particles per ml of plasma. Typical symptoms resemble that of mononucleosis including fever, fatigue, lymphadenopathy, headache and a maculopapular rash. Seminal work from Pedersen et al. report an association between a more severe clinical syndrome in HIV PI and rapid subsequent clinical course, which has been confirmed among several cohorts [113].

The first six months of HIV infection is referred to as Acute Infection Early Disease (AIED). The date of HIV infection is estimated using an algorithm proposed by the AIED Research Program sponsored by the National Institutes of Health (NIH) [114, 115]. Of note, the lack of HIV-specific clinical symptoms during PI and the existence of a 'window period' during which HIV-antibody testing may be negative for several weeks or months after exposure, makes HIV diagnosis quite challenging. HIV diagnosis therefore relies upon the combination of high clinical suspicion, HIV antigen and antibody tests and sometimes HIV RNA testing to diagnose new infections.

In the absence of ARV treatment, the initial spike in viremia settles at a level referred to as the VL setpoint that persists throughout the asymptomatic phase. Importantly, the magnitude of the VL setpoint correlates with the subsequent rate of disease progression [116-118]. This decline in viremia is thought to involve the dramatic loss of the main targets of HIV infection, memory $CD4^+CCR5^+$ T cells particularly in the gut-associated lymphoid tissue (GALT), and/or the appearance of HIV-specific immune responses [119-122].

Evidence in support of a role for HIV-specific CTLs in the decline of viral replication during PI include observations that viremia is not controlled in macaques depleted of $CD8^+$ CTLs who are undergoing SIV PI and the appearance of viral variants with escape mutations within CTL epitopes in both humans and non-human primates (NHP) [123-130]. During HIV PI CD4 counts and CD4 function may decline to levels that allow opportunistic infections (OIs) to develop [131-133]. Even though the absolute CD4 count rebounds after PI it rarely returns to normal baseline levels and in patients with clinical HIV disease progression, HIV-specific CD4 responses remain particularly impaired [134].

The intricate relationship between VL setpoint and subsequent disease progression has raised questions as to the benefits of early treatment. However, even though early ARV therapy may reduce viremia and preserve immune function, rapid control of viremia may also inhibit the full development of a mature virus-specific immune response [135, 136].

Chronic Infection (CI)

After AIED, a relative equilibrium between viral replication and the host immune response is reached and the quiescent chronic phase of HIV infection ensues. In the absence of ARV therapy the time between initial infection and development of AIDS averages 10 years [137]. Despite the relative clinical latency of this stage of HIV infection, viral replication and CD4 cell turnover remain active and the majority of infected persons progressively lose their CD4 lymphocytes at an average rate of 50-90 cells/mm^3 [138-141].

The train on a track analogy coined by John Coffin in 1996 has been useful in illustrating the independent perspectives gained from HIV VL and CD4 count information when painting a clinical picture of HIV during this phase. If the infected person is imagined as being on a train travelling toward a clinical event (such as the acquisition of an OI), the CD4 count provides information on the distance of the train from the destination and the VL provides information on the speed the train is travelling.

Acquired Immunodeficiency syndrome (AIDS)

According to the Centers for Disease Control and Prevention (CDC) criteria, AIDS is defined by either a diagnosis of one of the AIDS-defining conditions, or by the decline of CD4 levels to below 200 cells/mm^3. Progression to AIDS from time of infection occurs on average 2 years earlier when defined using laboratory criteria such as CD4 counts in comparison to the clinical identification of an AIDS-defining OI.

Table 1: The 1993 AIDS Surveillance Case Definition of the U.S Centers for Disease Control and Prevention (CDC) adapted from CDC 1993 Revised Classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults.

A diagnosis of AIDS is made whenever a person is HIV positive and:

- he or she has a CD4 $^{+}$ cell count <200 cells/µL, or
- his or her CD4 $^{+}$ cells account for <14% of all lymphocytes, or
- that person has been diagnosed with one or more of the AIDS-defining illnesses listed below.

AIDS-defining illnesses:

- Candidiasis of bronchi, trachea, or lungs
- Candidiasis, esophageal
- Cervical cancer, invasive *
- Coccidioidomycosis, disseminated
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (>1-month duration)
- Cytomegalovirus disease (other than liver, spleen, or lymph nodes)
- Cytomegalovirus retinitis (with loss of vision)
- Encephalopathy, HIV related $^{\#}$ (see Dementia)
- Herpes simplex: chronic ulcer(s) (>1-month duration) or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, disseminated
- Isosporiasis, chronic intestinal (>1-month duration)
- Kaposi sarcoma
- Lymphoma, Burkitt
- Lymphoma, immunoblastic
- Lymphoma, primary, of brain (primary central nervous system lymphoma)
- *Mycobacterium avium* complex or disease caused by *M kansasii*, disseminated
- Disease caused by *Mycobacterium tuberculosis*, any site (pulmonary *or extrapulmonary $^{\#}$)
- Disease caused by *Mycobacterium*, other species, or unidentified species, disseminated
- *Pneumocystis jiroveci* (formerly *carinii*) pneumonia
- Pneumonia, recurrent *
- Progressive multifocal leukoencephalopathy
- *Salmonella* septicemia, recurrent
- Toxoplasmosis of brain (encephalitis)
- Wasting syndrome caused by HIV infection $^{\#}$

Additional illnesses that are AIDS defining in children, but not adults

- Multiple, recurrent bacterial infections $^{\#}$
- Lymphoid interstitial pneumonia/pulmonary lymphoid hyperplasia

(a) Centers for Disease Control. 1993 Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Morb Mort Wkly Rep 1992; 41(RR-17):1-19.

* Added in the 1993 expansion.

$^{\#}$ Added in the 1987 expansion.

Table 2: CDC Categorization of HIV/AIDS adapted from CDC 1993 Revised Classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults.

The 3 CD4 count categories *	
Category 1	>=500 cells/µL or more
Category 2	200-499 cells/µL
Category 3	<200 cells/µL

*Categorization should be based on the lowest accurate CD4 count, not necessarily the most recent one. So someone whose CD4 count declined steadily over a period of months until it reached 180 cells/µL, but then rose above 200 cells/µL again and remained at that level (perhaps as the result of antiretroviral treatment), would be placed in category 3, not category 2.

Targeting HIV: The old and new players

Antiretroviral therapy (ART) changes the natural history of HIV infection by preventing clinical progression. With the development of effective (HAART) in the mid-1990s and the subsequent rapid widespread clinical use of HAART in developed countries, the death rate of HIV infected persons has decreased by two-thirds [142, 143]. Interestingly, current models estimate that an HIV-infected individual optimally treated with the appropriate ART regimen may have a life expectancy that approaches that of an HIV-uninfected person. To date, there are 25 ARV drugs approved for the treatment of HIV infection and successful treatment is associated with the continued durable suppression of HIV viremia. Since their initial development, ART regimens have

become markedly easier to administer, are less toxic and more potent and as a result, long-term adherence has improved dramatically [144-146].

Table 3: ARV Therapy Treatment Guidelines for Initiation of Therapy adapted from Antiretroviral Treatment 2010: Progress and Controversies adapted from Gulick et al. 2010.

	AIDS/Symptomatic HIV	CD4 Cell Count <200/uL	CD4 Cell Count 200–350/uL	CD4 Cell count 350–500/uL	CD4 Cell Count >500/uL
US Department of Health and Human Services (DHHS) '09[9]	Treat	Treat	Treat	Treat	Treat (optional)
International AIDS Society (IAS)-USA '10[10]	Treat	Treat	Treat	Treat	Consider treatment
British HIV Association (BHIVA) '08[11]	Treat (except tuberculosis)	Treat	Treat	Refer to a clinical trial	Refer to a clinical trial
European AIDS Clinical Society (EACS) '09[12]	Treat	Treat	Treat	Treat in certain patients	Defer treatment
World Health Organization '10[6]	Treat	Treat	Treat	Do not treat	Do not treat

Significant progress has been made in the development of agents to treat HIV infection. Shown below are the six different classes of ARV agents currently approved by US Food and Drug Administration (US-FDA) for the treatment of HIV infection.

a. Nucleoside reverse transcriptase inhibitors (NRTIs)
b. Nonnucleoside reverse transcriptase inhibitors (NNRTIs)
c. Protease inhibitors (PrIs)
d. Integrase inhibitors (IIs)
e. Fusion inhibitors (FIs)

f. Chemokine receptor antagonists (CRAs)

The use of these agents in clinical practice depends on the ease and complexity of their use, side-effect profile, clinical based efficacy and practice guidelines.

Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI)

Upon the approval of Zidovudine (ZDV), Retrovir, formerly Azidothymidine (AZT) in 1987, NRTIs became the first agents available for the treatment of HIV infection. There are seven commercially available NRTI, and although this class of drugs is less potent that NNRTI and PrI they remain an integral part of the current standard of care [147]**.** NRTI interrupt the HIV replication cycle via competitive inhibition of HIV RT and termination of the DNA chain [148]. NRTI are structurally similar to DNA nucleoside bases. However once they become incorporated into the proviral DNA, chain termination results [148].

Resistance to NRTI occurs by one of two mechanisms: either impaired incorporation into the proviral DNA chain or removal from the proviral chain of the NRTI [149]. Drug resistance mutations usually occur gradually and sequentially with the exception of the M184V mutation, which confers high-level resistance to lamivudine (3TC) and emtricitabine in one single step. Thymidine analog mutations (mutations associated AZT resistance) remove NRTI from the DNA chain by fostering a conformational change in the RT enzyme that allows for the addition of Adenosine tri-phosphate (ATP) or pyrophosphate to the end of the proviral DNA chain thus permitting continued elongation of the DNA strand [149]. The adverse effects of the NRTI class of

ARVs include mitochondrial toxicities, peripheral neuropathy, hepatic steatosis and lipoatrophy [150].

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)

NNRTI were first introduced in 1996 after the approval of nevirapine. All NNRTI non competitively bind to the hydrophobic pocket of the RT enzyme and induce a conformational change that alters the active site of the enzyme and limits its activity. Etavirine is the only second generation NNRTI, an inherently more flexible molecule that maintains the capacity to bind to RT in spite of drug resistance mutations, which hinder the ability of first-generation NNRTIs to bind to the hydrophobic pocket of RT [151]. Even though milder side effects have been associated with NNRTI administration, all first-generation NNRTI cause some degree of hepatoxicity [152].

Protease Inhibitors (PrI)

HIV PrI were first introduced in 1995 and form an integral part of all treatment regimens for HIV infection. PrI function as competitive inhibitors for the viral Pro enzyme by directly binding to it and preventing the subsequent cleavage of the *gag-pol* polypeptide precursor [153]. Resistance to PrI typically occurs via the development of major mutations that produce conformational changes in the Pro-binding site followed by secondary mutations that enhance enzymatic activity [154]. All eight PrI exhibit the same mechanism of action but differ in their pharmokinetic, efficacy, and side-effect profiles. PrI exhibit substantial inter- and intra-patient variability in pharmacokinetics. Metabolic

complications are very common in patients on PrI therapy and represent an important consideration when selecting PrI for inclusion into ARV regimens. In 1997, the FDA required that all PrI include labeling regarding the potential for hyperglycemia and diabetes mellitus with therapy [155].

Integrase Inhibitors (II)

In 1994, the crystal structure of HIV IN was first described and led to the identification of a novel class of inhibitors for HIV replication. As there are no human homologs for the HIV IN enzyme, inhibitors targeting HIV IN were expected to have a lower frequency of adverse effects [156]. The FDA approved raltegravir as the first II in 2007 and a second II (elvitegravir) is undergoing clinical development in phase III trials. Both compounds competitively inhibit the strand transfer reaction of the proviral integration step by binding to metallic ions in the enzymes active site [157]. Three resistance pathways involving mutations in the IN gene have been associated with greater than a 100-fold decrease in susceptibility to raltegravir [158].

Fusion Inhibitors (FI)

The FI were the first class of ARV drugs aimed at targeting HIV replication extra-cellularly. Based on their unique mechanism of action, FIs provide additional options for therapy in patients highly resistant to other ARV drug classes. FIs interfere with an important step in the HIV fusion pathway by specifically binding to the HR1 of gp41. In doing so, FIs hinder the proper folding of HR1 and HR2 and thereby prevent the

conformational changes required of gp41 to complete the fusion process [43]. Resistance to enfuvirtide usually occurs in the HR1 domain of gp41 and results in a significant loss of enfuvirtide activity [159]. However, FI resistance can be minimized through combinations with ARVs for which cross-resistance is rare.

Chemokine Receptor Antagonists (CRA)

In 2007, a completely new class of HIV ARVs termed CCR5 antagonists was introduced to the armament of HIV ARV agents. Maraviroc selectively and irreversibly binds to the CCR5 co-receptor thereby blocking the V3 loop and preventing the insertion of gp41 into the host cell. Maraviroc selectively inhibits HIV CCR5 trophic viruses and has no activity against CXCR4 trophic or dual/mixed trophic viruses [160].

Although clinical experience with Maraviroc is limited, reports of treatment failure have been observed. Two resistance mechanisms have been described, amino acid substitutions in the V3 loop of gp120 that allow HIV to bind to the co-receptor despite the presence of Maraviroc and the outgrowth of previously undetected CXCR4 using virus in the presence of Maraviroc [161]. Very few side effects have been associated with the administration of Maraviroc although severe hepatoxicity can occur in patients predisposed to hepatic impairment [160].

HIV-1 Vaccines: trials and tribulations

The goal of an HIV vaccine

For 25 years, HIV has ravaged the lives of close to 60 million people worldwide the majority of which live in developing countries. The development of a safe and effective HIV vaccine is a critically important global health priority and would be the best solution for the ultimate control of the world-wide AIDS pandemic. Unfortunately, despite recent advances in the understanding of HIV pathogenesis and immunology, HIV vaccine development efforts have been met with significant hurdles. Prototype HIV vaccine candidates aimed at eliciting humoral and cellular immune responses have thus far failed to protect against HIV infection and/or reduce VL post infection in clinical efficacy trials.

An ideal vaccine should completely block infection and provide sterilizing immunity. However, because most licensed viral vaccines appear to function by controlling subclinical viral replication and preventing clinical disease, a more realistic goal for an HIV vaccine could be to substantially reduce VL and clinical disease progression after infection [162]**.** Moreover, because VL represents a principal determinant of HIV transmission, even though such a vaccine would not provide sterilizing immunity it is conceivable that the partial protection conferred by such as vaccine might have an impact on a population level.

The challenges facing HIV vaccine development

Unprecedented challenges face the development of a prophylactic HIV vaccine these include:

1. Extensive viral clade and sequence diversity
2. Establishment of latent viral reservoirs early in infection
3. Poorly-defined correlates of immune protection
4. Viral evasion of humoral and cellular immune responses
5. Type-specific antibody responses
6. Impediment to eliciting neutralizing antibodies (NAbs)
7. Lack of small-animal model
8. Safety concerns related to use of attenuated viruses in humans
9. Limited interest from the pharmaceutical industry

Some the most significant challenges facing the development of an HIV vaccine include the extreme world-wide diversity of the virus, the lack of clear definitive correlates of immune protection and the irreversible damage the virus causes to the immune system during the earliest phases of infection. The HIV M group has diversified into nine divergent clades and multiple CRFs. Additionally, amino acid sequences of the Env protein can differ by up 20% within a certain clade and by up to 35% between clades [163]. Candidate immunogens have the daunting task of contending with this high degree of viral diversity and their success is largely dependent upon the capacity to elicit responses cross-reactive against heterologous viral strains [164].

The lack of clear correlates of immune protection in humans represents another key issue confronting the HIV vaccine effort [165]. Even though viral challenge studies in NHP and studies in HIV infected slow progressors (SP) provide some suggestive evidence regarding potential immune correlates of protection, they will remain speculative until successful vaccine efficacy trials are completed in humans. Of note, natural immunity itself is far from protective since induced humoral and cellular immune responses do not lead to spontaneous clearance or protection from superinfection [166]. Therefore, generating an immune response similar to that which is generated in natural infection is not expected to be effective against HIV.

Virus-specific NAb titres represent key immune correlates of protection for most licensed viral vaccines. The development of immunogens that induce broadly neutralizing antibodies (bNAbs) is a key priority for the HIV vaccine field [167]. Proof of concept passive transfer studies in NHP demonstrate that high doses of bNAbs can afford sterilizing protection from infection. However, the extensive N-linked glycosylation surrounding the HIV Env glycoprotein trimer on the virion surface effectively shields many conserved epitopes from antibody recognition making the generation of bNAbs against HIV difficult to elicit [167].

Several lines of evidence demonstrate that virus-specific CTLs play a critical role in controlling HIV replication and therefore HIV vaccine development efforts have explored several concepts aimed at eliciting cellular immune responses [119, 120, 168]. Unfortunately, a major limitation to this approach is the accumulation of CTL escape mutations in T-cell epitopes that allow the virus to circumvent immune surveillance by CTLs. Additionally, vaccine-elicited CTL responses may be limited by

immunodominance constraints, which may preclude sufficient coverage of HIV diversity [169]. CTL escape coupled with the establishment of latent reservoirs severely hinder the capacity of vaccine-elicited T-cell responses to prevent infection long term [170, 171].

HIV vaccine trials and tribulations

Although traditional vaccine strategies including live attenuated viruses, whole killed viruses and protein subunits have proved to be very successful for the development of vaccines against other viruses, they all have limitations in terms of their utility for HIV. Obvious safety concerns prevent the use of live attenuated HIV viruses in humans despite the demonstration of protective efficacy against SIV challenge in rhesus macaques [172-175]. On the other hand, whereas whole killed viruses and protein subunits have no associated safety concerns, both strategies have failed to induce bNAbs and/or CTL responses [176-178].

Novel vaccine approaches employing gene-delivery technologies such as plasmid DNA vaccines and live recombinant vectors engineered to express HIV antigens are simple, versatile and extremely attractive approaches to HIV vaccine development. However, the administration of several high dose injections of DNA vaccines required to elicit detectable immune responses, necessitates the use of an appropriate adjuvant that has not been identified to date [179-181]. Viral vectors administered either alone or in the context of heterologous DNA prime/boost regimens represent most of the HIV vaccine candidates currently being tested.

In spite of the challenges facing HIV vaccine development efforts, two vaccine concepts have completed clinical efficacy studies in humans. The first used monomeric

HIV Env gp120 protein with the aim of inducing Env-specific humoral immune responses and the second concept involved replication-incompetent recombinant adenovirus serotype 5 (rAd5) vectors expressing HIV Gag, Pol and Nef. The major problem associated with the use of monomeric HIV Env gp120 immunogens was the elicitation of type-specific binding antibodies and not bNAbs [182, 183]. The biotechnology company VaxGen carried out two phase III efficacy trials also aimed at eliciting bNAb, but yet again the vaccine candidates afforded no detectable protective efficacy [177, 178]. It has become evident that these type-specific antibody responses are insufficient to protect against HIV infection in humans.

The second vaccine concept used a replication-incompetent rAd5 vector expressing HIV clade B Gag, Pol and Nef proteins aimed at eliciting protective cellular immune responses. Early phase clinical trials generated encouraging results regarding safety and immunogenicity [184]. Two large phase IIb 'proof of concept' efficacy studies were initiated to determine whether HIV specific cellular responses elicited by this vaccine regimen would prevent HIV infection or reduce VL post-infection; the HIV Vaccine Trials Network (HVTN) 502 or the 'STEP' study and the HVTN 503 or ' Phambili' study.

Disappointingly, on 18 September 2007, these phase IIb trials were terminated after the primary interim analysis demonstrated that the vaccine failed to protect against infection and reduce viral loads after infection. [185, 186] [8,9] . Furthermore, the vaccinees with pre-existing Ad5-specific NAbs exhibited an augmented rated of HIV acquisition

[8] Gray G. Results from the Phambili (HVTN 503) study: a multicenter Phase IIB test of concept study of the MRKadn5 HIV-1 gag/pol/nef vaccine in South Africa. AIDS Vaccine 2008, Cape Town, South Africa. Oct 13-16, 2008

[9] Fauci. A,S. The release of the new data from the HVTN 502 (STEP) HIV vaccine study. NIH News (http://www3.niaid.nih.gov/about/directors/news/step_11707.html (2007).

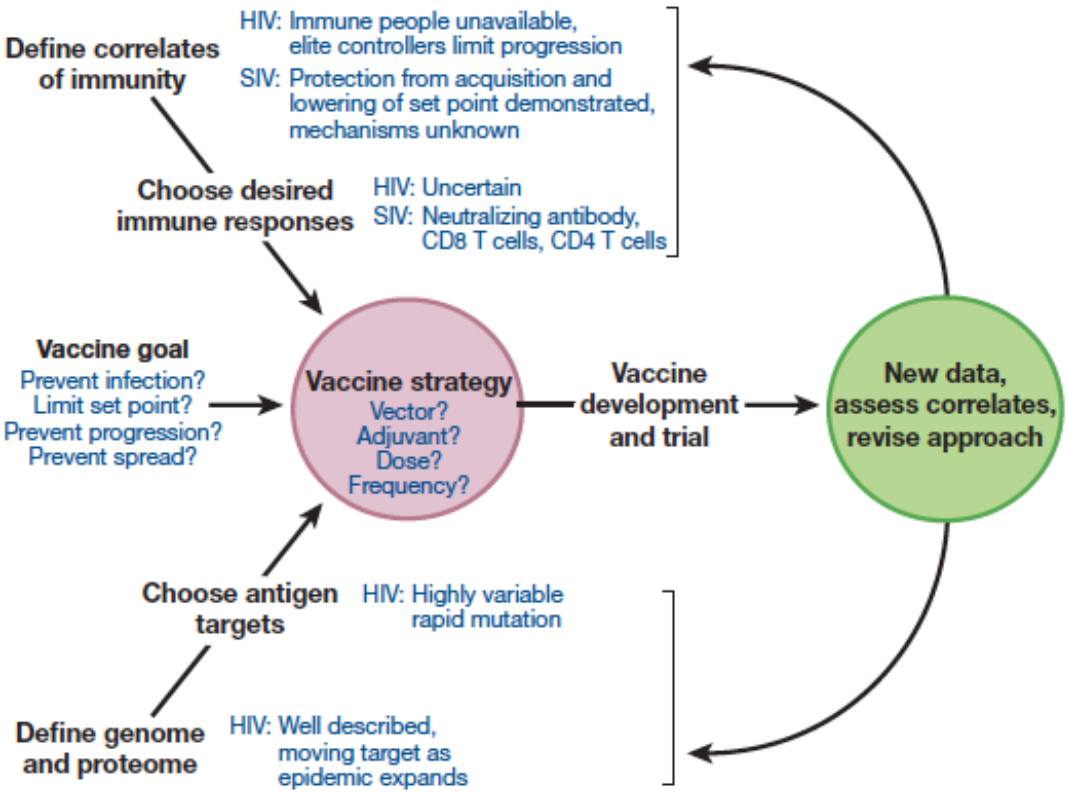
[187]. The impact of anti-vector immunity is predicted to be a severe limitation of rAd5 vectors because approximately 30-40% of individuals in the United States and Western Europe and 80-90% of the population in Sub-Saharan Africa have pre-existing Ad5-specific NAbs [188-190]. The disappointing results of the STEP trial has had a major impact every other HIV vaccine program and called for a substantial overhaul of all HIV vaccine concepts.

The way forward

Approaches to HIV vaccine concepts are being re-examined, A 'back to basics' approach has been recommended that focuses on 1) understanding basic immunological mechanisms that prevent the amplification and systemic spread of HIV from a limited nidus of infection, 2) elucidating the nature of the immune response needed for protection, 3) determining whether an induced response can maintain activity at relevant body surfaces, 4) understanding the influence of the virome and microbiome on vaccine responses and lastly 5) understanding the effect of pre-existing immunity[191]. Further insights may be gleaned from vaccine trials such as the phase III Thai ALVAC and AIDSVAX trials that demonstrated that induced protective immunity is indeed a feasible goal [192]. More robust findings from efforts to vaccinate against SIV corroborate the notion that a measure of protective immunity can be induced. Recently published data on the use of cytomegalovirus (CMV) as a vector to continuously deliver SIV antigens has shown exceptional promise by reporting protection from systemic infection in animals after intrarectal challenge with SIV [193].

Ultimately, to meet the unique challenges associated with HIV vaccination, the ideal HIV vaccine formulation will need to induce B and T cells that have and retain specific differentiated properties allowing them to function during the initial critical phase of infection in the intestine or genitourinary tract and control viral replication of established infection. Furthermore, by harnessing the appropriate adjuvants to both enhance the overall immunogenicity of the antigen and direct the differentiation of cells responding to the vaccine, the optimal protective response may be engineered.

Figure 5: An integrated approach to HIV vaccination and special challenges of HIV adapted from Virgin et al. Nature 2010.



HIV-infected Slow Progressors

Definitions and categories

The clinical course and outcome of HIV infection is characterized by extreme heterogeneity among treatment naïve infected individuals. A small subset of HIV infected individuals have a remarkably slower course of disease progression than the norm. Approximately 2-5% of the HIV infection population are defined as long term non-progressors (LTNP) who remain clinically asymptomatic and maintain a stable $CD4^+$ T-cell count of above 500 cells/mm^3 for 7 years or more without treatment [139, 194]. This initial definition for LTNP was constructed solely on an immunological basis, however as VL measurements became available in the mid 1990s it became evident that LTNP were a heterogenous population when VL was taken into consideration [195].

The quantification of HIV in plasma revealed that some LTNP consistently maintained extremely low plasma VL and led to the identification of a rare subset of untreated HIV-positive subjects [194, 196]. Major efforts have been made to identify and study larger numbers of these individuals such as the Canadian Cohort of HIV-infected Slow Progressors and the International HIV Controller Consortium (HIC) studies. The HIC focuses on the tail end of the spectrum of VL in untreated HIV infection with a particular emphasis on those who have maintained undetectable VL <50 copies/ml of plasma for at least 1 year. They are referred to as Elite Controllers (EC).

A variety of data indicate that risk of transmission of HIV is markedly reduced at plasma HIV RNA levels below 2000 copies/mL, therefore the HIC also provides a working definition for a group of viremic controllers (VC) who maintain VL between 50-2000 copies/mL for at least 1 year**.** Furthermore, all EC and VC are HIV seropositve and have stable amounts of viral DNA in their peripheral blood mononuclear cells (PBMC) [197]. The HIC estimates that EC represent approximately 1% of the HIV infected population, a statistic that is corroborated in other cohorts [197, 198].

Although the HIC has provided working definitions for EC and VC, there isn't an official standard for defining these subpopulations of HIV-infected subjects and variations in clinical definitions can significantly alter clinical outcomes. The EC and VC are mutually exclusive groups, however some EC and VC also maintain $CD4^+$ T cell counts above 500 cells/mm^3 whereas others have extremely low or declining $CD4^+$ T-cell counts [199-202]. Additionally, Okulicz et al. report that VC progress to AIDS more rapidly if they have low $CD4^+$ T cell counts versus those with CD4 counts within the normal range [203]. Therefore, superior control of HIV viremia as defined by undetectable VL in the absence of therapy does not necessarily correlate with the preservation of $CD4^+$ T-cell counts. The all-encompassing term HIV-infected Slow Progressor (SP) is used in this thesis to identify EC, VC and LTNP.

Several lines of evidence demonstrate that SP are distinct from other HIV-infected persons in terms of their natural immunity against the virus and therefore may serve as ideal models for the identification of the elusive correlates of immune protection. Even though much can be learned from the inherent capacity of SP to overcome

remarkable challenges such as HIV's antigenic variation and immune evasion, there are some viral features that may significantly contribute to their non-progession.

Viral factors associated with slow HIV disease progression

Undetectable VL and the accompanying low proviral DNA levels in EC suggests that they might be infected with attenuated viruses [196, 204-207]. However, even though mutations and deletions in various HIV gene products have been found in virus isolated from SP, none are common to all SP. Therefore infection with virus carrying deleterious mutations is not a major factor accounting for slow progression [208-210].

A notable exception of virus attenuation accounting for SP status comes from the Sydney Blood Bank cohort whose members were infected with contaminated blood transfusions from the same donor whose virus contained a large deletion in the *nef* gene [174, 211]. Six of the blood transfusion recipients became LTNPs and maintained stable $CD4^+$ T-cell levels and low VL for over 10 years [211]. Viruses from EC have a reduced viral replicative capacity (VRC) compared to those from progressors. This has been observed by inserting gag-protease and pol-integrase cassettes from EC and progressors into a common NL4-3 HIV backbone and comparing their VRC [212, 213]. Viruses from carriers of protective alleles such as HLA-B*57 have the lowest VRC [212]. These findings suggest that CTL selection pressure on these gene products alters viral fitness and may be an important factor in the VL control seen in EC [212].

Host Genetic Factors associated with HIV disease progression

Chemokine receptors

The most salient example of a host genetic factor that promotes resistance to acquisition and control of HIV infection is the CCR5Δ32 deletion mutant, which introduces a premature stop codon in the *CCR5* gene and results in the production of a truncated protein that is retained in the endoplasmic reticulum. Individuals who are homozygous for this deletion are completely resistant to R5-trophic HIV infection due to the abrogated surface expression of CCR5 on $CD4^+$ T cells [214]. CCR5 is normally expressed at low levels on the surface of naïve CD4+ T cells and is increased in activated CD4+ T memory T cells [215]**.**

The frequency of the *CCR5Δ32* allele differs between ethnicities but is more frequent in northern Europe and western Asia and completely absent in native Africans, East Asians, and American Indians [216]. Several studies report an increased frequency of LTNP who are heterozygote for the CCR5Δ32 in comparison to progressors and uninfected controls indicating that the carriage of one copy of the polymorphism results in the reduction of $CD4^+$ T cells expressing CCR5, decreased infection events which translates into slowing progression [214, 217-219].

HLA class I alleles

The most polymorphic mammalian genetic system is the MHC. The human MHC maps to the short arm of Chromosome 6 and contains the classical human MHC class I alleles (HLA-A, -B and -C), which encode for cellular surface molecules that are responsible for presenting antigens usually from intracellular pathogens to $CD8^+$ T cells. MHC class II alleles (HLA-DR, -DQ, and -DP) bind to and present antigens of extracellular origin to $CD4^+$ T cells. Several infectious and inflammatory conditions have shown strong genetic associations between HLA and differential disease outcome indicating that HLA plays a fundamental role in disease predisposition, of which HIV is no exception.

Expression of the maximum number of polymorphic HLA alleles is thought to generate the broadest immune response against infectious agents due to the increased capacity to present a wider repertoire of antigens to T cells [220, 221]**.** One explanation for the effect of heterozygozity on AIDS progression is that by being able to present a broader range of HIV peptides, heterozygotes prolong the time it takes for escape mutants to arise. Other explanations include frequency-dependent selection, where pathogens evolve under selection pressure from common HLA types to avoid a protective immune response. Therefore, individuals who express a frequent allele may be at a greater risk for AIDS progression versus those who carry at least one copy of an infrequent allele.

Correlations exist between certain HLA alleles and HIV disease progression. A number of convincing HLA class I associations with HIV have been identified and data

replicated in several independent cohort studies, strongly implicating the involvement of the alleles listed in Table 4 in protection or susceptibility to AIDS progression.

Table 4: Consistent HLA associations with HIV disease adapted from Carrington et al. Nat Rev Med 2003

HLA genotype	Model	Frequency		Epidemiological effect	Possible functional explanation
		Caucasians	African-Americans		
Class I homozygosity					
One locus	—	16.9%	14%	Rapid progression	Narrow range of HIV-1 peptides are presented to CTL; immune escape occurs rapidly
Two or three loci	—	5.5%	4.6%		
*HLA-B*27*	Dominant	5.1%	0.72%	Slow progression	Presents a conserved immunodominant epitope that is under structural constraint
*HLA-B*57*	Dominant	3.9%	6.1%	Slow progression	Broad cross-reactivity against HIV peptide variants
*HLA-B*35*	Codominant	7.7%	7.7%	Rapid progression*	Unknown
HLA Class I mismatching in HIV transmission	—	—	—	Protection from infection	Allogenic immune response against HIV and donor cells

HLA–B*57

Among the protective HLA effects to date, one of the most consistent genetic epidemiological associations has been between HLA-B*57 and slower HIV disease progression. Not surprisingly, increased frequencies of HLA-B*57 have been reported among HIV-infected SP [222-228].

HLA-B*57 has been associated with low VL set-point and long-term non progressive CI, which suggests that carriage of HLA-B*57 influences the quality and efficiency of immune responses generated against HIV [229]. Recently, studies have begun to address the functional significance of the HLA-B*57 genetic association with slow progression to AIDS by revealing unique characteristics of the molecule and HIV-specific response restricted by this allele.

Given the crucial role cellular immune responses generated during acute HIV infection play in subsequent disease progression, Altfeld et al. determined the impact of HLA-B*57 on the magnitude, breadth and maintenance of virus-specific $CD8^+$ T-cell response in acute infection and transition into chronic infection [230]. Expression of HLA-B*57 was significantly associated with the absence of a symptomatic HIV seroconversion syndrome and $CD8^+$ T cell responses restricted by this allele dominated the initial HIV-specific response and responses during the chronic phase of the infection [230]. $CD8^+$ T-cell responses restricted by HLA-B*57 molecules target multiple HIV peptides with predominant responses observed against Gag and RT motifs [231, 232].

The Caucasian HLA-B*5701 and closely related African B*5703 molecules both exhibit broad cross reactivity against common and rare variants of a dominant Gag epitope [233]. HLA-B*57 alleles demonstrate extreme plasticity in peptide binding and

strong HLA-B*57 restricted $CD8^+$ T-cell responses are generated against overlapping peptides of different lengths [234]. This broad peptide recognition specificity may explain part of the protective effect of HLA-B*57. Additionally, LTNP positive for a HLA-B*57 allele have the majority of their dominant HIV-specific $CD8^+$ T-cell response restricted by this allele [232, 235]. Furthermore, the protective effect of HLA-B*57 was observed in a study comparing LTNP and rapid progressors carrying HLA-B*57 [236]. $CD8^+$ T-cell responses from LTNP were primarily directed toward HLA-B*57-restricted Gag peptides, whereas HLA-B*57-positive progressors displayed a broader response to Gag peptides [236]. Collectively, these data suggest HLA-B*57-restricted $CD8^+$ T-cell responses may place significant constraints on viral escape, which translates into better VL control. However, recent work by Miura et al. dispute this by demonstrating that HLA-associated mutations are commonly found in EC [237].

The most effective immune responses might be those that target the regions of the virus where escape mutations inflict the largest fitness cost to the virus. A virus crippled by immune escape mutations could result in lower VL and delayed disease progression. A series of studies report that HLA-B*57-restricted $CD8^+$ T-cells target conserved regions of HIV thereby placing severe constraints on viral escape. So much so that when escape mutant viruses are transmitted to a new host that does not share the same HLA-B*57 alleles as the donor, they revert to the wild-type virus, which is reflects the fitness impact of the escape mutation [213, 238-240]. Work from Brockman et al. using recombinant viruses expressing plasma HIV RNA derived Gag-Protease sequences demonstrated that during CI, VRC correlates with the presence of known compensatory mutations such as the HLA-B*57 associated Gag T242N mutation [213]. This suggests

that the rescue of fitness defects occurred through mutations at secondary sites. The study concludes that HLA-B*57 and perhaps other protective alleles contribute to the selection of detrimental mutations in Gag during the acute/early phases of infection, but the effects of these mutations wane as compensatory mutations accumulate in the chronic phase of infection [213].

Not all alleles that belong to the same HLA supertype influence HIV disease progression in a similar manner, among the most divergent HLA associated clinical outcomes is the relatively good prognosis in HLA-B*5801 expressing persons and poor prognosis in HLA-B*5802 individuals [241-243]. These two alleles differ by only three aa in the regions involved in HLA-peptide recognition [244]. Yet the magnitude and breadth of HIV-specific $CD8^+$ T-cell response is significantly lower in HLA-B*5802 carriers [241, 243]. Therefore, minor differences in the HLA sequence can have a major impact on epitope recognition and subsequent viral containment and not all epitope specific responses contribute to immune containment [241].

Other HLA alleles associated with HIV disease progression

Epidemiological observations link carriage of HLA-B*27 to slow HIV disease progression .The mechanism underlying this association likely relates to areas of the virus that are targeted by HLA-B*27-restricted $CD8^+$ T-cell responses. An immunodominant response to a conserved HIV epitope in Gag p24 occurs in patients who carry HLA-B*27.

HLA-B*35, which is almost always found in haplotypic association with HLA-Cw*04, is the allele most consistently implicated in susceptibility to AIDS progression

[220, 245-247]. HLA-B*35 subtypes can be divided into two groups according to peptide-binding specificity namely: 1) the HLA-B*35-Py and 2) the broadly reactive HLA-B*35-Px [224]. HLA-B*35-Px is the group associated with rapid HIV disease progression.

Genome-wide association studies

One striking and largely unexplained variation in HIV infection is the level of plasma viremia during the chronic phase of infection. The VL setpoint can vary among individuals as much as 5 logs [248-251]. The first genome-wide association study (GWAS) aimed at identifying human genetic differences that influence this variation found associations between three single nucleotide polymorphisms (SNPs) and low levels of HIV viremia [252, 253]. All three SNPs mapped closely to HLA-B and in a non-coding site 35kb upstream of HLA-C, the *HCP5* gene which is in high linkage disequilbrium (LD) with the allele HLA-B*5701 [254]. These findings confirmed and emphasized the central role of the MHC in HIV control and the polymorphsims identified accounted for nearly 15% of the variation in VL setpoint among HIV-infected individuals [252, 253].

Recently, the HIC reported a GWAS aimed at identifying the genetic basis for EC status. The analysis was performed in a multiethnic cohort of EC and progressors and the effects of individual aa within HLA alleles was assessed. Of all the genome-wide SNPs identified, (n=313) that achieved significance were located within the MHC and none elsewhere. Three aa in HLA at positions 67, 70 and 97 had even stronger

associations with VL control than any single HLA allele including HLA-B*5701. As these three aa positions are located in the peptide-binding groove, they likely contribute to conformational differences that influence peptide presentation and through this influence the protective or susceptible nature of the various HLA-B allotypes.

Focusing on position 97 of the peptide-binding groove, the study also finds that depending on the aa present within the HLA-B allele, opposite disease outcomes result. Collectively, data from this seminal study links the major genetic impact of host control of HIV to specific aas involved in the presentation of viral peptides in infected cells. These results provide insights into the association of HLA alleles with disease progression by suggesting that it is likely related to the conformation of the peptide within the MHC class I binding groove. Thus, the nature of the HLA-viral peptide interaction is a predominant factor modulating durable control of HIV infection [255].

KIR/HLA genotypes and HIV disease progression

Further underlining the intricacies of host immunogenetics and HIV pathogenesis are epidemiological observations that link certain Killer Immunoglobulin-like Receptor (KIR)/HLA combined genotypes with beneficial outcomes such as time to AIDS and VL setpoint. Flores-Villanueva and colleagues noted that HIV infected EC were more likely to be homozygous for a Bw4-allele than those who could not control infection [256]. Alleles at the HLA-B locus can be dichotomized into HLA-B Bw4 or HLA-B Bw6 alleles based on aa between positions 78 and 83 of the heavy chain [223]. The Bw4 group also contains a number of HLA types known to confer protection against HIV infection

such as HLA-B*27 and HLA-B*57 and excludes those alleles associated with accelerated disease progression such as the HLA-B*35Px variant [224].

Survival analyses reported by Martin et al. examining the effects of HLA-B Bw4 alleles with an isoleucine at position 80 (Bw4-Ile80) in the absence or presence of the Natural Killer cell (NK) receptor KIR3DS1, revealed an epistatic interaction between KIR3DS1 and Bw4-80Ile and time to AIDS and were the first to report a synergistic effect of KIR and HLA within the context of HIV. The authors presented a model whereby a protective response involving the activation of KIR3DS1 expressing NK cells upon interactions with their putative HLA-B Bw480Ile ligands occurred within the context of slow disease progression [257]. Qi et al examined the same cohorts used by Martin et al and demonstrated that this compound genotype also confers protection against the development of AIDS defining opportunistic infections but not AIDS defining malignancies [258].

Interestingly, an increased proportion of KIR3DS1 homozygotes (hmz) has been reported in a cohort of HIV-exposed seronegative persons, highlighting the effect the presence of this activating receptor, with or without its putative ligand, have on outcomes following HIV exposure/infection [259]. However, Gaudieri and colleagues examined KIR and HLA genotype effects on pretreatment VL, rate of $CD4^+$ T-cell decline and progression to AIDS in a cohort of 174 Caucasians and observed an association between carriage of genes within the KIR region B haplotype (eg: KIR2DS2) and a more rapid $CD4^+$ T cell decline. Interestingly, the authors report that carriage of KIR3DS1/HLA-B Bw480Ile increased the chance of reaching the 1987 criteria for AIDS diagnosis ($CD4^+$

T –cell counts of <200 cells/mm^3) and suggest that KIR and HLA act both independently and in some cases synergistically to modify HIV disease progression [258]. Differences in cohort sizes and clinical follow-up time may account for the discrepancies in the findings of Martin et al versus studies from other cohorts [260] [261].

In a subsequent study from Martin et al. the effects of inhibitory KIR3DL1 subtypes in combination with HLA-B allelic groups on HIV disease progression and VL were explored. NK cell inhibitory capacities of individual KIR3DL1 allotypes are closely linked to their cell surface expression [262-265]. The study revealed a continuum of synergistic protective effects between certain KIR3DL1 allotypes and their HLA-Bw4 ligands relative to Bw6 hmz who do not express any ligands for KIR3DL1 receptors. The KIR/HLA combined genotype associated with the most favorable outcomes with respect to disease progression and VL was that which paired KIR3DL1*h/*y with HLA-B*57 (*h/*y*+B*57). The protective effect of HLA-B*57 in the presence of distinct KIR3DL1 groups indicates that the overall protection conferred by HLA-B*57 does not stem solely from its function as a $CD8^+$ T-cell restricting element in the adaptive immune response. [266].

The same *h/*y+B*57 combined genotype is reported to influence disease progression in a cohort of Zambian patients and is overrepresented in an exposed uninfected (EU) population compared with an HIV susceptible population in primary infection, suggesting that this particular compound genotype that favorably influences disease progression may also lower the risk of acquiring HIV [267, 268].

Functional data in support of these epidemiological observations further implicate NK cell receptor genes and their HLA ligands in modulating HIV pathogenesis. Alter et

al. used an *in vitro* viral replication inhibition assay to demonstrate that NK cells from carriers of KIR3DS1/HLA-B Bw480Ile strongly inhibit viral replication in autologous $CD4^+$ T cells in a dose and contact-dependent manner. These findings provide functional evidence that variation at the KIR locus influences the effectiveness of NK cell activity and that licensed NK cells were especially effective at containing HIV viral replication [269]. Alter and colleagues also demonstrate that during acute infection, KIR3DL1/3DS1$^+$ expressing NK cells are specifically expanded when they are from individuals who co-express HLA-B Bw480Ile alleles, thereby illustrating a ligand-dependent preferential expansion of licensed NK cell subsets within the context of HIV infection [270]**.**

Adding to a growing body of functional data in support of a link between KIR/HLA genotypes and NK cell function within the context of HIV, recent studies examined whether the KIR/HLA compound genotypes linked to better HIV outcome would confer a superior NK cell quality (functional potential). Upon stimulation with MHC class I devoid K562 targets, Boulet et al. report that the functional potential of NK cells from individuals carrying the *h/*y+*B57 combined genotype previously associated with favorable clinical outcomes, exhibit enhanced tri-functional potential (ie: positive for three markers of antiviral function) compared to carriers of any other KIR3DL1/HLA-B combined genotype or Bw6 hmz [271]. The study attributes the increased levels of tri-functional NK cells from *h/*y+*B57 individuals to the expression of potent high expression inhibitory receptors that are licensed for enhanced antiviral function when the inhibitory signal is disrupted as is the case during interactions with HLA-devoid K562 target cells or HIV-infected cells with reduced expression of HLA-A and -B [271]. One

of the studies' drawbacks was the inability to rule out the possibility that the increased NK cell tri-functional potential was solely an HLA-B*57 mediated effect due to the paucity of HLA-B*57 persons positive for the KIR3DL1*l/*x combined genotype.

Chapter three of this thesis extends the findings of Boulet et al. by assessing the functional potential of NK cells from HIV infected SP with defined KIR3DL1 receptors and HLA ligands. In accordance with the licensing theory, we show that SP who express at least one copy of an HLA-B Bw4 allele had elevated tri-functional potential in comparison to Bw6 hmz. Furthermore, we demonstrate that poly-functional NK cells are more potent for each individual function than mono-functional NK cells. We report that among Bw4 carriers, NK cells from HLA-B*57 and KIR3DL1*h/*y co-carriers have higher poly-functional responses than those who express either the receptor or the ligand alone or neither, thereby confirming the requirement of both the HLA-B*57 ligand and receptor in the KIR3DL1*h/*y genotype for the observed increased functional potential among these individuals. Within the context of HIV infection, the protective effect of HLA-B*57 may be mediated through mechanisms that involve NK cells in addition to T cells.

Collectively, these data from human studies reporting variations in NK cell potency according to KIR/HLA combined genotypes, support the NK cell licensing theory. These functional data illustrate that variations at KIR and HLA govern NK cell responsiveness and compliment epidemiological observations linking certain KIR/HLA compound genotypes with differential clinical outcomes by implicating enhanced NK cell activity in certain persons as a mechanism underlying their slower disease progression or protection from infection.

Adaptive immune response to HIV infection

The generation and maintenance of a successful primary and secondary immune response to viral infection involves the co-coordinated effort of both humoral and cellular immune responses that collectively constitute the host adaptive immune response. In comparison to the rapid responses to invasion by foreign antigens by the innate immune system, adaptive immune responses to pathogens are delayed and dependent on the presentation of pathogen-derived peptides in the context of MHC class I or II molecules by professional antigen presenting cells (APCs).

T cells in HIV infection

$CD8^+$ CTL

Antigen specific T cells are crucial components of the adaptive immune response to viral infection. The temporal association between the emergence of HIV-specific $CD8^+$ CTL and the resolution of peak viremia to a set-point in the acute phase of HIV infection was one of the first lines of evidence in support of the importance of CTL in the initial defense against HIV [119, 120]. Studies in animal models for HIV infection such as Rhesus macaques infected with SIV strongly implicate a role for CTL in control of viremia. Depletion of $CD8^+$ T cells in infected macaques prevented the resolution of viremia and led to rapid disease progression until reconstitution of the $CD8^+$ T cell subset

[124, 272]. Furthermore, studies examining the correlation between plasma viremia and CTL response levels during the chronic phase of HIV infection indicate a pivotal role for CTL in controlling viral replication and were substantiated with data demonstrating that CTL can efficiently inhibit viral replication *ex vivo* [273-277].

The association between certain MHC class I alleles and different rates of disease progression established significant support for CTL in the containment of HIV [169, 223, 250, 252, 278, 279]**.** HIV-specific CTL responses arise as viremia approaches its peak during the acute phase of infection [119, 120]. This CTL response peaks 1-2 weeks later as the viremia declines. Recent work from Fisher et al. report traces of immune escape in samples collected from patients during very early stages of acute infection suggesting that immune pressure is present and effective earlier than previously reported. In addition, Fisher et al. quantified the loss of the founder virus and directly implicate CTL responses in viral containment [280]**.** Collectively, these data provide compelling evidence for CTL in driving HIV diversity within the individual and containing viral replication [280-282]**.**

Intra-epitope escape mutations subvert CTL recognition by reducing peptide affinity for MHC class I binding or for T cell receptor recognition. Extra-epitope mutations (occurring within the epitope flanking residues) alter antigen processing and affect epitope recognition by changing the relative abundance of the peptide, the structure of peptide-MHC class I complexes seen by the CTL and the ability of the CTL clonal repertoire to recognize variants [283, 284].

Functional properties of protective CTL responses

$CD8^+$ T cell quality and poly-functionality

T cells respond in several ways to antigenic stimulation. These include the production and release of various cytokines and chemokines and the exocytosis of pre-formed cytolytic granules containing perforin and granzyme. The functions that have been shown to be relevant in antiviral immunity are proliferative capacity, IL-2, IFN-γ, TNF-α, and MIP-1β cytokine and chemokine secretion and cytotoxicity as measured by perforin/granzyme B expression and CD107a/b mobilization as a marker of degranulation [285-288].

To effectively characterize the complex $CD8^+$ T cell response to HIV, assessments of T cell function and phenotype have been employed. A fundamental characteristic of an HIV-specific T-cell response is its magnitude, which is commonly represented as the frequency of antigen-specific T cells. Another characteristic is its breadth, which is the number of HIV epitopes recognized. A third descriptor of T cell responses is the number of effector functions the T cell is capable of also referred to as the quality of the T cell response. The magnitude and breadth of a T-cell response as measured by a single function do not capture the full potential of the antigen-specific T cell response.

Flow cytometry can be exploited to concurrently characterize a combination of T cell functions and enables the assessment of the magnitude, breadth, phenotype and functional capacity (quality) of antigen-specific T cells. Measuring the qualitative aspects of HIV-specific T-cells has increasingly become the focus of attempts to identify reliable correlates of immune protection against HIV. Initial studies characterized the quality of HIV-specific T-cell responses by assessing IL-2 and IFN-γ production. Despite the clinical relevance of dual secreting HIV-specific T cell responses, a substantial fraction

of the antigen-specific population was missed by focusing on two functions only [289, 290].

Studies comparing HIV-specific CTL responses from HIV infected treatment naïve LTNP and progressors have begun to address some of the functional attributes and signatures associated with $CD8^+$ T cell mediated control of viral replication. Betts and colleagues applied a multi-parametric flow cytometry approach to assess the quality of the HIV-specific $CD8^+$ T cell response by measuring 5 T cell functions (degranulation, IFN-γ, MIP-1β, TNF-α and IL-2) in chronically HIV-infected progressors and LTNP. Poly-functional T cells were defined as a subpopulation of HIV-specific $CD8^+$ T-cells that retained antigen specific proliferative capacity by secreting IL-2 in addition to carrying out effector functions such as degranulation, IFN-γ, MIP-1β, and TNF-α secretion. The authors reported limited poly-functionality in progressors compared to LTNP. Importantly, rather than focusing on the frequency of the HIV-specific $CD8^+$ T-cell response, they assessed the relationship between functionality and VL by correlating the percent of the HIV-specific T cell response positive for 5 functions (5+ response) with VL in progressors. They found these parameters to be inversely correlated, whereas the absolute frequency of the HIV-specific $CD8^+$ T cell response did not correlate with VL [291]. The work by Betts et al. prompted a debate on whether the assessment of phenotypic markers was sufficient to predict protective capacity. Their results argued against this tenet since poly-functionality was not restricted to a particular memory phenotype [291]. Even though the frequency of HIV-specific $CD8^+$ T cells positive for more than one effector function is increased in situations of controlled viremia, the

elusive functions or combination of functions that bestow $CD8^+$ T cells with the capacity to eliminate virally infected cells have yet to be determined.

The fact that multi-functional T cells are associated with slower HIV disease progression is not simply explained by the fact that each cell is capable of a broader repertoire of functions. Interesting work from studies examining T cell responses following immunization of mice with non-live vaccines encoding *Leishmania major* antigens and humans with Mycobacterium bovis bacillus Calmette-Guerin (BCG) vaccine noted a 10-fold increase in the mean fluorescence intensity (MFI) of IFN-γ for multi-functional $CD4^+$ T-Helper 1 (Th1) cells compared to mono-functional Th1 cells. This attribute is not unique to multi-functional $CD4^+$ Th1 cells since poly-functional $CD8^+$ T cells also secrete more IFN-γ on a per cell basis and therefore have a higher MFI for IFN-γ compared to mono-functional $CD8^+$ T cells. Furthermore $CD8^+$ T cells that secrete IFN-γ and TNF-α have enhanced cytolytic activity and mediate more efficient killing compared to single cytokine secreting $CD8^+$ T cells for either function [291-293]. In chapter 4 we will show that poly-functional NK cells also exhibit more potent function on a per cell basis than mono-functional NK cells.

Phenotype

The most powerful phenotypic tools to enumerate antigen specific T cells are peptide-MHC class I/ and or class II tetramer complexes. For the past 20 years a multitude of phenotypic cell surface markers have been used to define different populations of antigen specific $CD4^+$ and $CD8^+$ T cells and these markers have been

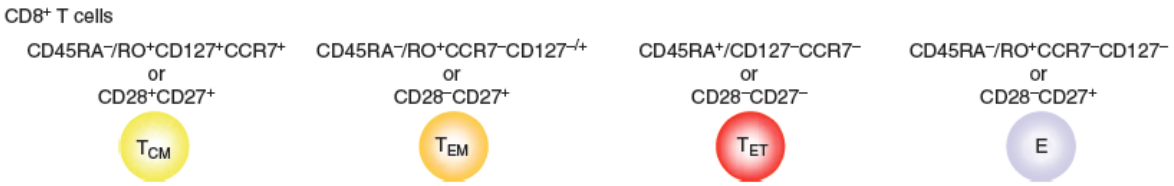
proposed to identify functionally distinct T-cell populations undergoing different stages of T-cell differentiation. Virus-specific $CD4^+$ and $CD8^+$ T cell responses display great phenotypic and functional heterogeneity among antigen experienced T cells. Antigen experienced/specific T cells are referred to as memory T cells and were traditionally defined as antigen-specific T cells following antigen elimination or effective clearance. However, current definitions of the term memory also refer to antigen-specific T cells under conditions of high antigen load associated with the lack of pathogen elimination and/or control.

The combined use of CD62L, CD45RO, CD45RA, CD27, CD28, CD7, CD57, CD127 and CCR7 has allowed the identification of antigen-specific T cells undergoing different stages of differentiation. On the basis of proposed differentiation models, three distinct subsets of memory $CD8^+$ T cells were distinguished using combinations of the CD28 and CD27 markers [294-296].

1. Central memory (T_{CM}) $CD8^+$ T cells: $CD28^+CD27^+$ or $CD45RA^-CCR7^-$
2. Effector memory (T_{EM}): $CD28^-CD27^+$ or $CD45RA^-CCR7^-$
3. Terminally differentiated (T_{ET}): $CD28^-CD27^-$ or $CD45RA^+CCR7^-$.

The addition of markers such as CD127 (Il-7 receptor-α chain) and CD57 (associated with T cell senescence) improves the definition of memory subsets somewhat by reducing the level of overlap between the different subsets of memory $CD8^+$ T cells listed above [294-304]. Current operational definitions of memory $CD8^+$ T-cells at early stages of differentiation are shown in Figure 6

Figure 6: Phenotypically distinct populations of memory and effector $CD8^+$ T cells identified using combinations of CD45RA/RO, CCR7, CD127, CD28 and CD28 adapted from Harari et al 2006.



There is a growing body of evidence that particular viral infections can induce divergent memory $CD8^+$ phenotypic profiles [296, 305-308]. Appay et al. were among the first to report phenotypic differences among antigen specific $CD8^+$ T cells during the chronic phases of infection with HIV EBV, and HCV and attribute these differences to distinct characteristics of virus infection such as antigen load and persistence [296].

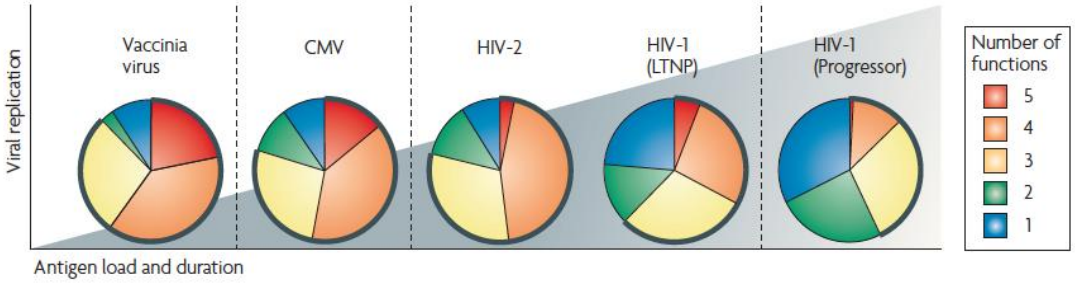
Changes in the expression of cell-surface differentiation antigens on $CD8^+$ T cells reflect the signals that have acted on these cells *in vivo*. Factors that determine the quality of T-cell responses are the amount and duration of exposure to antigen, the type of APC delivering signals 1 and 2 to the T-cell and the innate cytokine milieu. In addition, the heterogeneity of memory $CD8^+$-T cell responses is thought to correspond to different levels of viral replication and disease activity. Viral infections (and/or stages of different viral life cycles) can be classified into four models according to differences in viral

replication, antigen exposure and persistence. The four models are; antigen clearance, protracted antigen exposure and persistence with low antigen load; antigen persistence and high antigen load and acute antigen exposure/ and or re-exposure with high antigen load.

Studies have found a correlation between phenotypically defined $CD8^+$ T cell memory subsets and enhanced control of viral replication within the context of viral infections that represent the four models of viral infection such as non-progressive HIV, progressive HIV and CMV infection [300, 309, 310]. HIV specific $CD8^+$ T cells during primary HIV infection usually acquire a T_{EM} phenotype. However progression into the chronic phase of infection is associated with a failure to maintain T_{EM} cells and/or transition into the T_{CM} phenotype. Therefore unlike in other chronic infections, the HIV-specific $CD8^+$ T cells appear to be 'frozen' in a $CCR7^-CD27^{dull}CD28^-CD45RO^{dull}CD57^-$ phenotype [288, 294-296, 311-313]. Interestingly, in LTNP subjects there is evidence of some level of differentiation to T_{EM}, which may be related to a certain level of viral control among these individuals [296, 314].

Different patterns of cytokines have been associated with specific subsets of memory T cells. T_{CM} have been associated with IL-2 secretion and enhanced proliferative capacity, whereas T_{EM} cells primarily secrete IFN-γ. The enhanced ability of T_{CM} to produce IL-2 confers improved protection compared to T_{EM} to a systemic viral challenge [302]. However, functional output is not predictive of memory and therefore combined phenotypic and functional analyses allow for greater insights into whether a response is protective rather than either measurement alone [315].

Figure 7: Correlation of antigen-specific $CD8^+$ T cell quality with viral load adapted from Seder et al. Nature Reviews Immunol. 2008



Collectively these data strongly suggest that there exists a $CD8^+$ T cell subset with a distinct functional profile that is associated with enhanced antiviral capacity and may serve as an immunological correlate of protection against HIV. As there is no small animal model for HIV infection, most studies examining this issue have relied upon investigations in LTNP. This has led to an extensive debate surrounding the attribution of directionality to the issue of cause-and-effect. Do HIV-specific poly-functional $CD8^+$ T-cells actually drive the low viremia observed in LTNP or is their poly-functional nature a byproduct of an already low antigen load. This debate is far from being resolved.

However, studies examining the effect of ARV treatment on HIV-specific $CD8^+$ T-cell poly-funtionality demonstrate that the artificial control of viral replication can restore the generation of HIV-specific $CD8^+$ T cells. These findings indicate that antigen load has a dominant influence on the phenotypic and functional profile of HIV-specific $CD8^+$ T cells [316]**.** However, upon cessation of treatment, virologic failure ensues despite improved function [317-319]. Therefore, the relationship between phenotype, function and antigen load within the context of HIV infection is still being delineated.

Inhibitory co-receptor expression

Recently, a role for inhibitory markers belonging to the B7-CD28 family in several viral infections has been extensively examined. The B7-1 (CD80)/B7-2 (CD86)-CD28/CTLA-4 pathway is the best-characterized T-cell co-stimulatory pathway and is crucial for both T-cell activation and tolerance. B7-1 and B7-2 provide important co-stimulatory signals to augment and sustain a T-cell response via interactions with CD28. CD28 transmits a signal that synergizes with the T cell receptor (TCR) signal to promote T-cell activation. CD28 signaling regulates the threshold of activation and significantly decreases the number of TCR engagements required for effective T-cell activation [320]. CTLA-4 engagement delivers a negative signal inhibiting TCR and CD28 mediated signal transduction by inhibiting IL-2 synthesis and progression through the cell cycle [321]. The outcome of a T-cell response involves the balance between CD28-mediated T-cell activation and CTLA-4-mediated inhibition.

Programmed death (PD)-1 is a member of the CD28 family and is expressed on both $CD4^+$ and $CD8^+$ T cells. During chronic HIV infection, part of the functional impairment of HIV-specific $CD8^+$ T cells is in part due to the up-regulation of PD-1. The increased expression of PD-1 correlates positively with both VL and negatively with cytokine production. Of key interest, this phenomenon is unique to HIV-specific $CD8^+$ T cells as those specific for CMV in the same subjects do not up-regulate PD-1 and maintain the capacity to secrete high levels of cytokine. Furthermore, blocking PD-1 engagement with PD-1 ligand (PD-1L) enhances survival of HIV-specific $CD8^+$ T cells, proliferation and cytokine production [322].

$CD4^+$ T cells and HIV infection

$CD4^+$ T cells play a central role in immune protection. They do so through their capacity to help B cells make antibodies, induce macrophages to develop enhanced microbicidal activity and recruit neutrophils, eosinophils and basophils to sites of infection. Through their production of cytokines and chemokines $CD4^+$ T cells orchestrate a panoply of immune responses. In 1986, Mossmann and Coffman identified two subsets of activated $CD4^+$ T cells, a T-helper-1 (Th1) population that secreted IFN-γ as the signature cytokine and an IL-4 secreting population called T-helper-2 (Th2) [323]**.** Each subset represents a distinct lineage of $CD4^+$ T cells with different functions. Th1 cells are critical for immunity to intracellular pathogens whereas Th2 cells play a dominant role in the elimination of extracellular pathogens. Our understanding of $CD4^+$ T cell subsets and the mechanisms they use to achieve their differentiated state has

expanded during the last decade. After activation, naïve $CD4^+$ T cells differentiate down four distinct pathways that are determined by the signals they receive during their initial interaction with antigen. They can become Th1, Th2, Th17 and induced regulatory T-cells (iTreg) [324].

CD4+ T-cell subsets and HIV persistence

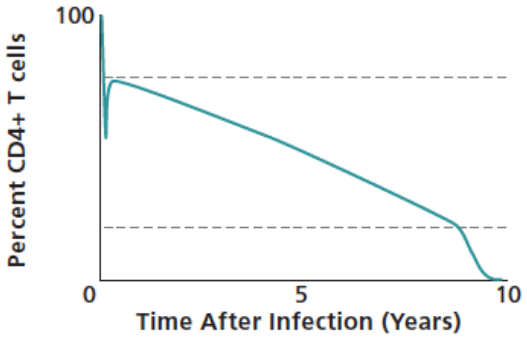
Similar to $CD8^+$ T-cells, the $CD4^+$ memory T-cell pool is primarily composed of two main compartments, central memory (T_{CM}) and effector memory with distinct effector functions and homing capabilities [300, 325]**.** In comparison to T_{EM}, T_{CM} have an increased capacity to survive and proliferate after activation and home to secondary lymphoid organs via the expression of chemokine receptor-7 (CCR7). T_{EM} are endowed with immediate effector function upon antigen stimulation and ensure the development of a rapid antigen-specific immune response to invading pathogens [326]. T_{CM} can differentiate into T_{EM} after TCR triggering and in certain circumstances in response to IL-7 and IL-15 [327]. A transitional (T_{TM}) memory subset that expresses both CCR7 and CD27 (a member of the tumor necrosis factor superfamily), has also been identified that displays characteristics intermediate to those of T_{CM} and T_{EM} [328]. The generation of memory T cells from effector T cells during HIV infection is suspected to be a major determinant for the establishment of a latent reservoir for the virus [326].

Massive $CD4^+$ T cell depletion in HIV infection

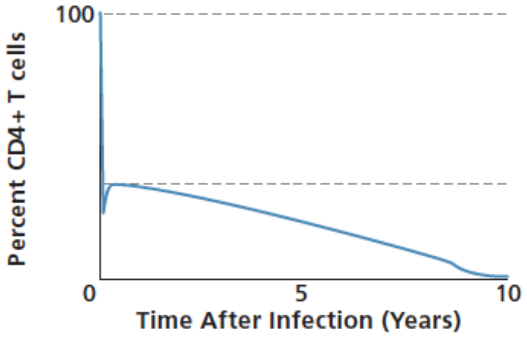
Brenchley et al demonstrated that the bulk of $CD4^+$ T-cell depletion occurred within the first weeks of infection of Rhesus macaques with SIV and was predominantly localized to the gastrointestinal (GI) tract where 80% of memory $CCR5^+CD4^+$ T cells reside [122]. GI memory $CD4^+$ T cells were more frequently infected than peripheral blood memory $CD4^+$ T cells with a frequency that differed by 10 fold in some cases [122, 329, 330]. This severe depletion of the memory T-cell population has profound repercussions for subsequent disease course [122, 330-332].

Figure 8: Traditional (A) and revised views (B) of HIV disease course adapted from Douek et al. HIV disease progression: Immune activation, microbes and a leaky gut.

A



B



Brenchley and colleagues further demonstrated that consequences of this severe depletion of the GI $CD4^+CCR5^+$ T-cell memory pool was a significant breach in the mucosal barrier permitting the translocation of gut-derived microbes or microbial products into the systemic circulation in the absence of overt bacteremia [122]. By quantitating the levels of lipopolysaccharide (LPS) as an indicator of microbial translocation they detected significantly higher levels of plasma LPS in HIV infected patients with progressive disease in comparison to EC and HIV uninfected individuals**.** They also found an association between the reduction in plasma LPS and $CD4^+$ T-cell reconstitution with individuals treated with HAART and demonstrated that that plasma LPS levels correlated with other markers of immune activation such as the increased frequency of HLA-DR^+CD8^+ T cells [122, 199]**.** Collectively these data provide mechanistic insights into the underlying causes of HIV $CD4^+$ T-cell depletion and critically link the pathological changes in the GI tract during the acute phase of infection to microbial translocation, which is associated with immune activation.

$CD4^+$ Th17 cells in HIV infection

The intestinal mucosal immune system is designed to cope with invading microbial pathogens and control inflammation and immune activation following the initial clearance of infection [333, 334]. Enteric pathogens infecting mucosa induce the preferential differentiation of naïve T cells into Th17 cells. The production of large amounts of IL-17, IL-21 and IL-22 by Th17 cells helps maintain GI integrity and the generation of a rapid response to microbial pathogens at mucosal sites [333, 335] Cytokine production by Th17 cells promotes neutrophil activation and recruitment and

the induction of antimicrobial peptides that contribute to the containment or clearance of enteric pathogens [335]. Of key importance, Th17 derived IL-17 and IL-22 are involved in the renewal and maintenance of the intestinal epithelial barrier by supporting the production of tight junctions. Th17 cells therefore have a direct role in maintaining GI tract integrity and coordinating mucosal innate and adaptive immune functions [335].

Notably, mucosal recovery is associated with a pronounced restoration of the Th17 subset [336]. Therefore, the massive depletion of mucosal Th17 cells during the acute phase of HIV infection creates severe immune vulnerabilities that are difficult to recover from because of the specific protection Th17 cells confer at this important interface.

$CD4^+$ T cell function in HIV infection

It is generally believed that the primary role of $CD4^+$ T cells is to co-ordinate different arms of the adaptive immune response so as to shape an effective response against invading pathogens and regulate deleterious immune related pathology. Even though the clearance of certain chronic viral infections such as HCV, EBV and CMV appears to be highly dependent upon the presence of antigen-specific $CD4^+$ T cells, limited attention has been paid to the impact of HIV-specific $CD4^+$ T-cell responses in the control of viral replication compared to that of HIV-specific $CD8^+$ T cells [337, 338].

The cytokine secretion patterns of antigen-specific $CD4^+$ T cell responses during acute infection are characteristic of an effector response with IFN-γ and TNF-α secretion dominating at the expense of proliferative capacity and IL-2 secretion [339]. During the

chronic phase, this $CD4^+$ T cell effector profile persists. However, the $CD4^+$ T-cell cytokine secretion profile of LTNPs or successfully treated patients is more poly-functional. It is characterized by higher frequencies of dual IFN-γ and IL-2 secreting $CD4^+$ T cells than is seen in progressors. Therefore, suppression of viral replication may be associated with the presence of poly-functional HIV-specific $CD4^+$ T cells with proliferative capacity [289, 290, 340-342]. These observations suggest that increased levels of viral replication obstruct the generation of IL-2 secreting HIV-specific $CD4^+$ T cells endowed with proliferative capacity [289, 290, 304, 341, 342]. Antigen-specific poly-functional $CD4^+$ T cells have also been observed in other chronic viral infections under conditions where viremia is effectively controlled such as EBV, CMV and (herpes simplex virus) HSV [341, 343].

Immune activation

Lessons from nonprogressive and progressive (SIV) infections

Both SIV infection of Asian macaques and HIV infection of humans almost invariably result in a chronic infection and progression to AIDS if left untreated. In contrast, SIV infection of natural African NHP hosts such as sooty mangabeys (SM), African green monkeys (AGM), mandrills and chimpanzees is typically non-progressive. Nonpathogenic SIV infection of natural hosts is characterized by preserved immune competence in the face of high levels of viremia [344-346].

What distinguishes non pathogenic from pathogenic SIV infection is the lack of immune activation associated with the early mucosal $CD4^+$ T cell depletion in SM and AGM and the reconstitution of the mucosal $CD4^+$ T compartment during the chronic phase of infection. This preservation of the mucosal compartment results in the maintenance of GI tract integrity, a lack of microbial translocation into the intestinal lumen and subsequent systemic circulation of microbial products [347].

Therefore, the two primary clinical markers of disease progression in HIV infection, VL and $CD4^+$ T-cell count cannot account for the non progressive nature of SIV infection in SM and AGM.

Immune activation and HIV prognosis

The establishment of a state of chronic, generalized immune activation is a characteristic feature of pathogenic HIV infection. Ordinarily immune activation reflects the mounting of antiviral immunity and is beneficial in cases of an infection with any other pathogen. However data from a series of studies have reported that T-cell activation levels as measured by the expression of activation markers on T cells are predictive of an adverse prognosis for HIV infected patients.

Seminal work from Giorgi et al. report that elevated frequencies of $CD38^+CD8^+$ T-cells and the intensity of CD38 expression on $CD8^+$ T cells have strong prognostic value for progression to AIDS that is independent from VL [348]. CD38 is a multifunctional transmembrane glycoprotein that is up-regulated during the early phases of T-cell activation [349, 350]. Physiologically, CD38 expression and or/ligation has been associated with increased cell-to-cell adhesion, increased levels of cytokine

production and more rapid $CD4^+$ T-cell proliferation [351-353]. Importantly, CD38 expression on T cells strongly correlates with other markers of cellular activation [354-356].

The immunologic and virologic events that occur during the earliest stages of HIV infection have an impact on subsequent disease progression. Given the impact of immune activation on disease outcome, Deeks et al. assessed the relative contributions of viremia and T-cell activation to the rate of $CD4^+$ T-cell loss in a prospective cohort of acutely and recently HIV-infected adults. In doing so, Deeks et al. also resolved the questions as to whether immune activation continues to escalate during progression into the chronic phase of infection or whether similar to VL the level of immune activation plateaus at a steady state called the immune activation set point [357]. We have generated data presented in chapter 2 of this thesis supporting the concept of immune activation setpoint.

Although lower plasma HIV RNA levels in the absence of ARV therapy predict slower rates of clinical progression, the extent of viral replication cannot fully account for all the variability in the rates of subsequent $CD4^+$ T cell decline and progression to AIDS among HIV-infected patients [358, 359]. Indeed, some subjects with undetectable plasma HIV RNA levels in the absence of therapy have experienced clinical progression indicating that factors other than level of viral replication contribute to immunodeficiency [200, 201, 203, 360-362].

Hunt et al. investigated whether within the context controlled viremia elevated T cell activation contributed to immunodeficiency. [199]. They reported abnormally high levels of T-cell activation levels in EC (as measured by the frequency of $CD4^+$ and $CD8^+$

T cells that co-expressed CD38 and HLA-DR) compared to HIV-uninfected persons but lower levels compared to patients with detectable viremia. Among EC, lower $CD4^+$ T counts were associated with higher percentages of activated $CD4^+$ and $CD8^+$ T-cells and higher LPS levels positively correlated with the frequency of activated $CD38^+$HLA-DR^+CD8^+ T-cells [199]. Consistent with the hypothesis that T cell activation drives $CD4^+$ T cell decline, the authors concluded that in a setting of controlled viremia, T-cell activation is associated with $CD4^+$ T-cell decline, indicating that T-cell activation plays a significant role in immunodeficiency independent of viremia. However, T-cell activation is unlikely to be the sole mediator of $CD4^+$ T cell decline because T cell activation declines dramatically after initiation of ARV therapy long before there is a significant restoration of $CD4^+$ T cells in the peripheral blood or tissues [199]. Longitudinal studies examining mechanistic link between chronic inflammation and progressive immunodeficiency within the context of controlled viremia should be more definitive in outlining whether T cell activation is a cause rather than a consequence of $CD4^+$ T-cell depletion in EC [199].

Chapter 2 presents results on the assessment of this immune activation marker in a group of 34 EC, 25 of who had sufficient longitudinal follow up to obtain an annual rate of CD4 decline. We found that 7 of 25 EC had declining CD4 counts and that the percent $CD38^+$HLA-DR^+CD8^+ T-cells could not distinguish those with falling versus stable/increasing CD4 counts. These results call into question the cross sectional results reported by Hunt et al. described above.

Humoral Immunity in HIV

Successful protective immunity against intracellular pathogens requires a concerted effort from both the T and B cell compartments of the immune system. In addition to the progressive depletion and dysfunction of $CD4^+$ T cells, HIV infection is accompanied by an aberrant humoral immune response due to deficiencies in the frequency, phenotype and function of HIV-specific B cells.

B-cell dysregulation in HIV infection

B cell hyperactivity in a disease characterized by immune deficiency is a hallmark of HIV infection. AIDS patients have B cell dysfunctions such as spontaneous activation of IgG secreting B-cells, hypergammaglobulinemia, increased polyclonal B-cell activation, increased cell turnover and expression of activation markers (CD70 and CD71) and co-stimulatory molecules (CD80 and CD86) [363-367].

Neutralizing antibodies (NAbs) and their cognate neutralizing epitopes on HIV

HIV infected individuals develop Abs to HIV Env as early as 50 days after infection. These anti-HIV Abs do not resolve natural infection and vaccine candidates that stimulate the production of NAbs have failed to protect against the acquisition of infection. These failures have led to rigorous efforts aimed at understanding the reasons behind the inability of Abs against HIV viral proteins to confer protection from infection.

Only Abs specific for the surface Env viral protein are capable of neutralizing HIV [368, 369]. The Env protein manifests a vast amount of antigenic diversity that adversely impacts Ab reactivity and importantly, most of the anti-Env Abs generated during natural infection are directed to regions of gp120 or gp41 that are not exposed on the mature functional virus Env spike [370-372]. As a result, a large proportion of the Abs detected by gp120 or gp41 ELISA assays are unable to bind to and/or neutralize the virus. Even though approximately 20% of HIV-infected patients can generate Abs against HIV that are capable of a moderate breadth of HIV neutralization and 5% generate bNAbs, these Abs are rarely able to neutralize autologous comtemporary virus [373, 374].

The HIV epitopes targeted by bNAbs are categorized into groups based upon the specific regions of the Env viral protein they recognize. The CD4 binding site (CD4bs) region of gp120 is recognized by bNAbs such as b12, VRC01 and VRC03. More than 50% of clade B viral isolates and 30% of non-clade B isolates can be neutralized by b12; approximately 90% of viral isolates tested are neutralized by VRC01 [375, 376]. Complex glycans that seclude a conserved vulnerable site on gp120 are recognized by bNAbs such as 2G12 [372, 376-378]. The induced CD4 site (CD4i) is recognized by bNAbs such as b17b. However, b17b and related Abs that bind to this site all have

limited neutralization activity against primary isolates perhaps because this epitope is exposed very briefly after viral attachment [379]. The membrane proximal region (MPER) on gp41 is recognized by bNAbs such as 2F5, 4E10 and Z13[380-382]. By binding to the MPER, NAbs interfere with viral fusion to the host cell and block viral entry [383]. Some studies have suggested that the shared homology between the MPER and self-proteins may result in self-tolerance mechanisms selecting against MPER-reactive antibodies as a plausible explanation behind the rarity of these NAbs in natural infection [384]. The last category of NAbs includes the novel bNAbs PG9 and PG16 that have been recently found to recognize the Env spike [385, 386].

Despite the initial encouraging data on neutralization potency and cross-reactivity of these bNAbs, attempts to elicit them by immunization have been disappointing. Although Abs to HIV can appear very early after infection, NAbs to HIV develop slowly and are rarely seen before several months of infection [387]**.** Furthermore HIV rapidly escapes from NAb pressure. Therefore NAbs directed to these variable regions of the Env protein may be ineffective in controlling initial viremia because of their limited neutralization activity towards autologous escape variants [388-390]. In addition, studies conducted in EC examining whether NAbs contributed to their VL control have been inconsistent in their findings [391-393].

It has become increasingly evident that a better understanding of the humoral immune response to HIV infection is crucial in order to identify the parameters associated with the generation of an ‘optimal’ Ab response to HIV such that it can be replicated in a therapeutic or prophylactic vaccine setting.

Antibody-Dependent Cellular Cytotoxicity (ADCC)

NAbs bind to epitopes on the virus and inhibit the viruses' ability to infect. However, once a cell is infected the role for NAbs becomes marginal. Luckily, neutralization is not the only host defense strategy available to virus-specific Abs. HIV-specific IgG1 binds to viral epitopes that are expressed on the cell membranes of infected cells. The exposed Fc region of these bound Abs interacts with FcγIII receptors on NK cells, monocytes and neutrophils that are capable of mediating antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cell-mediated viral inhibition (ADCVI) activity. The antigen-specific binding site of the Ab enables these immune cells to specifically target virally infected cells. Assessments of ADCC and ADCVI effector activity use assays that measure the number of HIV-infected cells that are killed or virus inhibition for ADCC and ADCVI respectively [394].

It is extremely rare for one antibody to mediate both the ADCC and ADCVI effector functions as well as neutralize the virus as these activities are mediated by different specificities of antibodies that do not necessarily overlap. In spite of the paucity of NAbs in the sera of HIV-infected patients, HIV-specific serum ADCC Abs are detected in a large majority of HIV-infected individuals [395]. Furthermore, studies of ADCC activity report higher serum ADCC titers in LTNP in comparison to progressors, indicating ADCC may have positive impact on viral control [396]. A recent study investigating a role for ADCC in viral control reported that unlike NAbs, EC have higher serum titers of Abs with ADCC function than viremic HIV-infected subjects. Forthal et al. also report that serum ADCVI antibodies in individuals from the Vax 004 clinical HIV vaccine trial correlated inversely with the rate of acquiring HIV infection [397].

Collectively these data demonstrate that in HIV infection, humoral immunity can play a role in natural host defenses by targeting several immunogenic epitopes, supporting various effector functions mediated by cells of the innate immune system and contribute to the a protective adaptive immune response. A successful HIV vaccine will have to incorporate an immunogen that effectively stimulates as many protective specificities and functions of HIV-specific Abs as possible [398].

The Innate Immune response to HIV

The innate immune response is an early line of defense against invading pathogens. It relies on cellular and soluble factors to work in tandem to mount a rapid response following the initial recognition of pathogen associated molecular patterns (PAMPS). The full appreciation of anti-microbial innate immune responses came with the discovery that a conserved family of PAMP recognition receptors (PRR) in *Drosophila* called Toll-Like Receptors (TLR), were responsible for determining susceptibility to fungal infection [399]. TLR are PPRs for ligand molecules derived from microbes or host cells. When triggered, TLR play a pivotal role in initiating an immediate anti-microbial innate response. The human homologues of TLR discovered by Medzhitov in 1997, are differentially located on the cell surface or intracellular vesicular compartments of the majority of innate immune cells [400].

The innate immune response to HIV infection involves the coordinated activation of innate immune cells either through triggering of PRRs, the detection of aberrant

expression of self-MHC class I molecules or stimulation by early pro-inflammatory cytokines. Plasmacytoid and myeloid dendritic cells (pDC and mDC) and NK cells are among the first cells to sense HIV infection and transmit this information to other innate cells in the periphery. Their activation in response to TLR signaling or otherwise results in the rapid secretion of Type-1 interferons (IFN) such as IFN-α and the creation of a pro-inflammatory environment via the secretion of IFN-γ and TNF-α that promote the recruitment and activation of macrophages, monocytes and neutrophils to mucosal sites. The release of pro-inflammatory cytokines and chemokines and innate soluble mediators combat the early onslaught of the virus and shape the adaptive immune response to HIV.

Receptors involved in the innate immune response to HIV infection

PRR: TLR and Nod like receptors (NLR)

Membrane anchored TLR and cytosolic NLR are the two receptor families that mediate immediate responses to invading pathogens or tissue injury. The mammalian TLR family consists of 11 members. Cell surface TLR include TLR 1, 2, 4 and 6 that recognize microbial membrane lipid motifs, whereas TLR 3, 7, 8 and 9, which are specific for microbial nucleic acids are located on endolysosomes. Intracellular localization avoids responses to self-nucleic acids [401]. To date, ligands for only eight of the TLR have been identified.

TLR 3, 7, 8 and 9 have been implicated in antiviral defense. TLR3 recognizes double stranded RNA (dsRNA) an almost universal viral intermediate generated during most viral replication cycles. Stimulation of TLR3 induces multiple cellular antiviral

responses including production of IFN-α/β and the up-regulation of the innate host cell restriction enzyme APOBEC3G [402, 403]. Single-stranded RNA (ssRNA) from HIV acts as a ligand for TLR7 and 8. Signaling through these TLRs activates pDC and monocytes in an MyD88 dependent manner [404].

The possible role for TLRs in the antiviral defense against HIV has provided the impetus for investigating whether these innate immune receptors can be used in novel HIV treatment strategies. A number of TLR agonists are presently undergoing clinical or preclinical trials such as the TLR3 agonist poly I:C 12U [405].

Human innate restriction factors: the APOBEC3 family and Tetherin

APOBEC 3

Restriction factors are natural cellular proteins that protect individual cells from infection by restricting viral infectivity through the hypermutation of viral cDNA, inhibition of reverse transcription or integration. All seven members of the APOBEC3 family (APOBEC3A-H) of DNA cytidine deaminases that deaminate viral cDNA cytidines to uridines have been shown to have varying levels of antiviral activity against HIV [406]. Unfortunately, HIV avoids viral restriction by APOBEC3 proteins through the action of its accessory protein Vif which decreases the encapsidation of certain APOBEC3 proteins and prevents neutralization of HIV in budding virions. However, a correlation between higher APOBEC3G expression and improved clinical status has been reported and the APOBEC3-Vif axis identified as a potential therapeutic target [407, 408]

Tetherin

Human cells possess an antiviral activity that prevents the release of retroviral particles. This antiviral activity can be constitutively expressed or induced by IFN-α and consists of protein based tethers that were termed "tetherins" by Stuart J.D Neil and colleagues. [409]. Tetherin (CD137), is specifically antagonized by the HIV viral accessory protein Vpu. Viruses defective in Vpu, assemble and mature but remain tethered to the surface of infected cells [409]. The molecular mechanisms underlying the induction of viral retention by tetherin and how this action is thwarted by HIV Vpu have not been fully elucidated.

Cellular mediators of the innate immune response to HIV infection

DC

DCs are the sentinels of the immune system. Poised in peripheral tissues to recognize and capture a wide range of micro-organisms, DCs relate information about invading pathogens to lymphoid organs and act as a link between the innate and adaptive arms of the immune system to orchestrate optimal immune responses. DCs exist in two distinct functional states, under steady-state conditions. DCs in the peripheral tissues are immature (iDC) and characterized by the localization of MHC class II molecules on the late endosome-lysosomal compartment, a low surface expression of co-stimulatory molecules and chemokine receptors such as CCR7 and an enhanced capacity to sequester, but not process antigen. Maturation of DCs via TLR triggering or the detection of pro-inflammatory cytokines, leads to the upregulation of MHC class II molecules, co-

stimulatory molecules and chemokine receptors that promote migration from the periphery to the draining lymph node in preparation for T cell priming in the secondary lymphoid tissue. In the T cell areas of the lymph nodes mature DCs present pathogen derived-epitopes to $CD4^+$ and $CD8^+$ T cells and activate antigen specific T cells [410].

Humans have two major DC subtypes: the mDC and pDC [411]. Although pDC comprise only 0.2-0.5% of the circulating PBMC they are exceptionally potent IFN-α producers. The role of pDC within the context of HIV infection is complex. The loss of pDC function is associated with HIV disease progression as is the impaired capacity of pDCs to interact with other immune cells during cross-talk between innate and adaptive immunity. However, chronic activation of pDCs through TLR7 can have adverse effects such as the upregulation of TNF-α related apoptosis inducing ligand (TRAIL) on T cells due to an aberrant increase in the levels of IFN-α [411]. Therefore, optimal pDC stimulation in response to HIV infection must delicately balance beneficial stimulatory signals with those that lead to pathologic chronic activation in order to attain viral control.

mDC sequester exogenous antigens for cross-presentation of MHC class I restricted epitopes to $CD8^+$ T cells.

The interaction of HIV infection with DC

DCs located at mucosal sites sample the antigenic environment. This DC function makes them the first potential cellular target for HIV infection during a transmission event. However, the productive infection of DC is 10-fold lower than that of $CD4^+$ T

cells because of the armament of factors that create a non-permissible environment within the DC [412-414].

DCs have the unique capacity to capture and internalize HIV in an infectious form in the absence of viral fusion. The latter involves the uptake of HIV viral particles via binding of the C-type lectin Dendritic Cell Specific ICAM3-grabbing Non-integrin (DC-SIGN) to the viral envelope protein (gp120) and their subsequent endocytosis. Viral particles can survive in an infectious form within a vesicular compartment of the DC and are released upon transfer to $CD4^+$ T cells in a process termed trans-infection.[415] The "Trojan horse" hypothesis was formulated to describe how this trans-infection process could initiate the uncontrolled spread of HIV infection. Like 'Helen of Troy' HIV successfully hijacks iDCs that express high levels of DC-SIGN, escapes degradation and is maintained in a vesicular compartment. In this form HIV is ready for efficient transport and entry into the secondary lymphoid tissue's T cell rich zones where it can infect many T cells.

The 'Trojan horse' hypothesis may be an oversimplification of HIV transmission events mediated by DCs. Recent data has demonstrated that iDCs are capable of rapidly degrading captured viral particles as opposed to retaining whole infectious virus, whereas the sequestration of whole virus is favored by mature DCs [416]. Therefore, the 'Trojan exosome hypothesis' has been proposed and stipulates that HIV exploits a pre-existing antigen exosome dissemination cellular pathway of antigen up-take and retention which is a specific function of mature DC. This pathway does not rely on the recognition of viral envelope protein, but rather ubiquitous signals for *trans*-infection and allows for the retention of whole virus [416, 417].

DC-NK cross-talk

The bi-directional cross-talk between NK cells and DCs affects the functional regulation of both cell types leading to the modulation of both innate and adaptive responses to infection. NK cells contribute to innate immunity by lysing tumor and virally infected cells without prior sensitization. The cytolytic activity of NK cells is profoundly enhanced by IFN-α produced by the pDC. [418]**.**

Activated NK cell play a significant role in DC immunoregulation. Depending on the ratio of NK:DCs, NK cells can selectively lyse iDC through the engagement of the NK cell receptor (NKp30) by ligands on iDCs. By eliminating their precursors, activated NK cells in turn limit the generation of mature DC [418]. The reciprocal activation of DC and NK results in the production of IFN-γ from NK cells and IL-18 from DCs, which both serve to augment IL-12 secretion from accessory cells such as macrophages, thereby favoring the development of a Th1 adaptive immune response.

Natural Killer cells

NK cells constitute an important arm of the innate immune response to virally infected and transformed cells [419-421]. They were first described in 1975 as a lymphocyte subset capable of cytotoxicity against leukemia cells *in vitro* without prior sensitization [422]. NK cells make up 5-15% of circulating lymphocytes but exist in

higher frequencies in tissues where they have specialized functions such as in the liver and pregnant uterus. Mature NK cells are phenotypically defined by the surface expression of CD56 and CD16 on lymphocytes that lack CD3 and functionally as an important source of immunoregulatory cytokines and chemokines. Through the release of pre-formed granules containing perforin and granzyme, NK cells are capable of directly lysing target cells as well as mediating ADCC via interactions between membrane receptors such as FcγRIII (CD16) and the Fc portion of IgG [423-425].

NK cells are distinct from T and B lymphocytes because they do not express germ-line rearranged receptors. Instead, the net balance of signals transmitted through an array of stochastically expressed inhibitory and activating receptors governs NK cell activation while maintaining tolerance to self [426].

NK cell subsets

The CD56 and CD16 phenotypic markers for NK cells

Two populations of NK cells are distinguished based on the density CD56 and CD16 cell surface expression. Circulating human NK cells express a low density of CD56 and high levels of CD16 and are referred to as the $CD56^{dim}$ subpopulation; and the remaining ten percent are $CD56^{bright}$ $CD16^{dim/neg}$. The functional significance of the CD56 molecule remains unknown, however the bright or dim expression of CD56 correlates with the expression of other surface markers that confer unique functional properties to the $CD56^{bright}$ and $CD56^{dim}$ subsets [427].

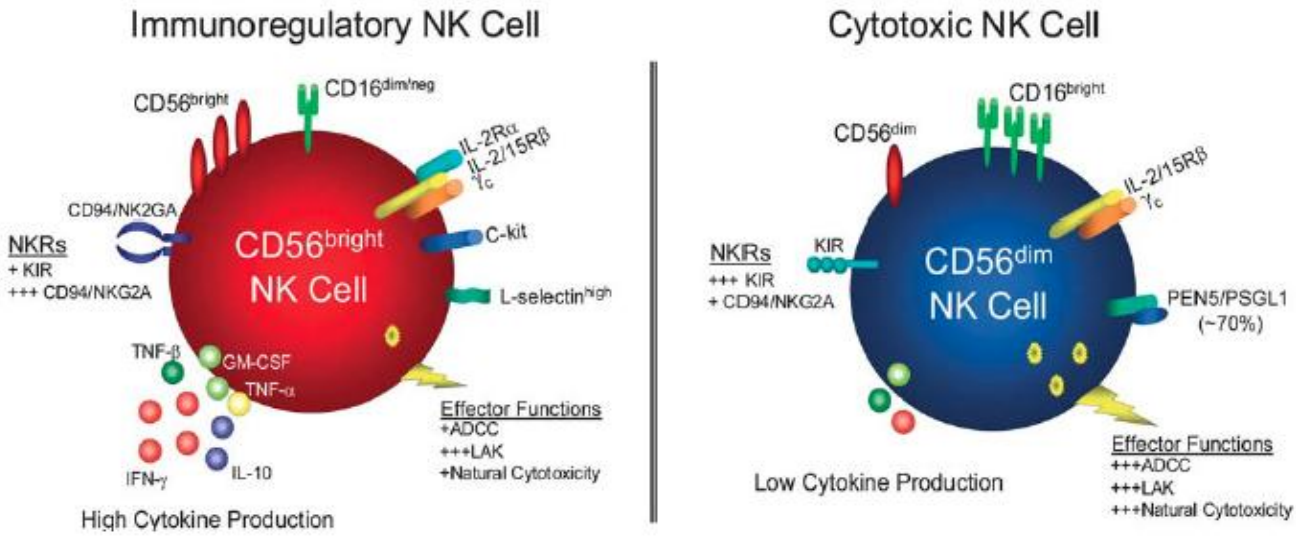
$CD56^{bright}$ NK cells are the main subset producing the immunoregulatory cytokines IFN-γ, TNF-α and TNF-β, granulocyte stimulating factor (GM-CSF), IL-10, and IL-13. CD8 is expressed on a minority of $CD56^{bright}$ NK cells and a population of $CD8^{+}CD56^{bright}$ NK cells produce copious amounts of type-2 cytokines. Only $CD56^{bright}$ NK cells constitutively express the high affinity heterodimeric IL-2 receptor (IL-2Rαβγ), which allows them to expand in culture to low concentrations of IL-2. All other NK cells express the intermediate affinity heterodimeric IL-2 receptor (IL-2Rβγ) and therefore proliferate to only to high nanomolar concentations of IL-2 [428, 429].

$CD56^{bright}$ NK cells express adhesion molecules such as L-selectin (CD62L) and high levels of the CC-chemokine receptor 7 (CCR7), which enhance interactions with the vascular endothelium and permit homing to secondary lymphoid organs through high endothelial venules. Fehringer et al. find a disproportionately higher percentage of $CD56^{bright}$ NK cells in parafollicular (T cell regions) of the lymph node than in the peripheral blood where they use their high affinity IL-2 receptors to respond to T-cell derived IL-2 and engage in cross-talk with T-cells [430].

$CD56^{dim}$ NK cells are more 'naturally' cytotoxic against NK-sensitive targets than the $CD56^{bright}$ subset and secrete negligible amounts of cytokines and/or chemokines. However upon stimulation with IL-2 both NK cell subsets have comparable levels of cytotoxicty. In addition, $CD56^{dim}$ NK cells lack the expression of both L-selectin and CCR7 but express high levels of leukocyte function associated antigen (LFA-1). These differences in the expression pattern of adhesion molecules between the two subsets suggests that $CD56^{bright}$ NK cells and $CD56^{bright}$ NK cells traffic to different sites *in vivo*.

Consistent with differences in their functional capacities, $CD56^{dim}$ NK cells express FcγRIII (CD16) that enable them to function in ADCC, whereas $CD56^{bright}$ NK cells lack or have low density expression of FcγRIII. The expression patterns of important NK cell receptors such as the KIRs and CD94/NKG2A also differ between $CD56^{bright}$ and $CD56^{dim}$ NK cells. Figure 9 summarizes some of the features that distinguish the $CD56^{bright}$ and $CD56^{dim}$ NK subsets.

Figure 9 : Human NK cell subsets as defined by CD56 expression adapted from Farag et al. 2005. Human Natural Cell development.



NK-T cells

NK-T cells are a subset of T lymphocytes that share both NK and T cell markers. In humans the major NK-T cell population exhibits a restricted T cell receptor repertoire of Vα24 preferentially paired with Vβ11 and is called the invariant NK-T cell (iNK-T). [430]. Unlike conventional T cells, NK-T cells do not recognize peptides in the context of MHC class I or II molecules, but glycolipids presented by MHC class II-like CD1d molecules that are expressed on mature DC. Once activated, NK-T cells secrete large amounts of IFN-γ, TNF-α, IL-4 and IL-13 and display cytolytic activities through the release of perforin and granzyme B [431]. High levels of CD4 and CCR5 expression on NK-T cells make them prime targets for HIV infection. Accordingly, $CD4^+$ NK–T cells are rapidly depleted in some patients during primary infection and the reduced percentages of NK-T cells inversely correlate with HIV VL [431].

NK cell receptors

NK cells stochastically express a variety of activating and inhibitory germ line encoded receptors. Activating receptors bind to stress-induced proteins encoded by the host genome or pathogen-encoded molecules expressed on the surface of infected cells.

Inhibitory receptors often use MHC class I molecules as ligands. In this way they do not respond to normal cells but rather detect aberrant expression of MHC class I molecules due to tumor transformation or viral infection. NK cell receptors that are specific for MHC class I molecules are broadly categorized into two main classes of structurally distinct molecules namely; the C-type lectin or the immunogloblulin-(Ig)-like families of receptors depending on features of their extracellular domains. Additional inhibitory receptors specific for molecules that are not HLA are also involved in cellular interactions between NK cells and their targets.

MHC class I specific NK cell receptors

KIR

The first human KIRs were discovered in the early 1990s as novel surface 'triggering' molecules that exerted a regulatory role in the control of a subset of $CD3^-$ $CD16^+$ activity [432, 433]**.** To date the highly polymorphic KIR gene cluster includes up to 17 KIR genes or pseudo-genes with individual genes exhibiting allelic variability and individual haplotypes differing in gene content [426]. KIR genes encode for both inhibitory and activating receptors, which are determined by the sequence of their intracellular domains.

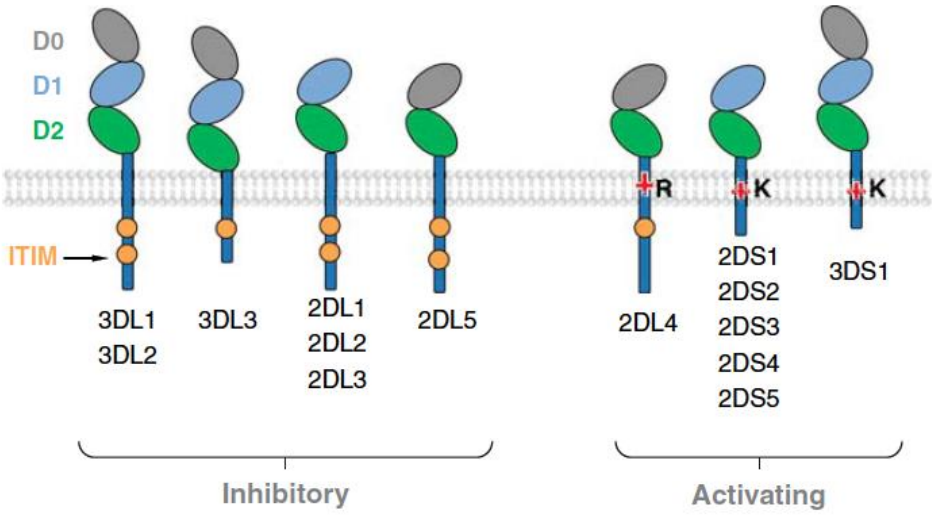
KIR structure and nomenclature

KIR molecules have an extracellular region formed by Ig-like domains, a stem and a cytoplasmic tail. While the majority of KIR genes encode membrane proteins, there are some exceptions to this including the KIR cDNA for KIR2DL4, KIR2DS4 and the 2DL5A/3DP1 hybrid, which are not expressed on the cell surface but may be secreted [434-436].

Figure 10 depicts how KIR nomenclature is based upon the structural features of their extracellular domains and cytoplasimic tails. KIR2D or 3D refers to the number of extracellular Ig-like domans, L or S denotes whether the KIR has a short or long cytoplasmic tail and p indicates a putative pseudogene. KIR receptors display structural and sequence similarities allowing for distinct KIRs to be defined as type 1 or 2 depending on the configuration of their Ig-like domains termed D0, D1 and D2. Type 1 KIRs include all KIR3D receptors and certain KIR2D receptors with a D1-D2 configuration from the N to C terminal whereas Type 2 KIRs have a D0-D2 configuration. The cytoplasmic tail predicts the functional activity of the KIR. Long-tailed KIR are predominantly inhibitory (except for KIR2DL4) and short-tailed KIR are activating. The long cytoplasmic tails of inhibitory KIRs contain one or two

immunoreceptor-tyrosine based motif(s) (ITIM) and a transmembrane portion with only non-polar amino acids [437]**.** KIRs with short cytoplasmic tails on the other hand possess a transmembrane stem with positively charged amino acids required for pairing with the immunoreceptor-tyrosine based activation motif (ITAM)- containing adaptor DAP12 [438]

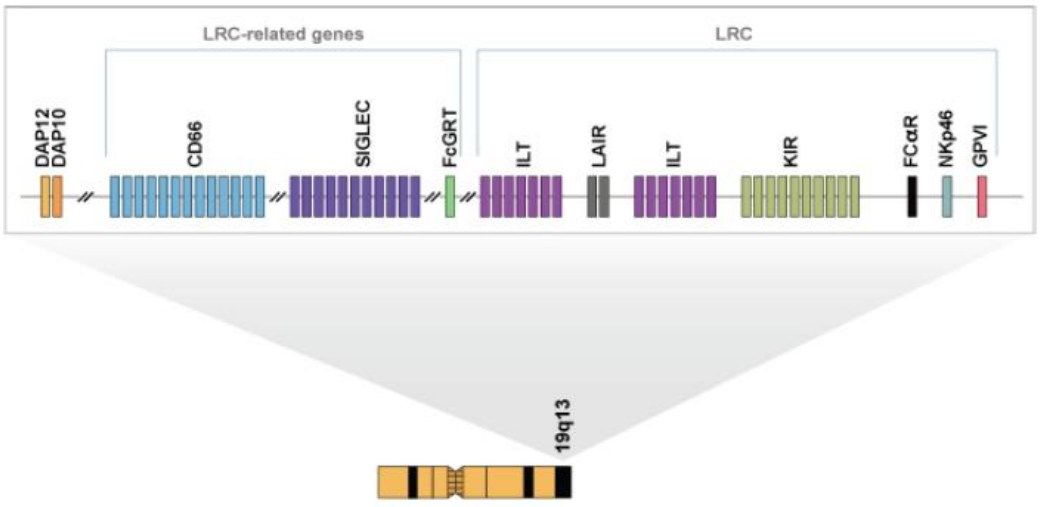
Figure 10 : Killer-Immunoglobulin-Like domain organization adapted from Bashirova et al Annual Rev Genomics and Human Genetics 2006.



KIR gene structure

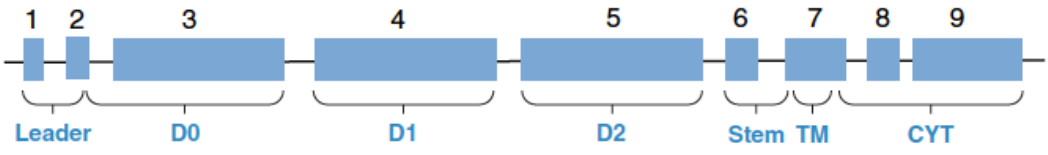
The KIR gene cluster maps onto a 150 kilo base-pair (kb) region of the leukocyte region complex (LRC) on human chromosome 19q13.4 (see figure 11). In addition to KIR, the polymorphic LRC includes genes that code for at least 30 members of Ig-like receptors such as the leukocyte Ig-like receptor family (LILR), leukocyte associated inhibitory receptor (LAIR-1 and LAIR-2), Fcα receptor and collagen binding receptor (GPV1) [439].

Figure 11: Map of the Leukocyte Receptor Complex adapted from Carrington et al. 2003 The KIR gene cluster.



Overall, KIR genes are highly homologous with a basic nine exon unit structure shown in Figure 12. Despite this extensive degree of sequence similarity, SNPs between KIR sequences translate into KIR alleles that encode for proteins with diverse characteristics in terms of transcriptional regulation, ligand binding, cell surface expression, intracellular signaling and protein folding. This diversity is crucial for efficient NK cell respones to a variety of pathogens.

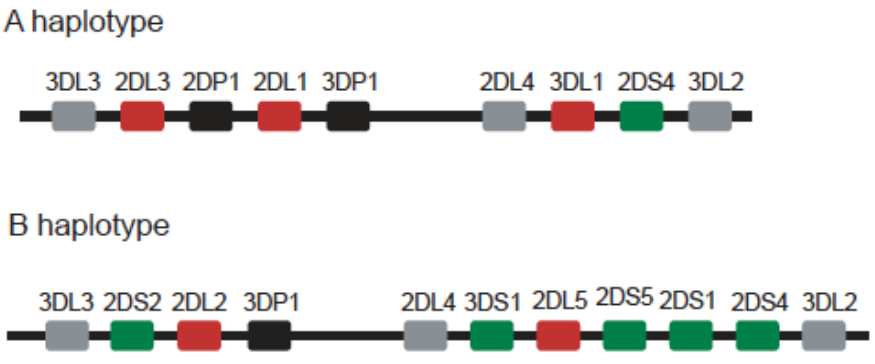
Figure 12: The nine exon structure of the ancestral KIR gene adapted from Bashirova et al. Ann Rev Genomics .2006.



Variation in gene content is a key characteristic of the KIR complex. Segregation analyses and sequence data have identified two broad haplotypes [440, 441] [442]. Examples of the organization of KIR region haplotypes A and B are shown in Figure 13 They differ from each other in gene content. Haplotype A includes genes that encode predominantly inhibitory receptors; it contains nine KIR genes of which only one is the activating KIR2DS4 and four are framework genes. B haplotypes exhibit extreme diversity both in terms of gene content and allelic polymorphism.

The four KIR genes, KIR3DL3, 3DL2, 3DP1, and 2DL4 are defined as framework genes because they are present on all haplotypes. Furthermore, because all haplotypes contain the genes encoding KIR2DL2/2DL3 and KIR3DL1/3DS1, these alleles are also considered to be framework genes.

Figure 13: Organization of the KIR gene cluster showing gene orders for haplotype A and a representative haplotype B. Green, red and grey boxes indicate activating KIR, inhibiting KIR and framework genes, respectively adapted from Hongchuan li et al. 2008



Genetic control of KIR expression

NK cells and subsets of T cells are the only lymphocytes that express KIR genes. Analyses of mRNA and protein expression demonstrate that each NK cell clone expresses a stochastic combination of the KIR genes present in an individual's genome [443]. Therefore, within one person there exists a diverse repertoire of NK cells with an array of different KIR receptors on their surfaces determined by the KIR region genes they carry.

Even though KIR promoter regions are 90% homologous, the transcriptional regulation of KIR genes differs. Several factors determine the frequency of KIR gene activation and the percentage of NK cells expressing a given KIR. These include presence of a cognate ligand, epigenetic mechanisms and polymorphisms within promoter sequences [265, 444, 445] Studies investigating the mechanisms controlling KIR receptor expression demonstrate that expression is controlled by a stochastic mechanism and the probability of co-expression of two distinct inhibitory receptors is equal to the product of their individual frequencies [446].

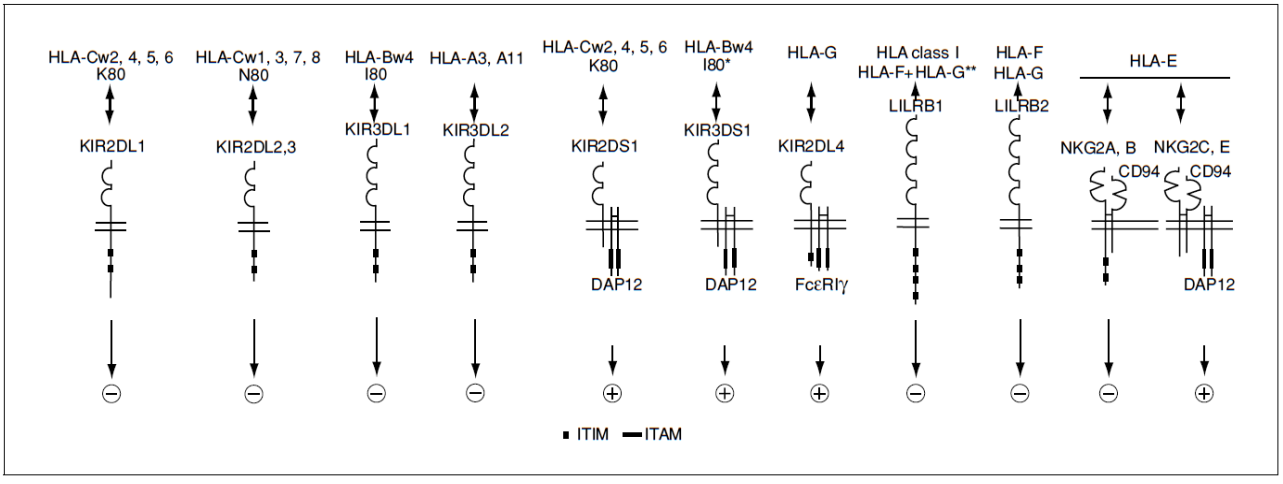
DNA methylation in the 5' area of KIR promoters correlates with silencing of KIR transcription. Active KIR genes are hypomethlylated whereas silent genes are methylated [447-449]. Multiple promoters, some of which are bi-directional, are present

within KIR genes. Several functionally relevant polymorphisms clustered around inhibitory KIR family members affect the strength of competing sense and anti-sense activities from a bi-directional promoter. These control the probability of gene activation and frequencies of receptor expression.

KIR ligands

The extracellular domain of KIR receptors is responsible for defining their ligand specificity. To date only MHC class I molecules have been identified as ligands for KIR and the common mode of KIR recognition involves the region around position 80 of the MHC class 1 alpha helix.

Figure 14: HLA Class I specific NK cell receptors and their ligands adapted from Biasonni et al. Human Natural Killer Receptors, Co-Receptors and their ligands. 2009



Crystallographic studies indicate KIR/HLA interactions are stabilized by peptide. Furthermore, the peptide amino acid residues present at positions 7 and 8 play key roles in stabilizing interactions between KIR and their HLA ligands. The presence of residues with a small side chain at position P8 of the peptide bound to HLA is of particular importance**.** The ligand binding area of KIR comprises six surface loops near the interdomain hinge region. Three of the loops (A'B, CC' and EF of the D1 domain) interact with the alpha (α) 1 helix of the HLA, whereas the other three BC, FG loops of the D2 domain and the hinge loop contact the (α) 2 helix of the HLA. In contrast to TCR/HLA interfaces that are characterized by Van der Waals and hydrogen bonding, the KIR/HLA interface displays striking complimentarity. [450, 451]

Much of the research used to understand KIR/HLA interactions has concentrated on inhibitory KIR that recognize HLA-C. The first crystal structure of a KIR/HLA complex was that of KIR2DL2 bound to HLA-Cw3 and a monomeric self peptide with the sequence GAVDPLLAL (GAV) from importin [450]. It is now known that KIR2DL2/3 and 2DL1 recognize two mutually exclusive groups of the HLA-C allotypes group 1 (HLA-C1) and group 2 (HLA-C2), respectively [452-454]. HLA-C1 alleles have an Asparagine at position 80 and include HLA-Cw1, Cw3, Cw7, Cw8, Cw13, Cw14 and Cw16 whereas HLA-C2 alleles have a Lysine at this position and include HLA-Cw2, Cw4, Cw5, Cw6, Cw17 and Cw18. Crystal structures of both KIR2DL2-C1 and KIR2DL1-C2 complexes demonstrate that position 44 in the D1 domain of KIRs and

position 80 of HLA-C are important for determining KIR/HLA-C binding specificity [455].

HLA-A allotypes and HLA-B allotypes that share the serologically defined Bw4 public epitope are ligands for KIR3DL1 receptors and are putative ligands for the allelic activating KIR3DS1 receptor [262]**.** Mutually exclusive HLA-Bw6 allotypes do not serve as ligands for KIR3DL1 receptors although some low affinity binding to KIR3DL1 receptors can occur [456]. Among the HLA-Bw4 allotypes, those with an Isoleucine at position 80 are the preferred ligands for KIR3DL1 receptors because they show the strongest inhibition of NK cell mediated lysis by KIR3DL1 [262]. KIR2DL4 has been shown to bind to HLA-G, the non classical MHC class I molecule that is specifically expressed on fetal trophoblasts, thymic endothelial cells and the cornea.

Interactions between KIR and their ligands can be altered by the presence of peptide loaded into the peptide-binding groove of the HLA molecule [457-460]**.** For example, the binding of KIR3DL2 to HLA-A3 and -A11 allotypes is only detected when a specific EBV peptide is folded into the HLA tetramers, indicating that some KIR/HLA interactions have strong peptide selectivity [461]. The interaction between KIR3DS1 and its putative ligand Bw4-Ile80 is speculated to be another example.

No activating KIR has been shown to bind to any HLA molecules as strongly as its inhibitory counterpart. The activating receptors, KIR2DS1, KIR2DS2 and KIR3DS1 share sequence similarity with the inhibitory KIR2DL1 KIR2DL2/3 and KIR3DL1, respectively, but the binding to KIR2DS1 to HLA-C2 allotypes is much weaker than KIR2DL1. KIR2DS2 binding to HLA-C1 is very difficult to demonstrate [462-464]. The low affinity binding of activating KIR to HLA is thought to have evolved to ensure the

self-tolerance of NK cells that express activating KIR without inhibitory KIR. Even though activating KIR recognition of HLA molecules loaded with stress-related peptides or other disease specific peptides has been suggested, the overall biological relevance of these low affinity interactions remains largely unknown.

Other MHC class I specific NK cell receptors:

C-type Lectin Family: CD94/NKG2 Receptors

The *CD94* and *NKG2* family of genes are located within the NK complex on human chromosome 12p12.3p13.2 and encode receptors that recognize nonconventional MHC class Ib human HLA-E molecules. A single *CD94* gene is closely linked to four NKG2 family genes namely; the NKG2A, C, E and F genes [465, 466]. Unlike the KIR genes, the CD94/ NKG2 genes have limited polymorphism and minor allelic variants that have not been shown to affect function.

CD94 and NKG2 encode type II membrane proteins of the C-type lectin-like family. CD94 can be expressed on the cell surface as a disulphide-linked homodimer or as a disulphide-linked heterodimer with NKG2A or NKG2C. CD94/NKG2A heterodimers function as inhibitory receptors because the cytoplasmic tail of NKG2A contains an ITIM [467, 468]. CD94/NKG2C heterodimers serve as activating receptors and require association with a DAP12 adaptor protein for stable expression on the cell surface and for signaling. CD94/NKG2 receptors are expressed on most NK cells and $\gamma\delta$ TCR^+ T cells and a subset of effector/memory $CD8^+$ $\alpha\beta$ TCR^+ T cells [469, 470].

HLA-E binds peptides derived from the leader sequences of HLA-A, B, C or G. In this way CD94/NKG2A senses alterations in overall MHC class I expression that may occur in virus infected or transformed cells.

LILR

The human *LILR* family of genes is located on human chromosome 19q13.4 just centromeric of the *KIR* genes. Of the 13 LILR genes only two are MHC class I specific namely the inhibitory LILRB1 (ILT2/LIR1/CD85j) and LILRB2 (ILT4/LIR2) [471]. LILRBI is a cell surface glycoprotein with four Ig-like domains in the extracellular region and four ITIMs in the cytoplasmic tails. LIRRBI expression is not restricted to NK cells. Rather this receptor is highly and uniformly expressed on B cells, monocytes and a subset of T cells [471]. LILRBI binds with low affinity to a conserved region of the $\alpha 3$ domain of practically all HLA class I glycoproteins. The LIRB2 gene codes for the CD85D, LIR2/ILT4 receptor that is involved in the recognition of the non-classical MHC class I molecules HLA-F and HLA-G [472, 473].

Natural Cytotoxicity Receptors (NCRs)

The major NK cell receptors with the inherent capacity to induce NK-mediated killing are the natural cytotoxicity receptors (NCRs) NKp46 (NCR1, CD335), NKp30 (NCR3, CD337) and NKp44 (NCR2, CD336), which belong to the Ig-like superfamily and the lectin-like receptor NKG2GD (CD314, KLRK1) [474].

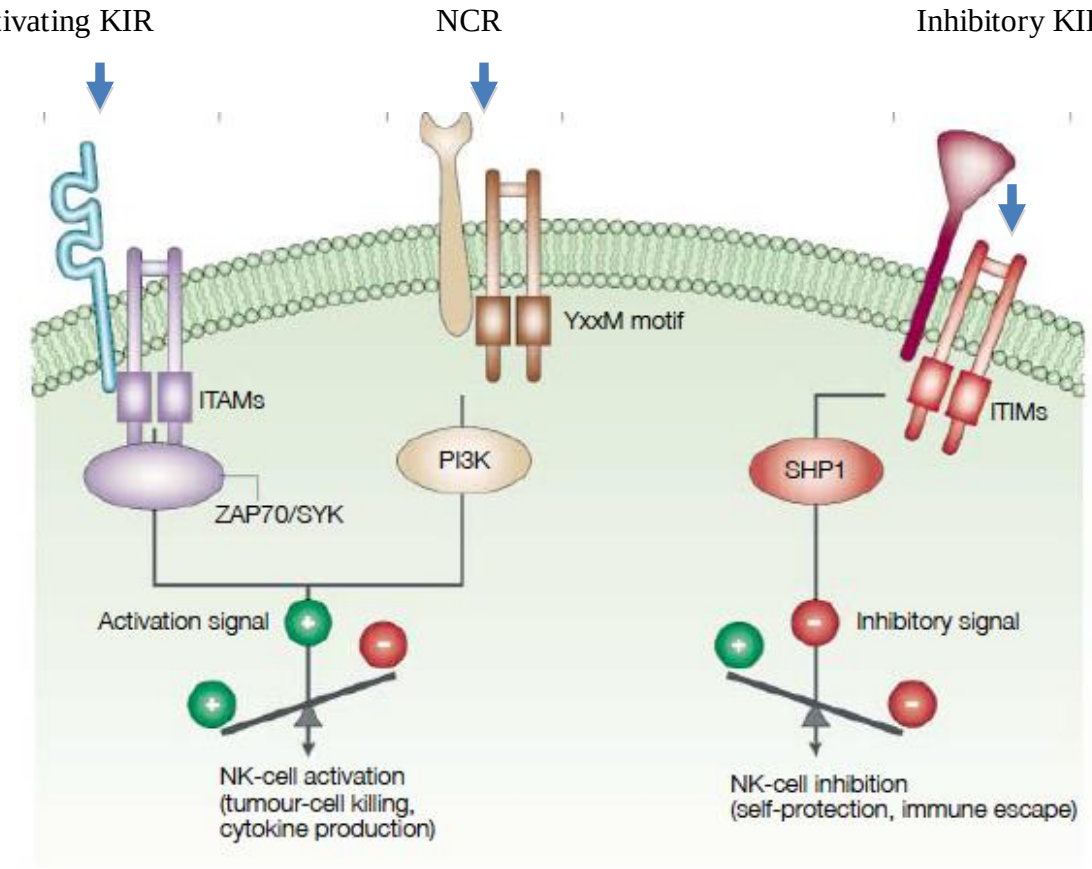
NK receptor signal transduction

The cytoplasmic tails of NK cell receptors determine the inherent signaling capacities and what signaling pathways each NK cell receptor will utilize upon binding to its ligand. All inhibitory NK cell receptors contain within their cytoplasmic domains one or two copies ITIMs. These are phosphorylated by a Src family kinase upon ligand binding.

The phosphatases, SH2 containing protein tyrosine phosphatase (SHP)-1 and SHP-2 or the SH2-containing inositol polyphosphate 5-phosphatase (SHIP) are subsequently recruited to the receptor and depending on the specific phosphatase used, cellular signaling proteins such as FcεRIγ, ZAP70, Syk, PLCγ2, Shc are dephosphorylated. Upon SHIP recruitment to the receptor, phosphatidylinositol-3,4,5-tri-phosphate (PI-3,4,5-P_3) is degraded to (PI-3,4,5-P_2), which subsequently leads to the prevention of Ca^{2+} dependent signaling. The overall effect of phosphastase recruitment to the receptor is the dampening or prevention of NK cell effector functions such as cytotoxicity and cytokine and chemokine production.

Several activating receptors share a common signaling pathway with T cell and B cell receptors and use adaptor proteins containing ITAMs for signal transmission upon ligand binding. NK cells express three ITAM-containing adaptor proteins FcεRIγ, CD3ζ and DAP12 [475-477]. These associate with several activating NK cell receptors leading to phosphorylation of the ITAM. Syk kinases and ZAP70 are recruited through their SH2 domains to stimulate downstream events such as Ca^{2+} influx, degranulation, and transcription of cytokine and chemokines genes (See Figure 15).

Figure 15: General paradigms of NK cell receptor signaling that control NK cell activation



NK cell recognition of 'missing self' and licensing

NK cells distinguish normal from aberrant cells by recognizing the 'absence of the expected' rather than the 'presence of the unexpected'. This is the premise of the missing self hypothesis, a notion pioneered by Klas Karre that revolutionized the field of NK cell research [425, 478, 479]. Karre observed that targets with normal expression of MHC class I molecules were tolerated and resistant to killing by NK cells and that MHC molecules on host cells served as potential markers of self. Karre hypothesized that MHC class I molecules negatively modulated NK activity and governed NK cell self-tolerance.

In his original 'effector inhibition' theory, Karre predicted that NK cells from a MHC heterozygous A/B F_1 hybrid would reject B/B parental cells because of the 'absence' of type A MHC molecules. He speculated that NK cell mediated rejection could be induced by a B/B recipient of a B/B graft by deleting MHC class I expression of the graft and introducing expression of type A MHC class I molecules. [480]. At the time Karre's missing self hypothesis was conceived, the experimental tools required to rigorously test his hypothesis had not been developed and importantly the existence of an NK cell receptor capable of receiving negative signals from MHC class I molecules had not been described.

According to the missing self hypothesis, NK cells developed in MHC deficient mice should be autoreactive. However, NK cells from MHC class I deficient mice remain hyporesponsive and demonstrate poor killing against MHC class I deficient targets. NK cells from humans with MHC class 1 deficiencies are not spontaneously over reactive [481, 482]. To reconcile these observations with the missing self hypothesis the NK cell maturation process termed 'licensing' was described. Sungjin Kim and Wayne Yokoyama coined the term licensing to describe the MHC class I dependent maturation

process that confers functional competency to NK cells. NK cells that express self-MHC specific inhibitory receptors are endowed with functional competency during the licensing process. This ensures that tolerance to self is maintained because NK cells that lack self-MHC specific inhibitory receptors remain unlicensed and therefore incapable of reacting against MHC class I deficient targets [483, 484].

Seminal data using MHC class I transgenic mice has demonstrated a role for MHC class I molecules in licensing by showing that the responsiveness of mature NK cells can be altered by changes to the MHC class I environment [482, 485, 486]. Even though the specific molecular mechanisms underlying the interactions between MHC class I molecules and NK cell inhibitory receptors during licensing events have remained elusive, a functional ITIM in the inhibitory receptor is required.

The four prevailing theories of NK cell licensing include; the arming, disarming, cis-interaction and rheostat models. The arming model posits that signaling from inhibitory receptors promotes functional maturation and that in the absence of arming the NK cell remains hyporesponsive. The disarming model proposes that NK cells are activated by default and in the absence of self-MHC class I specific inhibitory receptor expression NK cell becomes hyporesponsive. In the cis-interaction model, inhibitory receptors bind to MHC class I molecules on the same cell membrane thereby sequestering the re-location of inhibitory receptors to the immunological synapse. The balance between activating and inhibitory signals tips in favor of the activating signal and the NK cells become more responsive as a consequence. This model has limited applicability to humans as it remains to be determined whether human inhibitory NK cell receptors can interact with MHC class I molecules in *cis*.

The rheostat model of NK licensing postulates that NK cell education by MHC class I molecules is a quantitative and dynamic process whereby the strength of the signals delivered by MHC class I molecules is proportional to a threshold of activation that is set in the NK cells. Therefore, the different degrees of inhibitory input quantitatively tune the activation pathways of the NK cell and translate into a corresponding level of responsiveness. The higher the inhibitory input during education, the more likely the NK cell will pass the threshold required to respond to stimulation [487]. In support of this quantitative model of NK cell licensing, recent data from studies in humans report higher levels of potency among NK cells expressing two versus one self-MHC class I specific inhibitory KIR [488, 489]. Brodin and colleagues liken the licensing process to a dynamic rheostat that is tuned up or down depending on the net inhibitory input transmitted to the NK cell from the environment thus allowing the NK cell to remain 'optimally' responsive. This is a particularly appealing aspect of the model because it means that NK cells are capable of adjusting their level of responsiveness in accordance with changes in MHC class I expression that occur during infection, inflammation and cellular stress versus the steady state [490].

The common denominator among the four models of NK cell licensing is the requirement for inhibitory signaling from self-MHC class I specific NK cell receptors. Additionally, all models take into account that the net inhibitory input received by the NK cell must be balanced out by activating signals. However, none of these models can fully account for the biological relevance of maintaining a hyporesponsive unlicensed NK cell population. Furthermore, Fernandez et al. report that NK cells that lack all known self-MHC class I-specific inhibitory receptors are self-tolerant, have a normal

phenotype and upon stimulation with pharmacologic agents *in vitro* exhibit functional activity comparable to licensed NK cells [491]. Moreover, recent reports by L Lanier and CH Tay describe a protective role for unlicensed NK cells in the early control of murine CMV infection [492, 493]. In summary, several mechanistic details that underlie the licensing process still need to be elucidated.

NK cells in HIV infection

Pathologic redistribution of NK cell subsets

Although NK cells represent a fundamental component of the innate immune response to viral infection by acting as effector and regulatory cells, they remain unable to contain the spread of HIV and are defective in the clearance of HIV infected $CD4^+$ T cells [494-496]. Increased VL impairs NK cell mediated killing of tumor cell targets, cytokine production and disrupts cross-talk with DC [497, 498]**.** For several years, the hypothesis that ongoing HIV replication resulted in a decrease in the absolute number of total circulating NK cells was used to account for defects in NK cell antiviral activities. The redistribution of NK cells in the peripheral blood of HIV infected subjects and the appearance of an aberrant NK cell subset has been associated with NK cell functional deficiencies following HIV infection [1, 499, 500].

Mavillio and colleagues were the first to examine and characterize the expansion of a dysfunctional/anergic Siglec-7^{neg} and/or $CD56^-CD16^+$NK cell subset during the course of HIV infection. From their work, the concept that the sequential deregulation of

NK cell subset distribution rather than a decrease in total NK cell numbers was used to explain NK cell functional defects in HIV infection. Studies from LTNP and EC have confirmed the association between HIV viremia and the expansion of peripheral $CD56^-$ NK cells by demonstrating that individuals with low VL did not display an increased frequency of peripheral $CD56^-$NK cells [499]. Furthermore, the suppression of HIV VL with ARV leads to the slow restoration of CD56 expression on the surface of NK cells [501]. Accordingly, Mavillio et al. postulate that the increased expression of Siglec-7 in conjunction with the loss of CD56 can be used as biomarkers of disease progression [501].

Other Innate immune cells

γδ T cells

The majority of circulating human γδ T cells are VγVδ2 cells that reside in the epithelial mucosae. Upon activation these VγVδ2 cells secrete Th1 and Th2 type cytokines and exert either cytotoxicity or induce the apoptosis of HIV infected target cells via the perforin/granzyme pathway or the Fas/FasL pathway respectively, thus enabling them to be involved in both innate immunity and mucosal protection [502-504].

Macrophages/Monocytes

Macrophages also reside in the mucosae and constitute the first line defense against viral infection. Their location makes them primary targets for HIV infection and

reservoir establishment. Depending on the cyokine milieu and microenvironment, macrophages can become differentially activated. In the presence of pro-inflamatory Th-1 type cytokines, monocyte-derived macrophages (MDM) are polarized toward a M1 phenotype whereas anti-inflammatory Th-2 cytokines promote the generation of M2 macrophages. The differentiation stage of monocytes/macrophages profoundly modulates permissiveness of HIV infection. Generally monocytes are less susceptible to viral infection than differentiated macrophages and the capacity of macrophages to support HIV infection is influenced by cytokine microenvironment [505]. Recent studies have investigated the effects of cytokine-mediated polarization of macrophages on HIV infection susceptibility and report non polarized MDM are the most vulnerable to infection [506].

Infection with HIV rapidly activates several components of the innate immune response before the development of adaptive immunity. These early constituents of the innate immune response can directly contribute to the control of viral replication before the host succumbs to the viral onslaught. Genetic resistance and susceptibility to HIV is likely to involve a complex array of immune-response genes. Epidemiological data linking variations in HIV disease course with genetic background provide insights into the genetic determinants of HIV disease. Functional studies that affirm these epidemiological associations provide an unprecedented depth of understanding of the mechanisms behind some of these associations and provide pertinent clues as to the types of immunity an HIV vaccine should engineer.

RATIONALE

Protective HLA alleles such HLA-B*57 are overrepresented in cohorts of HIV-infected SP and several studies support the notion that the protective effect of HLA-B*57 is

mediated through $CD8^+$ T-cells. Epidemiological studies have associated the expression of certain KIR/HLA combined genotypes with slower progression to AIDS and lower viral load and implicate a prominent role for NK cells in HIV pathogenesis. Thus, HLA-B*57 may mediate it protective effects on HIV infection through NK cells as well as through $CD8^+$ T cells.

CHAPTER TWO

Association of T Cell Activation with carriage of protective HLA or KIR/HLA genotypes in HIV-infected Elite Controllers

Association of T Cell Activation with carriage of protective HLA or KIR/HLA genotypes in HIV-infected Elite Controllers

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Running head: T cell activation in Elite Controllers.

ABSTRACT

Background: Elite controllers (EC) are a rare subset of HIV infected individuals who control viral load below 50 copies/ml of plasma without treatment.

Methods: Thirty four EC were studied. The slope of CD4 count change was available for 25 of these subjects. All EC were typed for major histocompatibility complex class I alleles and killer immunoglobulin-like receptor (KIR)3DL1 generic genotypes. We assessed immune activation by measuring the percent of $CD38^{+}HLA\text{-}DR^{+}CD8^{+}$ T cells in the EC group and comparing it with that in 24 treatment-naïve HIV disease progressors and 13 HIV uninfected healthy controls.

Results: Compared to HIV uninfected subjects, EC had a higher percent of $CD38^{+}HLA\text{-}DR^{+}CD8^{+}$ T cells ($p<0.001$) that was lower than that observed in progressors ($p<0.01$). Fifteen of 25 EC had a slope of CD4 count change that was not significantly different from 0 while 3 had a positive and 7 a negative CD4 count slope. Although immune activation did not distinguish EC subsets with stable/increasing versus declining CD4 counts, the percent $CD38^{+}HLA\text{-}DR^{+}CD8^{+}$ T cells was higher in EC expressing the protective HLA-B*57 allele and the KIR3DS1/HLA-Bw4*80I allotype associated with slower time to AIDS.

Conclusions: Elevated immune activation in EC is not associated with a faster rate of CD4 decline but may reflect the ability to mount immune responses capable of controlling viremia.

INTRODUCTION

Untreated HIV infection is usually characterized by viral replication and chronic generalized immune activation, which is thought to be an important driver of CD4 decline in HIV infection [1-6]. Markers of immune activation such as CD38 can be found on a high proportion of the $CD8^+$ T cells in HIV infected individuals. CD38 an ectoenzyme involved in transmembrane signaling and cell adhesion, is ubiquitous in its distribution among cells of the immune system and is a marker of both activation and differentiation [7]. HLA-DR is a human major histocompatability complex (MHC) class II antigen that is expressed on macrophages, monocytes, B cells and on activated T and NK cells. The co-expression of CD38 and HLA-DR on $CD8^+$ T cells has been used to detect immune activation in HIV infected individuals with low-level viremia and to distinguish populations that spontaneously control VL from those successfully treated with anti-retroviral drugs [8, 9].

While stimulation of the immune system by HIV likely induces anti-viral immunity that plays a role in suppression of viral replication, chronic immune activation of non HIV-specific T cells reflects rapid cell turnover due to increased expansion and contraction of antigen stimulated T cell clones [2]. This process leads to $CD4^+$ T cell depletion and immune exhaustion [4, 8, 10].

Less than 1% of those infected with HIV maintain VL below the level measured by standard assays, i.e <50 copies/ml plasma long term without treatment and are called Elite Controllers (EC) or Elite Suppressors [10-15]. Despite VL control some EC have

low or declining CD4 counts [8, 9, 14, 16, 17]. Certain MHC class I (or HLA) alleles and Killer Immunoglobulin-like Receptor (KIR)3DL1/3DS1-HLA-B receptor-ligand combinations have been associated slower disease progression in the context of HIV infection [12, 18-22].

Here, we assessed the percent of $CD38^{+}DR^{+}CD8^{+}$ T cells in 34 EC and compared these values to that seen in chronically infected HIV progressors and uninfected healthy controls. For 25 EC there were a sufficient number of longitudinally collected CD4 count determinations to calculate the annual rate of CD4 count change. Since immune activation is implicated in HIV disease progression and varied among EC, we questioned whether EC with stable or increasing CD4 counts would have lower immune activation levels than those with declining CD4 counts. We also examined whether the level of immune activation would differ in EC with or without protective HLA alleles or KIR/HLA combinations. We confirmed previous studies reporting abnormally high immune activation levels among EC compared to healthy uninfected controls and lower levels than HIV infected progressors in the chronic phase of infection [8, 23, 24]. We found that that immune activation measures were similar in EC with stable/increasing versus declining CD4 counts. EC expressing the protective HLA-B*57 allele or the protective KIR/HLA combination KIR3DS1+HLA-Bw4*80I had a higher percent of $CD38^{+}DR^{+}CD8^{+}$ T cells than EC expressing non protective HLA or KIR/HLA combinations.

MATERIALS AND METHODS

Study population

The study population included 58 untreated HIV-infected individuals (34 EC, 24 progressors) and 13 HIV-negative healthy controls. Informed consent was obtained from all participants and the research conformed to all ethical guidelines of the participating institutions. 28 EC were recruited from the Canadian Cohort of HIV Infected Slow Progressors, which recruits HIV-infected individuals from several community and university-based hospital clinical centres in Canada; six were from a cohort of HLA-B*57 positive viral controllers followed at the National Institutes of Health [12]. EC were defined as having HIV RNA levels below the level of detection by an ultrasensitive VL assay (<50 copies/mL) on at least 3 occasions for at least 1 year. VL was undetectable at the time point immune activation was assessed. HIV disease progressors were infected for at least 1 year with evidence of declining $CD4^+$ T cell counts that fell below 500 cells/mm^3 and VL >10,000 copies/ml. None of the study subjects had evidence of concurrent infections at the time immune activation was assessed. For comparison, 13 healthy uninfected controls were also studied.

Laboratory testing

Plasma viremia was measured using the Versant HIV-1 3.0 RNA assay (bDNA) (Bayer Diagnostics, Tarrytown, NY) with a detection limit of 50 HIV-1 RNA copies/ml of plasma. HLA typing was done on genomic DNA extracted from PBMC or EBV-transformed cells using a QIAamp DNA blood kit (QIAGEN, Inc., Mississauga, Ontario, Canada). HLA typing was done by sequencing using kits from Atria Genetics (South San

Francisco, CA). Assign software was used to interpret sequence information for allele typing (Conexio Genetics, Perth, Australia). KIR3DS1/L1 generic genotyping was performed by PCR with sequence-specific primers as described previously [25, 26].

Cells

Blood was obtained by either venipuncture into tubes containing EDTA anticoagulant or by leukapheresis as previously described [27]. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Ficoll-paque, Pharmacia Uppsala, Sweden) and cryopreserved in 10% dimethlyl sulfoxide (DMSO, Sigma-Aldrich, St- Louis, MO) 90% fetal bovine serum (FBS, Medicorps, Montreal, Quebec, Canada).

Flow Cytometry

Activation marker expression levels on T cells was measured on thawed PBMCs that were at least 80% viable by staining with fluorescein isothiocyanate (FITC) conjugated anti-CD8, phycoerythrin (PE) conjugated anti-CD38, allophycocyanin (APC) conjugated anti-HLA-DR, and peridinin chlorophyll protein (PerCP) anti-CD3 antibodies (BD Biosciences, Mississauga, Canada) for 30 minutes in the dark. In parallel, control samples were stained with PE- and APC-conjugated immunoglobulin isotype control antibodies (BD Biosciences) and used to set gates for defining positive staining. Analysis was performed on a FACSCalibur instrument (BD Biosciences). At least 100,000 events were acquired and analyzed using FlowJo software, version 8.8 (Tree Star, Inc, Ashland OR).

Statistics

GraphPad Prism software version 4.0a was used for graphical presentation and GraphPad InStat version 3.06 for statistical analysis. Mann-Whitney and Kruskal-Wallis tests with Dunn's multiple post-test comparisons were used to assess the significance of between group differences for comparisons of 2 and more than 2 groups, respectively. Linear regression was used to calculate CD4 count change. P-values <0.05 were considered significant.

RESULTS

Study population

Table 1 provides information on age, CD4 count, CD8 counts and $\log_{10}$VL at the time at which percent $CD38^+DR^+CD8^+$ T cells were assessed for each of the EC participants included in this study. It also presents information on the number of CD4 count assessment and follow up time used to calculate annual rate of CD4 count change with 95% confidence intervals (CI). As well, HLA-B alleles and generic KIR3DL1 genotype is shown for each subject in the EC group. Table 2 compares the gender composition, median (range) age, CD4 count, CD8 count, $\log_{10}$VL and duration of infection for the EC group with that of 24 HIV infected progressors. The ECs and progressor groups were similar to each other in age and absolute CD8 T cell counts ($p>0.05$; Mann-Whitney test). As expected based on the criteria used to define the study populations, EC had significantly lower $\log_{10}$VL and higher absolute CD4 counts compared to progressors at the time of immune activation assessment ($p<0.05$ for both comparisons; Mann-Whitney test). EC were infected for longer than progressors ($p<0.05$; Mann Whitney test). The control group of 13 healthy controls included 9 males and 4 females aged a median (range) of 27 (23, 51) yrs.

Assessment of immune activation in HIV-infected EC, progressors and healthy controls.

To address the reproducibility of the assessment of percent $CD38^+DR^+CD8^+$ T cells we tested 6 time points from the same HIV positive treatment naïve EC individual in duplicate on 2 occasions. The average intra- and inter-assay coefficients of variation (CV) were 3.7% and 12.62%, respectively. The CV for this measure determined 6 times

over 3 years of follow up was 14.9%. In contrast, the CV for percent $CD38^+DR^+CD8^+$ T cells observed among the individuals in the EC and progressor groups was 67.5% and 65.4%, respectively. Therefore, the intra- and inter-assay variability for assessment of this immune activation parameter did not exceed 13% providing a measure of the reproducibility of this measurement within and between experiments. The variability of this immune activation marker within a study subject followed 6 times over 3 years was less than the variability observed among unrelated HIV infected EC or progressors confirming the notion of an immune activation set point introduced by Deeks et al [10].

Figure 1 shows a scatter plot displaying the distribution of the percent of $CD38^+DR^+CD8^+$ T cells in the 3 study groups. Healthy controls, EC and HIV infected progressors had a median (range) of percent $CD38^+DR^+CD8^+$ T cells of 2.83 (0.9, 7.3), 12.6 (2.3, 37.3) and 39.8 (2.87, 77.4), respectively. Levels of this marker was significantly higher in EC than in healthy controls and lower than in progressors ($p<0.01$ and $p<0.001$ for both comparisons; Dunn's multiple comparisons test).

EC with declining CD4 counts do not have higher levels of percent $CD38^+DR^+CD8^+$ T cells than those with stable/increasing CD4 counts.

Previous studies have proposed immune activation to be an important driver of CD4 decline [2, 28]. Twenty-five EC were followed longitudinally for a minimum of 2 years with at least 4 CD4 count determinations. We used this information to calculate their annual rate of CD4 count change. The median (range) number of CD4 determinations per subject was 18 (4, 86) taken over 10 (1, 20) yrs. Overall, the rate of CD4 count change

was -6.04 (-48.9, 32.1) (Table 1). Since longitudinal CD4 count determinations for any one patient are not linear and biological fluctuations in CD4 count occur, leading to wide 95% CI for CD4 count slopes within any given patient, we categorized all CD4 count slopes having a 95% CI that crossed zero as not significantly different from zero or stable. According to this criterion 15 EC had stable CD4 count slopes, 3 had CD4 count slopes that increased and 7 that declined significantly. Figure 2 shows graphs plotting the CD4 count change for the 10 subjects with either increasing (Figure 2A) or decreasing (Figure 2B) annual CD4 slopes. Since the EC group described here exhibited higher immune activation levels than healthy controls, we questioned whether EC with declining CD4 counts would have higher immune activation levels than those with stable or increasing CD4 count slopes. The percent of $CD38^+DR^+CD8^+$ T cells for EC with declining and stable/increasing CD4 count slopes was 8.8 (3, 35.4) and 10.2 (2.4, 37.3) (p=0.92, Mann-Whitney test) (Figure 3). Therefore, EC with falling CD4 counts were indistinguishable from those with stable/increasing CD4 counts with respect to this measure of immune activation.

*Expression of HLA-B*57 or KIR3DL1+Bw4*80I genotypes is associated with elevated levels of percent $CD38^+DR^+CD8^+$ T cells in EC.*

EC are more likely than typical progressors or uninfected controls to express protective HLA alleles such as HLA-B*27, B*57 or B*58 [12, 21, 29, 30]. Twenty one of 34 (61.8%) ECs had at least 1 copy of a protective HLA allele. The percent of $CD38^+DR^+CD8^+$ T cells in EC with no versus at least 1 of these protective alleles was 7.1 (3, 37.3) and 14.6 (2.2, 35.4), respectively, a difference that did not achieve statistical

significance (p=0.13; Mann-Whitney test (Figure 4A). However, EC who were HLA-B*57 positive (n=13) had higher levels of percent $CD38^{+}DR^{+}CD8^{+}$ T cell than either the 13 EC expressing no protective HLA alleles or the 21 EC not expressing HLA-B*57 (17.5 [2.4, 35.4], 7.1 [3, 37.3] and 7.3 [2.4, 37.3], respectively; p=0.01 and p<0.01 for each comparisons; Mann-Whitney test) (Figure 4B).

Co-carriage of the KIR3DS1 with its putative ligand, HLA-Bw4 with an isoleucine at position 80 of the HLA heavy chain (Bw4*80I), is associated with slower time to AIDS [19]. Seven EC carried this KIR/HLA combination whereas 8 were Bw6 homozygotes (hmz). Bw6 hmz have no HLA alleles able to interact with KIR3DS1 and therefore serve as controls for any signaling that may occur through KIR3DS1 binding its ligand [31]. EC who were KIR3DS1+Bw4*80I had higher levels of the percent $CD38^{+}DR^{+}CD8^{+}$ T cell immune activation marker than Bw6 hmz (15.3 [2.4, 37.3] and 6.9 [3, 12.4], respectively; p=0.05; Mann-Whitney test).

In summary EC who carry the protective HLA-B*57 allele or the KIR3DS1+Bw4*80I genotype associated with slower time to AIDS have higher levels of percent $CD38^{+}DR^{+}CD8^{+}$ T cells than EC carrying no protective HLA or KIR/HLA combinations.

DISCUSSION

We confirmed previous reports of elevated levels of $CD8^+$ T cell immune activation among EC compared to healthy uninfected subjects [8, 16]. EC with a declining CD4 counts did not have elevated percent $CD8^+DR^+CD8^+$ T cell levels compared to those with stable or increasing CD4 counts. EC who expressed HLA-B*57 or the KIR3DS1/HLA-Bw4*80I combined genotype, had higher immune activation levels versus those without these genotypes.

High T-cell activation levels predict more rapid disease progression in untreated HIV infected individuals and decreased treatment mediated gains during anti-retroviral therapy independent of plasma HIV RNA levels [4, 5, 32-34]. The correlation between HIV VL and immune activation has made it difficult to resolve the relative contributions of immune activation independently of viremia on disease progression. Although spontaneous control of viremia predicts slower HIV disease progression, VL alone only explains a fraction of the variability in rate of HIV disease progression [35].

Even in EC, undetectable VL is not always accompanied by maintenance of CD4 counts above 500 cells/mm^3 and a stable CD4 count slope, suggesting that some EC are exhibiting evidence of HIV disease progression [8, 9, 13, 14, 16, 36, 36, 37]. We hypothesized that in a setting of controlled viremia it would be possible to determine whether immune activation is driving the rate of CD4 count change. Although there have been several reports of EC exhibiting low or declining CD4 counts despite VL control to

below the limit of detection of standard assays, the cross sectional nature of some of these analyses [8], small sample size [9, 16, 36] and failure to take 95% CI into consideration in assigning a negative value to the slope of CD4 count change [14, 37] may have limited their ability to determine whether immune activation is driving CD4 decline. The results presented here add to this body of knowledge by reporting that in a group of 25 EC with a median (range) follow up time of 10 (1,20) yrs and 18 (4,86) CD4 count determinations 7 (28%) EC exhibited a negative slope of CD4 count change. Since those with declining CD4 counts did not have higher levels of immune activation than those with stable or increasing CD4 counts our results support the interpretation that the level of immune activation as determined by the percent of $CD38^{+}DR^{+}CD8^{+}$ T cell levels is not high enough in EC to drive CD4 decline.

Recently, it has been observed that most EC have low-level viremia detected by assays that are more sensitive than the standard VL assays [37-39]. A limitation of the results reported here is that we do not have access to sufficient volumes of plasma from these subjects to obtain VL information using more sensitive assays that detect VL levels below 50 copies/ml plasma to address this point. Therefore we cannot rule out that low level VL may be a determinant of immune activation as measured by assessment of percent $CD38^{+}DR^{+}CD8^{+}$ T cells.

Several MHC class I alleles such as HLA-B*27, *B57 and B*58 have been reported to be associated with slower time to AIDS and VL control [18, 21, 30]. There is an over representation of these alleles among EC [11, 12, 40, 41]. We noted that EC carrying

HLA-B*57 have higher levels of immune activation than those with no protective HLA alleles. We also observed a non-significant trend towards carrying at least 1 protective HLA allele and higher immune activation. It is likely that this non-significant trend is driven by percent $CD38^+DR^+CD8^+$ levels from EC carrying HLA-B*57. If carriers of a protective HLA allele other than B*57 are compared to those with no protective HLA alleles for percent of $CD38^+DR^+CD8^+$ T cells, no between-group differences are seen (not shown).

MHC class I alleles present pathogen derived epitopes to $CD8^+$ T cells. In individuals undergoing acute infection and in EC carrying one of these protective alleles a large proportion of the HIV-specific response is restricted by protective HLA alleles [42] (our unpublished observations). In HLA-B*57 positive subjects undergoing acute infection, the $CD8^+$ T cell responses restricted by this allele seem to be particularly effective at controlling the initial VL spike [43]. HIV isolates from HLA-B*57 and B*58 carriers develop CTL escape mutations early in infection that have a negative impact on viral replicative fitness [44-46]. These findings suggest that the $CD8^+$ T cell responses restricted by protective MHC class I alleles are important in viral control. Finding higher levels of $CD8^+$ T cell immune activation markers on carriers of HLA-B*57 may reflect a level of immune activation that is effective at controlling VL in a setting in which HIV disease progression is limited [47].

We also observed that carriers of KIR3DS1+HLA-Bw4*80I had higher levels of immune activation than Bw6 hmz expressing no ligands for KIR3DS1. KIR3DS1 is an activating

receptor expressed on Natural Killer cells and certain $CD8^+$ T cell subsets [48]. Epidemiological studies and inhibition of viral replication experiments implicate interactions between KIR3DS1 and HLA-Bw4*80I alleles in control of HIV in early infection and in slowing time to AIDS [19, 49, 50]. HLA-B*57 is a Bw4*80I allele. Indeed, 5 or the 6 individuals in the KIR3DS1+Bw4*80I group were HLA-B*57 positive. Therefore, the elevated levels of immune activation in carriers of this KIR/HLA genotype may be due to the role the B*57 allele plays in HIV-specific T cell responses. The possibility that this allele may also function through interactions with activating receptors on $CD8^+$ T cell subsets warrants further investigation.

In summary, despite VL control, EC have higher $CD8^+$ T cell activation levels than uninfected healthy controls. Some EC have declining CD4 counts and thus appear to be exhibiting HIV disease progression. Immune activation as determined by percent CD38-HLA-DR^+CD8^+ T cell levels is not higher in the EC subset with falling CD4 counts. EC carrying the protective HLA-B*57 allele or the protective KIR3DS1+Bw4*80I genotype combination have higher levels of immune activation than those without these genotypes. This may reflect a $CD8^+$ T cell mediated immune response effective at controlling VL rather than one driving CD4 decline and disease progression.

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CHAPTER TWO LEGENDS

Figure 1. Distribution of $CD8^+$ T cell activation markers among HIV uninfected healthy controls, HIV infected Elite Controllers (EC) and HIV infected progressors

Shown is a scatter plot of the percent of $CD38^+DR^+CD8^+$ T cells in healthy controls (HIN-neg), HIV-infected EC (EC) and progressors (PROG). The line through each scatter plot indicates the median value for the group. The significance of between-group activation marker levels was assessed by comparing EC with healthy controls and with HIV infected progressors using a Kruskal-Wallis test with Dunn's multiple post-test comparisons. P-values shown correspond to comparisons performed between the 2 groups linked by the line under the p-values.

Figure 2. Rate of CD4 decline in Elite Controllers with stable/increasing and declining annual rates of CD4 decline. Each graph shows longitudinal absolute CD4 count determinations obtained through the period each study subject was followed. Time from start of follow up at which CD4 counts were assessed is shown on the x-axis, while the absolute CD4 count in cells/mm^3 is shown on the y-axis**.** Panel A show results for the 3 subjects with positive CD4 count slopes and B the 7 subjects with negative CD4 count slopes. The trend line through the points describes the annual slope of CD4 count change, which is also written over each graph. The arrows in each graph indicate the time from start of follow up at which immune activation was measured.

Figure 3. Percent of $CD38^+DR^+CD8^+$ T cells does not distinguish EC with stable/increasing versus declining CD4 counts. Shown is a scatter plot comparing the percent of $CD38^+$HLA-DR^+CD8^+ T cells in the EC group with stable or increasing versus decreasing CD4 counts. The bar through each scatter plot indicates the median value for the group. P-values shown correspond to between-group comparisons performed using a Mann-Whitney test.

Figure 4. Comparison of the percent of $CD38^+$HLA-DR^+CD8^+ T cells in Elite Controllers with or without protective HLA alleles or KIR/HLA genotypes. Shown are scatter plots comparing $CD38^+$HLA-DR^+CD8^+ T cell levels in carriers of no versus at least 1 protective HLA allele (Panel A), no protective HLA alleles versus at least 1 HLA-B*57 allele (Panel B) and Bw6 homozygotes versus carriers of a KIR3DS1+Bw4*80I genotype. The line through each scatter plot indicates the median value for the group. P-values shown correspond to between-group comparisons performed using a Mann-Whitney test.

CHAPTER TWO FIGURES

Figure 1

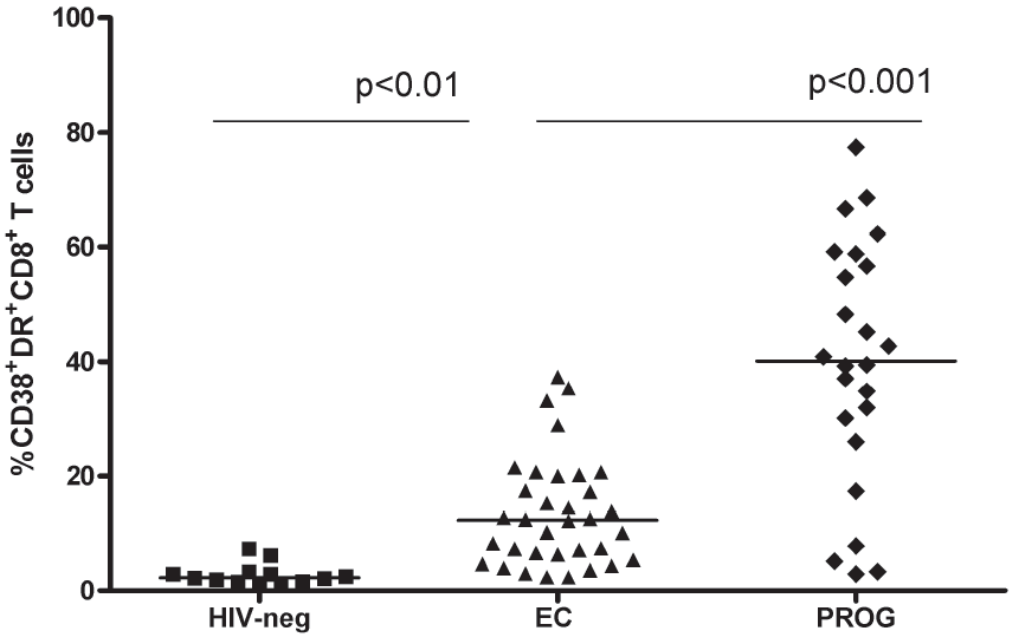


Figure 2

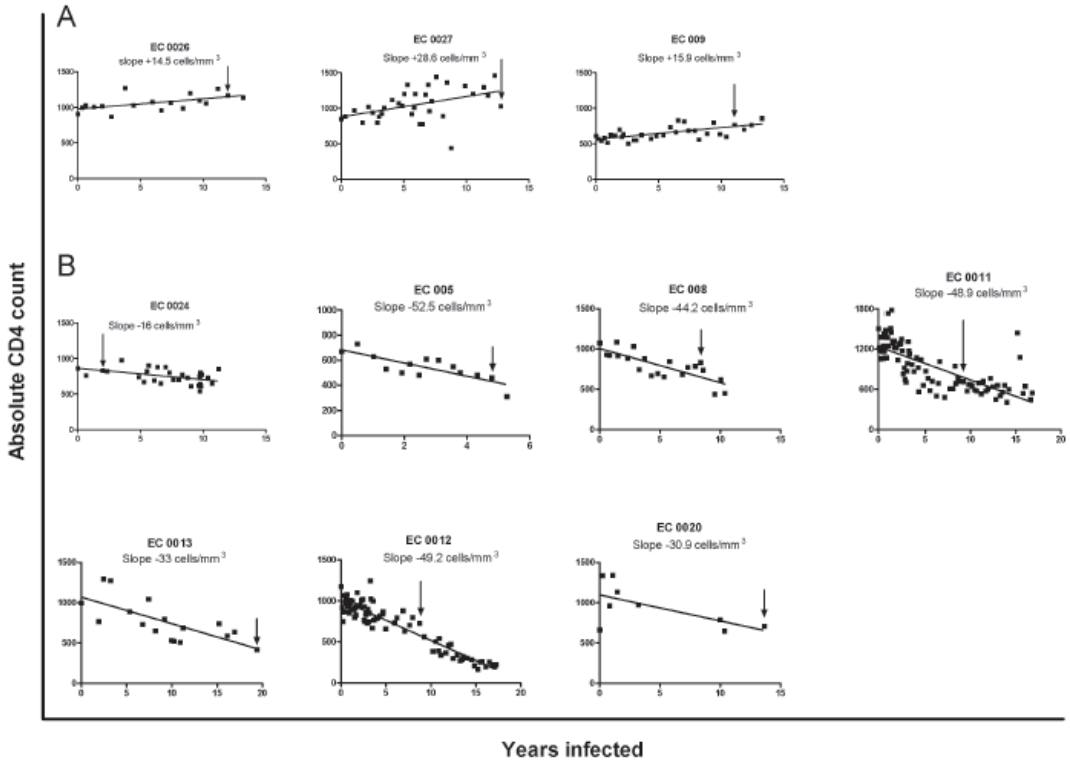

A
EC 0026
slope +14.5 cells/mm 3
EC 0027
Slope +28.6 cells/mm 3
EC 009
Slope +15.9 cells/mm 3
B
EC 0024
Slope -16 cells/mm 3
EC 005
Slope -52.5 cells/mm 3
EC 008
Slope -44.2 cells/mm 3
EC 0011
Slope -48.9 cells/mm 3
EC 0013
Slope -33 cells/mm 3
EC 0012
Slope -49.2 cells/mm 3
EC 0020
Slope -30.9 cells/mm 3
Absolute CD4 count
Years infected

Figure 3

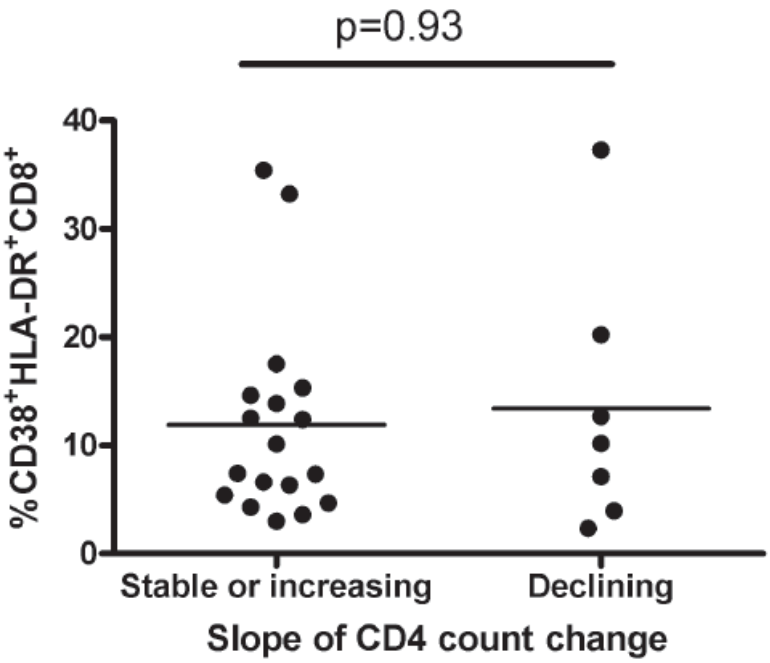
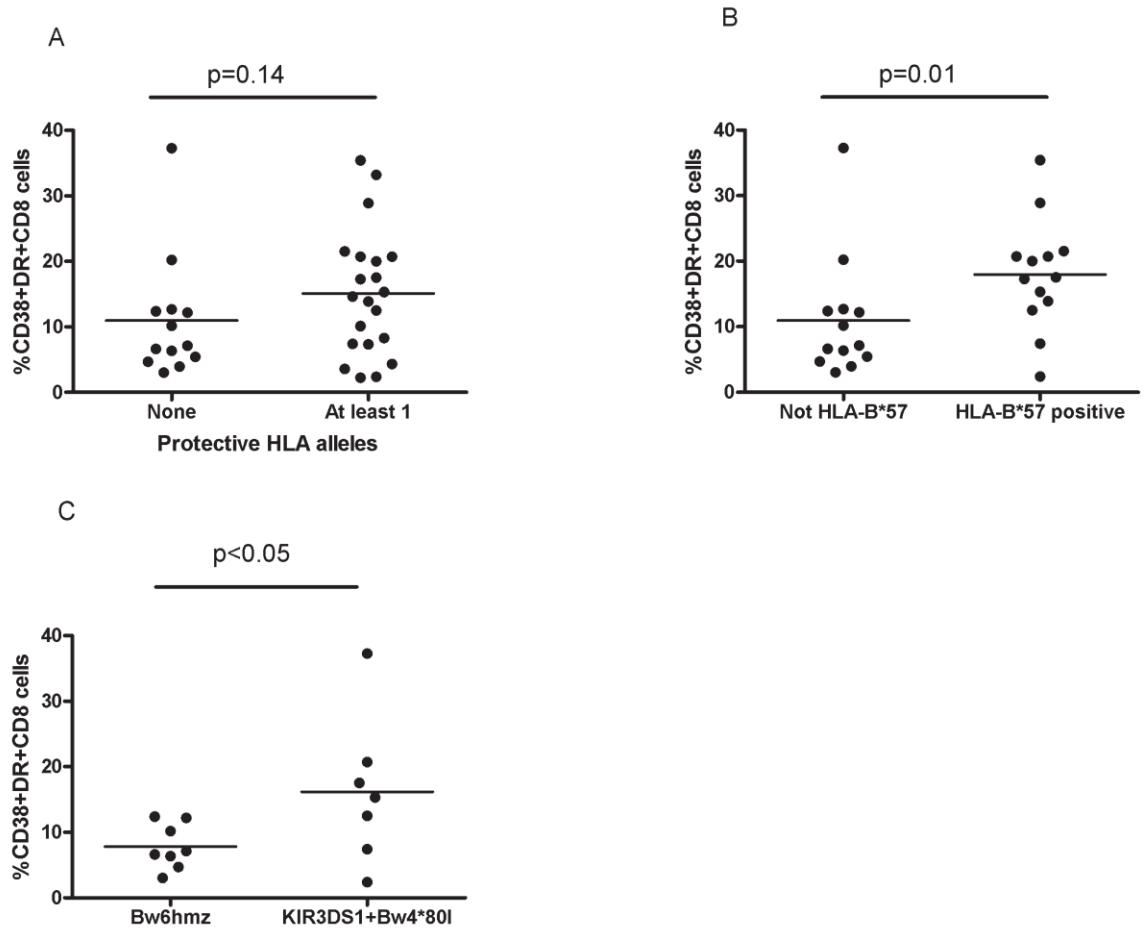

p=0.93
40
30
20
10
0
%CD38+HLA-DR+CD8+
Stable or increasing
Declining
Slope of CD4 count change

Figure 4


A
p=0.14
%CD38+DR+CD8 cells
40
30
20
10
0
None
At least 1
Protective HLA alleles
B
p=0.01
%CD38+DR+CD8 cells
40
30
20
10
0
Not HLA-B*57
HLA-B*57 positive
C
p<0.05
%CD38+DR+CD8 cells
40
30
20
10
0
Bw6hmz
KIR3DS1+Bw4*80I

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CHAPTER TWO AND THREE LINKER

The data presented in Chapter two of this thesis demonstrated that in a setting of undetectable viremia immunological heterogeneity can occur among EC. Examinations of T immune activation EC showed that those carrying the protective HLA-B*57 allele or the protective KIR3DS1+Bw4*80I genotype combination have higher levels of immune activation than those without these genotypes and created the impetus for me to formally investigate a role for KIR/HLA combined genotypes and NK cells in slow disease progression in Chapter three.

CHAPTER THREE

Receptor-ligand requirements for increased NK cell poly-functional potential in *h/*y+B57 HIV-1 infected Slow progressors

Receptor-ligand requirements for increased NK cell poly-functional potential in *h/*y+B57 HIV-1 infected Slow progressors

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Running head: NK cell function in HIV infected slow progressors.

ABSTRACT

Carriage of the Natural Killer (NK) receptor genotype KIR3DL1*h/*y with its HLA-B*57 ligand (*h/*y+B57) is associated with slow time to AIDS and low viral load (VL). To provide a functional basis for these epidemiological observations, we assessed whether HIV-1 infected slow-progressors (SP) carrying the *h/*y+B57 compound genotype would have increased NK cell poly-functional potential in comparison to SP with other KIR/HLA compound genotype and whether this enhanced poly-functionality was dependent upon the co-expression of both the KIR3DL1*h/*y and HLA-B*57. The functional potential of NK cells was investigated by stimulating peripheral blood mononuclear cells with HLA-devoid targets or single HLA-transfectants. Multi-parametric flow cytometry was used to detect NK cells with 7 functional profiles representing all permutations of CD107a expression, IFN-γ and TNF-α secretion. NK cells from individuals carrying KIR3DL1 receptor-HLA-Bw4 ligand pairs had greater tri-functional responses than those from KIR3DL1 hmz who were Bw6 homozygotes. NK cells from subjects carrying the *h/*y+B57 genotypes exhibited the highest tri-functional potential and this was dependent on co-carriage of the NK receptor and its ligand. On a per cell basis, tri-functional cells secreted more of each function tested than corresponding mono-functional NK subset. Although VL influenced NK functionality, individuals with defined KIR/HLA genotypes exhibited differences in NK cell poly-functionality that could not be accounted for by VL alone. The protective effect of HLA-

B*57 on slow progression to AIDS and low VL may be mediated through its interaction with KIR3DL1 alleles to educate NK cells for potent activity upon stimulation.

INTRODUCTION

Natural Killer (NK) cells are key players in host innate immune defenses. They function in the early response to virus infected and transformed cells without prior sensitization [507, 508]. Through the release of pre-formed granules containing perforin and granzyme, NK cells can directly lyse tumour cells and virally infected targets. In addition, they can secrete large amounts of pro-inflammatory cytokines and chemokines, which promote interactions with Dendritic Cells, monocytes and granulocytes and influence the evolution of the adaptive immune response [509-511].

NK function is tightly regulated by the integration of inhibitory and activating signals transmitted through germ-line encoded cell surface receptors, which include alleles encoded by Killer Immunoglobulin-like Receptor (KIR) region genes. KIRs are expressed on NK cells and some T cell subsets [509]. The most polymorphic locus among KIR region genes is KIR3DL1, which encodes both inhibitory KIR3DL1 (henceforth 3DL1) and activating KIR3DS1 (3DS1) alleles [512]. 3DL1 alleles can be further classified according to their expression levels on the cell surface into high (*h), low/intermediate (*l) and null (*004) unexpressed alleles. [512-515]. Genotypes homozygous for 3DL1 can be divided into 2 groups: *h/*y (where *y can be either another *h allele or *004) with no *l alleles and *l/*x (where *x can be either another *h, *l or *004) with at least one *l allele [516]. Cell surface staining with DX9, a monoclonal antibody (mAb) specific for 3DL1, shows that 3DL1 levels are higher on NK cells from carriers of *h/*y than *l/*x genotypes [513].

The ligands for 3DL1 receptors belong to the Bw4 group of Major Histocompatibility Complex (MHC) class I B encoded alleles that are distinguished from

the Bw6 subset by amino acids present between position 77 and 83 of the HLA heavy chain [517-519]. Bw4 alleles can be further divided into those with an isoleucine at amino acid 80 (Bw4*80I) or a threonine at this position (Bw4*80T) of the HLA heavy chain [520]. Bw4*80I alleles are the preferred ligands for 3DL1 receptors [481, 521].

Epidemiological studies have shown an association between certain KIR/HLA combined genotypes with time to AIDS and viral load (VL) setpoint [516, 522]. Compared to Bw6 homozygotes (hmz) with no alleles that interact with KIR, the 3DL1/Bw4 receptor-ligand combination, which has the most potent effect on favorable HIV disease outcomes is the one that combines 3DL1*h/*y with HLA-B*57, (*h/*y+B*57) [516]. NK cells from HIV uninfected persons carrying the *h/*y+B*57 genotype respond to stimulation with the HLA-devoid K562 cell line with a higher frequency of functional cells exhibiting multiple functions than NK cells from Bw6 hmz [523]

B*57 is a protective allele in the context of HIV infection and has a higher frequency in HIV-infected slow progressors (SP) than in HIV disease progressors or healthy controls [252, 524, 525]. Part of its effect on slow time to AIDS and VL setpoint is mediated through the adaptive arm of the immune response via CD8+ T cells that recognize B*57 restricted epitopes [525-529]. However, because B*57 is a Bw4*80I allele and therefore a ligand for 3DL1 receptors, it has the capacity to participate in the MHC class I dependent NK cell functional maturation process termed ‘education’ or ‘licensing’ that renders NK cells functionally competent [530].

This study investigated the relationship between NK cell functional potential and KIR/HLA genotype in a population of HIV infected SP. We found significant differences in the percent contribution of NK cells with 3 functions (secretion of Interferon-γ [IFN-γ]

and Tumor Necrosis Factor-α [TNF-α] and expression of CD107a) following stimulation with K562 when from 3DL1 hmz who carried at least 1 copy of a Bw4 allele versus a Bw6 hmz genotype. Of those who carried the 3DL1 and Bw4 NK receptor-ligand combination, it was the carriers of the *h/*y+B*57 genotype that exhibited the highest NK functional potential. Neither expression of 3DL1*h/*y nor B*57 alone was sufficient to support the level of stimulated tri-functionality observed in NK cells from *h/*y+B*57 carriers. Each of the functions exhibited by tri-functional NK cells was more potent on a per cell basis than the same function in the mono-functional NK subsets. We used a panel of transfectants expressing single HLA alleles to further confirm that disruption of 3DL1 signaling was responsible for elevated functional levels in NK cells from *h/*y+B57 carriers stimulated with HLA devoid cells. Transfectants expressing the Bw4*80I alleles B*57:01 and B*27:02 suppressed the function of NK cells from *h/*y+B57 carriers while the transfectant expressing the Bw6 B*35:02 allele did not.

MATERIALS AND METHODS

Study population

The study population included 57 HIV-infected SP. Thirty-six SP were recruited from the Canadian Cohort of HIV Infected Slow Progressors and 21 were from a cohort followed at the National Institutes of Allergy and Infectious Diseases (NIAID) [531]. The term SP was used here to define treatment naïve HIV infected subjects who maintained absolute CD4 counts above 400 cells/mm3 for more than 7 years, Elite Controllers (EC- followed for at least 1 year with VL <50 copies/ml of plasma) and Viral Controllers (VC- followed for at least 1 year with VL <3000 copies/ml of plasma). Information on CD4 and CD8 T cell counts, VL and duration of infection at time of testing, 3DL1 genotype and HLA type of the study population is provided in Table 1. All participants were 3DL1 hmz. This allowed them to be classified as 3DL1*h/*y or *l/*x and eliminated the possible confounding influence of an activating 3DS1 allele on NK functional potential [259, 513, 516]. Informed consent was obtained from all study participants and research adhered to the ethical guidelines of the authors' institutions. For certain comparisons we also included results generated from 11 HIV-1 negative *h/*y+B57 carriers whose characteristics have been previously described [523] and 13 HIV-1 infected untreated viremic progressors recruited from the Montreal Primary Infection cohort. The descriptive characteristics of these 13 HIV infected viremic progressors are included in Table 1.

MHC and KIR typing

All subjects were typed for MHC class I alleles by sequence-based typing using kits from Atria Genetics (South San Francisco, CA) and using Assign software to interpret sequence information for allele typing (Conexio Genetics, Perth, Australia) as previously described [523]. HLA-B alleles were classified as either Bw4 or Bw6 and Bw4 alleles as either Bw4*80I or Bw4*80T depending on whether they had an isoleucine or threonine at position 80 of the HLA heavy chain. Bw6 hmz served as controls for the effect of NK education signals through 3DL1 on NK functional potential as Bw6 alleles do not interact with 3DL1 [519, 520]

3DL1/S1 genotyping was performed using two sets of primers specific for the 3DL1 and 3DS1 loci as previously described [259]. Subjects were subsequently 3DL1 allotyped by identifying single nucleotide polymorphisms (SNP) corresponding to high frequency 3DL1 alleles as previously described [523]. In our study we categorized 3DL1*005, *006, *007, *053, *054 as *l alleles, *001, *002, *008, *009, *015, *020 as *h alleles and *004 as a null allele.

Cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Paque Pharmacia Upsala, Sweden) from whole blood obtained by venipuncture into tubes containing EDTA anti-coagulant or by leukaphersis as previously

described [532]. Cells were cryopreserved in 10% DMSO (Sigma-Aldrich, St. Louis, MO) with 90% fetal bovine serum (FBS, Wisent, St. Bruno, Quebec, Canada).

Assessment of NK cell functional potential

NK cell activation

Cryopreserved PBMC were thawed and resuspended at 106 cells/ml in RPMI 1640 that contained 10% FBS, 2mM L-glutamine, 50 IU penicillin and 50µg/ml streptomycin (all from Wisent). Brefeldin A (5µg/ml, Sigma-Aldrich), Monesin (6µg/ml, Golgi-Stop; BD Biosciences, Mississauga, Ontario, Canada) and anti-CD107a–FITC mAb (BD Biosciences) were added to the cells.

PBMC (10^6 per condition) from 47 SP were stimulated with HLA-devoid K562 cells (American Type Culture Collection Manassas, VA). PBMC (106 per condition) from 10 of the SP carrying *h/*y+B57 were stimulated with the HLA-devoid Epstein-Barr Virus (EBV)-transformed 721.221 (221) cell line or 221 transfectants expressing single HLA alleles (221-B*57:01, 221-B*27:02 and 221-B*35:02). For all stimulations, the PBMC: target ratio was 5:1 for 6 hours at 37^oC in a humidified 5% CO_2 incubator. Included in all experiments were conditions where PBMC were stimulated with medium alone as a negative control and with 1.25µg/ml phorbol 12-myristate 13-acetate (PMA); 0.25µg/ml ionomycin, (Sigma Aldrich) as a positive control. Only data from cells that responded to the PMA; ionomycin positive control stimulus, were included in analyses.

NK cell staining for phenotype and function

After stimulation, cells were stained for viability using the Aqua LIVE/DEAD® fixable dead cell stain kit (Invitrogen, Burlington, Ontario, Canada) following manufacturer's instructions. Live PBMC were then stained for cell surface markers with anti-CD56-APC, anti-CD16-Pacific Blue (BD Biosciences), anti-CD3-ECD and CD158e-PE (ie: Z27-PE, Beckman Coulter, Mississauga, Ontario, Canada) for 30 min. After washing with phosphate buffered saline (PBS) containing 1% FBS (Wisent) and 0.1% sodium azide (Sigma Aldrich), cells were fixed and permeabilized using the Fix and Perm Kit (Invitrogen) and stained for intracellular cytokines using anti-IFN-γ-Alexa 700 and anti-TNF-α-PE-Cy7 mAbs (BD Biosciences). Cells were washed and fixed with a 1% paraformaldehyde solution (Fisher Scientific, Ottawa Ontario, Canada) and stored in the dark at 4^{o}C until acquisition.

In parallel one million unstimulated PBMCs were phenotyped for NK markers by staining for viability using the Aqua LIVE/DEAD® fixable dead cell stain kit (Invitrogen) and for cell surface markers with anti-CD56-APC, anti-CD16-Pacific Blue(BD Biosciences), anti-CD3-ECD and CD158e-PE (ie: Z27-PE, Beckman Coulter, Mississauga, Ontario, Canada) for 30 min. After washing with PBS containing 1% FBS (Wisent) and 0.1% sodium azide (Sigma Aldrich), cells were washed and fixed with a 1% paraformaldehyde solution (Fisher Scientific) and stored in the dark at 4^{o}C until acquisition.

Flow cytometry analysis

Cells were acquired on an LSRII flow cytometer (BD Biosciences). Between 400,000 and 500,000 events were collected per sample. Data analysis was performed using FlowJo software version 8.7.1 (Tree Star, San Carlos, CA). The functional profiles of NK cells stimulated with K562, 221 and the 221 HLA transfectant panel were determined using the gating strategy shown in Supplementary Figure 1. NK cells were defined as $CD3^{-}CD56^{+/-}CD16^{+/-}$. Boolean gating was used to identify seven NK cell functional profiles, i.e. tri-functional NK cells (CD107a+ IFN-γ+ TNF-α+), bi-functional NK cells (any combination of two of these functions) and mono-functional NK cells (any single one of these functions). The Z27 antibody was used to assess the frequency and percent contribution of each of the seven possible NK cell functional profiles within the 3DL1+ or 3DL1- NK response. To obtain the frequency of each functional subset and then the percent contribution of each NK cell functional subset to the total K562 stimulated response, the negative control (medium alone) value for each of these functional subsets was subtracted from the corresponding K562 stimulated functional subset response to correct for background. To support analyses where mean fluorescence intensity (MFI) of CD107a expression and IFN-γ and TNF-α was compared in NK cells having different functional profiles Ultra Rainbow Fluorescent particles (Spherotech, Cedarlane, Burlington, Ontario, Canada) were used to calibrate the LSRII flow cytometer. 500,000 events for acquired for the MFI comparisons.

Generation of 221 cells expressing individual HLA alleles

The cDNAs encoding HLA-B*57:01, HLA-B*27:02 and HLA-B*35:02 were amplified using PCR and cloned into a murine stem cell virus (MSCV)-based retroviral vector encoding the puromycin resistance gene under the control of an internal rybozyme entry site (IRES). The full-length insert was sequenced, and the sequence was verified against the reference sequences in the IMGT/HLA database. HEK-293T cells were co-transfected with the retroviral construct and plasmids encoding gag/pol and VSV-g, and retrovirus-containing supernatant was harvested 48 and 96 hours post-transfection. To generate 221 cells stably expressing individual HLA alleles, 10^5 221 cells were cultured in the retrovirus-containing supernatant for 3 days. Two days after this incubation, 1 μg/mL puromycin was added, and the cells were cultured until a stable polyclonal line was produced. The surface expression of the individual alleles was verified using flow cytometry, and following selection, all cells expressed the individual alleles.

Statistical analysis

GraphPad Instat 3.05 and GraphPad Prism 4.01 were used for statistical analyses and graphical presentations. Mann-Whitney U tests were used for between group comparisons in the percent contribution of tri-functional NK cells to the total NK cell response among SP. A Bonferonni correction was applied to situations where multiple comparisons were made. The Spearman correlation test was used to assess the significance of associations between the percent contribution of a response to the total NK response and its frequency and to test the significance of associations between VL and the percent contribution of the tri-functional NK response to the total NK cell

response. The percent of the parental 221 response for tri-functional 3DL1+ NK cells induced by each 221 HLA transfectant was calculated using the following formula: (frequency of Z27hi [i.e. 3DL1+] cells in a functional NK subset after incubation with a 221 transfectant/ frequency of Z27hi cells in that NK functional subset after stimulation with the parental 221 cell line) X 100. A paired t-test was used to assess the significance of the level of inhibition of the Z27hi tri-functional response between 221-HLA transfectants. A p-value of <0.05 was considered significant.

RESULTS

NK cells from 3DL1 hmz SP who carry at least 1 HLA-Bw4 allele have higher tri-functional potential than those from Bw6 hmz. To investigate whether NK cells from HIV-infected SP grouped according to whether their 3DL1 and HLA-B alleles were NK receptor-HLA ligand pairs differ in their NK cell functional potential, we measured the expression of CD107a and secretion of IFN-γ and TNF-α from K562-stimulated NK cells from 47 HIV-infected 3DL1 hmz SP using eight color multi-parametric flow cytometry. By measuring these three functions concurrently, we were able to assess the frequency of seven possible NK cell functional profiles that were positive responses to K562 stimulation and their percent contribution to the total K562 response. Supplementary Figure 1A shows the gating strategy that was used to obtain the frequency of each NK cell functional subset from which the percent contribution of each subset was calculated. Supplementary Figure 1B also shows examples of flow cytometry plots for NK cells from a representative *h/*y+B*57 SP stained with the isotype control for the Z27 monoclonal antibody that was used to set the gate for KIR3DL1+ NK cells (top row), an unstimulated control was used to set the gates for IFN-γ+, TNF-α+ and CD107a+ NK cells used to correct for background (middle row) and positive staining for IFN-γ, TNF-α, CD107a and Z27 in a K562 stimulated test sample (bottom row). Supplementary Figure 1B shows flow cytometry plots of the frequency of tri-functional NK cells from a representative *h/*y+B57 and Bw6 individuals.

Figure 1A shows the percent contribution of each NK cell functional profile in PBMC from 3DL1 hmz expressing HLA-Bw4, the 3DL1 ligand (n=40) with that from subjects who were Bw6 hmz (n=7). Of the seven NK cell functional profiles, only the

percent contribution of tri-functional NK cells to the total K562 response was significantly higher in the Bw4 positive compared to Bw6 hmz SP ((median, [range]) (2.42 [0.0-7.8]) and (0.52 [0.0-2.2]) for Bw4 positive and Bw6 hmz, respectively, corrected p=0.032; Mann-Whitney U test). When the data was analyzed using the frequency of each NK functional subset, only the frequency of tri-functional cells was significantly higher in the Bw4 positive versus Bw6 hmz SP. Since in all the analyses performed, the frequency of each NK cell functional subset was correlated with its percent contribution to the total response ($p<0.0001$; Spearman $r=0.93$, Figure 1B), we reported subsequent results as the percent contribution of a functional subset to the total NK cell response.

We next questioned whether, tri-functional NK cells were more potent than mono-functional cells for each function tested. We compared the MFI of IFN-γ and TNF-α secretion and CD107a expression in tri-, bi- and mono-functional NK cells. As seen in Figure 1C the MFI of IFN-γ secretion from tri-functional NK cells was significantly higher than that from bi-functional CD107a+IFN-γ+ NK cells and mono-functional NK cells secreting IFN-γ only ($p<0.001$ for both comparisons, Mann-Whitney U test). There was no significant difference in the intensity of IFN-γ secretion between the tri-functional NK subset and the one that secreted IFN-γ and TNF-α (Figure 1C). Figure 1D and 1E show the MFI of CD107 and TNF-α expression, respectively, in all the NK subsets exhibiting these functions. Tri-functional NK cells expressed more CD107a and TNF-α than the mono-functional NK subsets with these activities. ($p<0.001$, Mann-Whitney U test). The amount of CD107a and TNF-α secreted by tri-functional cells did not differ significantly from that secreted by the bi-functional subsets with these activities.

Collectively these results indicate that on a per cell basis, tri-functional NK cells exhibit multiple functions, each with a higher intensity than mono-functional NK cells.

*Receptor ligand requirements for increased NK cell tri-functional potential in *h/*y+B57 SP.*

The 3DL1/Bw4 combination with the most potent influence on time to AIDS and VL setpoint is the *h/*y+B57 genotype [516]. Our previous work in HIV uninfected subjects showed that NK cells from individuals with the *h/*y+B57 genotype had higher NK tri-functional potential than those from carriers of *h/*y+Bw4*80I, *h/*y+Bw4 or Bw6 hmz genotypes [523]. The low frequency of B*57+ subjects from which these HIV seronegative study subjects was drawn [533] and the even lower frequency of carriers of 3DL1*h/*y+B57 or 3DL1*l/*x+B57 precluded our being able to formally determine whether elevated tri-functional potential of NK cells from uninfected *h/*y+B57 carriers required the presence of both the NK receptor and HLA ligand [523]. B*57 is a protective allele in the context of HIV infection and is found at a higher frequency in HIV infected SP than in the uninfected population. We were therefore able to assemble a sufficient number of SP expressing *h/*y+B57, *h/*y with Bw4 alleles other than B*57 and *l/*x+B*57 carriers to address this point.

To assess the requirement for both carriage of a 3DL1*h/*y NK receptor genotype and HLA-B*57 ligand in elevated NK tri-functional potential, we compared the percent contribution of tri-functional NK cells from 23 3DL1 hmz who were B*57 positive to that of 18 3DL1 hmz who carried Bw4 alleles other than B*57 and found no significant between-group differences ($p>0.05$, Mann Whitney U test, Figure 2A). We

next divided the same SP subjects into two groups based on their 3DL1 allotype. No significant between-group differences were observed in the percent contribution of tri-functional NK cells to the total NK response between 24 subjects who were 3DL1*h/*y versus 17 subjects who were 3DL1*l/*x. ($p>0.05$; Mann-Whitney U test, Figure 2B).

The percent contribution of tri-functional NK cells from 14 *h/*y+B57 carriers was higher than that for 9 *l/*x+B57 carriers (4.1 [0.8, 7.8] versus 1.8 [0.0-6.4], $p<0.01$; Mann Whitney U test) (Figure 2C). This value was also higher than that seen in 12 individuals who carried the 3DL1*h/*y genotype with Bw4 alleles other than B*57 (*h/*y-Other Bw4) (1.49 [0.0-5.8]), or 7 Bw6 hmz (0.52 [0.0-1.68]), $p=0.05$ and $p<0.001$, respectively; Mann-Whitney U test) (Figure 2C). Therefore the enhanced NK tri-functional potential in *h/*y+B57 individuals depends on carriage of both 3DL1*h/*y and B*57 and supports the interpretation that neither the expression of the NK receptor nor the HLA ligand alone is sufficient for the elevated NK tri-functional potential observed in carriers of the *h/*y+B57 genotype.

The difference in tri-functional potential among these study groups was not due to differences in the percent or absolute number of total NK cells or NK subsets such as CD56dim and CD56bright cells between SP grouped by 3DL1/HLA-B (Supplementary Figure 3A-C). Nor did the percent of 3DL1+ NK cells differ between groups (not shown). The only exception to this is that *h/*y+B*57 SP had significantly lower levels of the anergic CD56-CD16+ NK subset when compared to SP with other 3DL1/HLA-B genotypes (Supplementary Figure 3D). In order to control for the possibility that increased tri-functional potential in NK cells from *h/*y+B57 carriers was due to lower percentages of anergic CD56-CD16+ cells, we performed a sub-analysis excluding the

CD56-CD16+ subpopulation from the comparison of the groups shown in Figure 2C. Removing CD56-CD16+ cells from the analyses did not affect the conclusion that a significantly higher percent contribution of tri-functional NK cells to the total NK response was observed when NK cells were from carriers of *h/*y+B57 in comparison to *l/*x+B57, *h/*y+Bw4 alleles other than B*57 and Bw6 hmz genotypes (not shown).

Influence of HIV VL on NK tri-functional potential.

3DL1 hmz SP stratified according to their 3DL1/HLA-B genotype exhibited subject to subject variation in their tri-functional potential (Figure 2C). It was reported previously that HIV-1 infection disregulates NK subset distribution and function [1, 499-501, 534]. Our definition of SP in the study population included subjects with detectable VL. We therefore questioned whether, there was evidence for any influence of VL on NK tri-functional potential. We observed a weak but significant negative correlation between the percent contribution of tri-functional NK cell responses to the total NK response and log10VL (r=-0.34, p=0.01; Spearman correlation test) (Figure 3A).

To address the possibility that EC with undetectable viremia were driving this negative correlation we re-analyzed the association excluding the EC subset. The significance of the correlation between Log10VL and NK cell tri-functional potential was lost (r=-0.03, p=0.88; Spearman correlation test) (not shown). No significant correlations were seen between the percent contribution of any of the bi- or mono-functional response to the total K562 stimulated response and $\log_{10}$VL (not shown). In order to address the possibility that between group differences in tri-functional potential could be accounted for by differences in VL, we compared VL in the 4 SP groups shown in Figure 2C. No

significant between-group differences in VL were observed (data not shown). To further address the concern that the differences in NK cell tri-functional potential in SP groups stratified according to their 3DL1/HLA-B genotypes could be attributed to differences in VL, we compared the tri-functional potential of NK cells from individuals with a VL below 3000 copies/ml plasma (EC and VC) that were *h/*y+B57 versus *l/*x+B57. We reasoned that the low levels of VL in these subjects would minimize the effect of VL on NK disregulation. Although no significant differences were detected in the VL between EC and VC with *h/*y+B57 and *l/*x+B57 genotypes, carriers of *h/*y+B57 had a significantly higher level of tri-functional NK cells in than *l/*x+B57 carriers (4.17 [0.86-7.83] and 1.90 [0.52-6.43], respectively; p=0.01, Mann Whitney U test) (Figure 3B).

We next compared NK cell tri-functional potential in 3 groups of individuals matched for the *h/*y+B57 combined genotype, 11 HIV uninfected subjects, 10 HIV infected individuals with VL <3000 copies/ml plasma (EC+VC) and 5 viremic HIV infected progressors. Whereas no significant differences were found between HIV negative individuals and EC+VC with this genotype combination (4.8% [0.7-11] vs 4.17 [0.86-7.83] p=0.3; Mann-Whitney U test), EC+VC had significantly higher levels of tri-functional NK cells versus viremic progressors (2.43 [1.62-2.85], p=0.02; Mann-Whitney U test) (Figure 3C). Furthermore, *h/*y+B57 viremic progressors had significantly higher levels of tri-functional NK cells than 8 Bw6 viremic progressors (0.66 [0.00-3.87], p=0.03; Mann-Whitney U test) even though these groups did not differ significantly from each other for VL (p>0.05; Mann-Whitney test) (Figure 3D). Together these results suggest that even though the negative impact of VL on NK function may contribute to

some of the variability in NK poly-functional potential, 3DL1/HLA-B genotype combinations are also determinants of NK functional potential.

*3DL1+ NK responses from *h/*y+B57 SP are inhibited by 221 cells bearing B*5701 and B*2702 alleles*

In order to determine whether the NK activation seen following stimulation with HLA devoid cells was related, in part, to the loss of suppressive signals mediated by 3DL1 interacting with its ligand, we stimulated NK cells from individuals expressing the *h/*y+B57 genotype with the HLA devoid 221 cell line and a panel of 221 transfectants expressing single HLA alleles (namely; the Bw4*80I alleles B*57:01, B*27:02, and B*35:02, a Bw6 allele). For these experiments we used the Z27 mAb to distinguish the 3DL1+ from 3DL1- NK cells.

Figure 4A shows flow cytometry plots of the frequency of 3DL1+ and 3DL1- NK cells secreting IFN-γ following stimulation of PBMC from a representative *h/*y+B57 carrier with media alone, the parental 221 cell line and 221 HLA transfectants. Stimulation with 221 induced 16% of 3DL1+ NK cells to secrete IFN-γ whereas 221-B*35:02 induced 11.4% of these cells to secrete this cytokine. Stimulation with 221-B*57:01 and 221-B*27:02 suppressed IFN-γ secretion to 1.46% and 5.75% of 3DL1+ NK cells, respectively. Within the 3DL1- compartment the extent of suppression of IFN-γ secretion by 221-B*57:01 and 221-B*27:02 compared to 221 and 221-B*35:02 target cells was much less.

Figure 4B, C shows pooled results for the percent contribution of IFN-γ+ secreting 3DL1+ and 3DL1- NK functional subsets to the total 3DL1+ and 3DL1- NK cell response, respectively, from 10 *h/*y+B57 carriers stimulated with the 221 panel. The percent contributions of IFN-γ+ 3DL1+ NK cells to the 3DL1+ NK cell response was significantly lower following stimulation with 221-B*57:01 and 221-B*27:02 than the parental 221 cell line ($p<0.01$ for both comparisons, Mann Whitney U test for). The percent contribution of IFN-γ+ secreting 3DL1+ to the 3DL1+ response did no differ between the 221-B*35:02 and the 221 parental line. None of the 221 transfectants suppressed IFN-γ secretion within the 3DL1- NK compartment compared to the parental 221 cell line. These results confirm that the loss of the interaction between 3DL1 receptors and their ligands is important to the enhanced function of NK cells from *h/*y+B57 carriers following stimulation with HLA-devoid cells. The presence of either B*57:01 or B*27:02 ligands on 221 transfectants that can interact with 3DL1 receptors is sufficient to suppress NK function in the NK subset expressing this receptor; in contrast 221.B*35:02 transfectants do not suppress NK cell function of 3DL1+ cells. Neither the 221 parental line nor any of the 221 transfectants suppressed NK function in the 3DL1- compartment.

We next examined the effect of the three transfectants on the 3DL1+ tri-functional NK cell response. In Figure 4D the effect of each HLA tranfectant on the 3DL1+ tri-functional subset is expressed as a percentage of the response to the parental 221 cell line, which is set at 100%. The 221-B*57:01 and 221-B*27:02 transfectants suppressed the tri-functional activity of 3DL1+ NK cells whereas the 221-B*3502 tranfectant did not.

Together these results indicate that interactions between cells expressing single HLA Bw4*80I alleles and 3DL1+ NK cells suppresses of NK activity.

DISCUSSION

Of the KIR/HLA combinations associated with slow time to AIDS and VL control *h/*y+B*57 is the most potent [516]. We report here that in 3DL1 hmz SP with at least 1 copy of a Bw4 allele, tri-functional NK cells contribute significantly more to the total K562 stimulated response than those from Bw6 hmz. *h/*y+B*57 carriers have the highest NK tri-functional potential compared with carriers of other 3DL1 hmz receptor genotypes and/or Bw4 alleles. Tri-functional NK cells have a higher functional potency than corresponding mono-functional cells. Although VL negatively impacts NK tri-functional potential, VL alone does not account for variations in NK functional potential. We were able to confirm that HLA devoid cell line stimulation of NK tri-functional potential in *h/*y+B57 carriers arises at least in part from the abrogation of inhibitory signals mediated by 3DL1 by showing that stimulation with 221 HLA transfectants expressing Bw4*80I alleles, but not Bw6 alleles suppressed the function of 3DL1+ NK cells from these individuals.

The more potent effector functions mediated by poly-functional NK cells compared to corresponding mono-functional subsets is reminiscent of that seen in poly-functional CD8+ T cells [291, 535]. Poly-functional CD8+ T cells are commonly observed in the setting of effectively controlled viral infections such as Cytomegalovirus, EBV, vaccinia, and influenza infections [536, 537]. The preferential maintenance of multi-functional HIV-specific CD8+ T cells in HIV-infected LTNP compared to progressors may be due to poly-functional CD8+ T cells having superior anti-HIV activity or the effect of low viremia limiting the functional exhaustion of these cells [165, 291]. We report here a similar phenomenon for tri-functional versus mono-functional NK

cells. We did not detect significant differences in the MFI of TNF-α secretion and CD107a expression between the bi-functional and tri-functional NK cell subsets. The bi-functional CD107a+IFN-γ+ NK subset however, secreted lower amounts of IFN-γ when compared to the tri-functional NK cells. Whether tri-functional NK cells are endowed with a superior capacity to suppress viral replication in infected targets than mono-functional NK cells warrants further investigation. In the work reported here we have not directly tested the anti-viral activity of NK cells from subjects with defined 3DL1/HLA-B genotypes since we did not use HIV infected cells as targets. Although the biological relevance of poly-functional antigen specific CD8+ T cells in HIV infection is not yet clear, they do serve as an indicator of an effective response to HIV. Our data illustrates that NK cells with poly-functional potential may also be of immunological relevance in this context.

The percent contribution of tri-functional responses to the total NK cell response varied within study populations stratified by 3DL1/HLA-B. It is possible that some of this variation is due to VL since VL was negatively, albeit weakly, correlated with tri-functional potential. The finding that *h/*y+B*57 HIV viremic progressors have lower levels of NK tri-functional potential than *h/*y+B*57 matched HIV uninfected and HIV infected EC+VC would be consistent with the disruption of NK cell function in HIV infected individuals with uncontrolled viremia as has been reported by others [1, 538] However, VL is unlikely to be the only determinant of NK functional potential since there were no significant differences in the VL of SP grouped by the 3DL1/HLA-B genotypes. Furthermore, in a subset of EC and VC where the effect of VL would be minimized we found significantly higher NK tri-functional potential when cells were

from *h/*y+B57 than *l/*x+B57 subjects. Our data does not dispute that HIV infection affects NK cell function but provides evidence that 3DL1/HLA-B genotype is also a determinant of NK functional potential. We were unable to examine and compare the NK tri-functional potential in successfully treated individuals matched for the *h/*y+B*57 combined genotype because we identified only 2 such individuals.

Functional heterogeneity of NK cell responses within *h/*y+B*57 carriers and other groups classified by their 3DL1/HLA-B genotype may arise through the contribution of KIR/HLA receptor-ligand combinations other than 3DL1/Bw4 to the NK functional repertoire [539, 540]. The number of inhibitory KIR to self HLA alleles a person carries, the HLA-B alleles carried (Bw6, Bw4 or Bw4*80I), the frequency of KIR3DL1+ NK cells and level of expression of KIR3DL1 on NK cells may all contribute to variability in NK functional potential within groups classified by 3DL1/HLA-B genotype [489, 540]. HLA and KIR region typing to identify the presence of other KIR/HLA combinations that have an impact on NK education would be a first step to addressing the contribution of other KIR/HLA to NK functional potential. Expanding the antibody panel to detect NK subsets expressing single or defined combinations of other NK receptors, use of blocking antibodies for NK receptors and a broader array of 221 transfectants will further aid in dissecting the contribution of other KIR/HLA combinations to stimulated NK function.

Interactions between HIV and NK cells lead to the pathogenic redistribution of NK cells in the peripheral blood of HIV infected subjects [1, 499, 500]. The expansion of a dysfunctional anergic CD56-CD16+ NK cell population has been associated with high VL during the chronic phase of HIV infection. In HIV infected persons with low VL, this

dysfunctional subset is smaller than that seen in progressors. Suppression of VL by effective anti-retroviral therapy also leads to a reduction in the size of this subset [1, 501]. The percent or absolute number of total NK cells, CD56dim, CD56bright and KIR3DL1+ NK cell subpopulations was similar in the SP groups categorized by 3DL1/HLA-B. However, we did find a significantly lower frequency of the anergic CD56-CD16+ NK cells in the *h/*y+B57 carriers than in SP with any other 3DL1/HLA-B genotype. Excluding this anergic subset in a sub-analysis confirmed that increased NK tri-functional potential was not due to the decreased frequency of CD56-CD16+ among *h/*y+B*57 SP.

As has been reported before for uninfected individuals, stimulated NK cells from HIV infected *h/*y+B57 positive SP have higher levels of tri-functional NK than carriers of other 3DL1/HLA-B combinations such as *l/*x+B57, *h/*y+other Bw4 alleles or Bw6 hmz [523]. In the case of the *l/*x+B57 combination, the strength of the interaction between KIR receptors and B*57 may be weaker than that between 3DL1 receptors and the HLA ligand in *h/*y+B57 subjects. Although this may depend on individual 3DL1 receptors and HLA ligands, in general the receptors in the 3DL1*l/*x genotype are expressed on the NK cell surface at lower levels and several members of the *l 3DL1 group have been shown to be weaker inhibitory alleles than common *h 3DL1 alleles [513, 541]. In the case of Bw6 hmz, none of the Bw6 alleles are ligands for 3DL1 receptors. Therefore NK cells from Bw6 hmz are not expected to receive educating signals through 3DL1. The difference in functional potential between NK cells from carriers of *h/*y+B57 versus *h/*y+other Bw4 alleles may reflect differences in the impact of B*57 alleles versus other Bw4 alleles in providing educating signals to NK

cells during development. Transgenic mice expressing single MHC class I alleles have shown that MHC class I alleles can differ in their impact on NK cell education [542-544]. This process is important for the development of self-tolerant NK cells and for endowing NK cells with the capacity to mediate cytokine secretion and cytolysis upon encountering virally infected cells or tumor cells with aberrant MHC class I expression or with ligands for activating NK receptors [481]. The strength of the inhibitory input during education is an important factor in determining the functional responsiveness of individual NK cells [490, 544]. One interpretation of the finding that carriers of the *h/*y+other Bw4 combinations had NK cells with a lower functional potential than those from *h/*y+B57 carriers is that B*57 differs from most other Bw4 alleles in terms of the strength with which it interacts with 3DL1 to educate NK cells. Although there is experimental evidence that Bw4*80I alleles interact with 3DL1 more strongly than Bw4*80T alleles there has been no direct examination of the strength with which B*57 binds any 3DL1 allele compared to other Bw4 alleles [519-521].

221 cells expressing single HLA alleles allowed us to confirm that NK activation following stimulation with HLA devoid cells was in part due to disruption of the inhibitory signal mediated by 3DL1 binding to its ligand. Stimulation of NK cells from *h/*y+B*57 carriers with both 221-B*57:01 and 221-B*27:02 Bw4*80I transfectants, but not the 221-B*35:02 Bw6 transfectant, suppressed IFN-γ secretion within the 3DL1+ but not the 3DL1- NK cell compartment. The 221 HLA devoid cell line and transfectant panel allowed us to confirm this for the 3DL1+ tri-functional NK cell response as well.

Host factors associated with the control of HIV infection may help identify important targets for vaccine design. In this report we measured NK functions that can mediate anti-

viral activities [292, 545-548]. Although we have not directly tested the anti-viral activity of NK cells from subjects carrying *h/*y+B57 since we did not use HIV infected cells to stimulate NK responses the results presented here may be relevant to HIV control. NK responses induced upon encountering HIV-infected cells with reduced HLA-A and -B expression may be one of the mechanisms underlying reduced risk of HIV infection and slower disease course in carriers of the *h/*y+B57 genotype who become infected [549, 550]. The results presented would be consistent with the possibility that SP who express the protective B*57 allele may be able to control HIV not only through CD8+ T cell recognition of HIV peptides restricted by the B*57 alleles but also through interactions between B*57 and 3DL1 receptors that educate NK cells for potent anti-viral activity.

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CHAPTER THREE FIGURE LEGENDS

Figure 1

Stimulated Natural Killer (NK) cells from KIR3DL1 (3DL1) homozygous HIV infected slow progressors (SP) have higher tri-functional activity when from carriers of an HLA-Bw4 allele than from Bw6 homozygotes (hmz).

Panel A. Comparisons of percent contributions of seven functional profiles to the total NK response by NK cells from 3DL1 hmz SP stimulated with K562. Results for 40 SPs carrying at least 1 Bw4 allele were compared to that of 7 Bw6 hmz. Dots below the x-axis refer to the presence of a measured functional marker (CD107a, IFN-γ and TNF-α). Bar heights refer to the median percent contribution of that functional subset to the total NK response for the group. Error bars refer to the upper range for the group. Significant between-group differences are shown with an asterisk (*) over the line linking the two bars representing groups being compared. Panel B. Correlation of results reported as the percent contribution of the tri-functional (CD107a+IFNγ+ TNFα+) NK subset to the total NK response with results reported as the frequency this functional NK subset for the 47 SP subjects in panel A. A Spearman correlation test was used to test the significance of these 2 ways of reporting results. Panel C. Comparisons of the mean fluorescence intensities (MFI) of IFN-γ secretion is plotted on the y-axis for tri-functional (CD107a+ IFNγ+TNFα+), bi-functional (CD107a+ IFNγ+ and IFNγ+ TNFα+) and mono-functional IFNγ+ NK cells. Panel D shows the MFI of TNF-α secretion plotted on the y –axis for tri-functional (CD107a+ IFNγ+TNFα+), bi-functional (CD107a+ TNF-α+) and IFNγ+ TNFα+) and mono-functional TNFα+ NK cells. Panel E shows the MFI of CD107a

expression plotted on the y-axis for tri-functional (CD107a+ IFNγ+TNFα+), bi-functional (CD107a+ TNF-α+) and (CD107a+ IFNγ+) and mono-functional CD107a+ NK cells. For panels C, D and E, the line through the scatter plots represents the median. Mann-Whitney U tests were used to test the significance of between group comparisons. P-values for these are shown above the lines linking 2 bars representing functional subsets

Figure 2

Higher percent contribution of tri-functional NK cells to the total NK cell response in *h/*y+B*57 than Bw6 homozygotes (hmz) SPs requires co-expression of the *h/*y 3DL1 receptor genotype and the HLA-B*57 ligand.

Scatter plots show the percent contributions of tri-functional NK cells to the total K562 stimulated NK response for HLA-B*57 (B*57) positive SP versus those that with Bw4 alleles other than HLA-B*57 (Non-B*57-Bw4) (A), for carriers of HLA-Bw4 and 3DL1*h/*y genotypes (*h/*y+Bw4) versus 3DL1*l/*x genotypes (*l/*x+Bw4) (B), and for carriers of a 3DL1*h/*y genotype with HLA-B*57 (*h/*y+B*57), a 3DL1*l/*x genotype with HLA-B*57 (*l/*x+B*57), a 3DL1*h/*y genotype with an HLA-Bw4 allele other than HLA-B*57 (*h/*y-Other Bw4) and Bw6 hmz (C). The line through each scatter plot is the median for the group. Mann-Whitney U tests were used to test the significance of between group differences. P-values for comparison are shown over the line linking 2 groups.

Figure 3

The influence of HIV viral load (VL) and KIR/HLA genotype on NK functional potential.

Panel A. Correlation between the percent contribution of tri-functional NK cells to the total K562 stimulated NK response and log10 VL. A Spearman correlation test was used to assess the significance of the association between these 2 parameters. Panel B. Scatter plots show the distribution of the percent contribution of tri-functional NK cells to the total K562 stimulated NK response in HIV infected individuals meeting the criteria for classification as either Elite Controllers (EC, <50 HIV copies/ml plasma) or Viral Controllers (VC, <3000 HIV copies/ml plasma) that carry a 3DL1*h/*y genotype with HLA-B*57 (*h/*y-B*57) versus a 3DL1*l/*x genotype with HLA-B*57 (*l/*x-B*57).

Panel C. Scatter plots show the distribution of the percent contribution of tri-functional NK cells to the total K562 stimulated NK response in HIV-1 negative individuals, HIV-1 infected EC+VC and untreated viremic progressors matched for the *h/*y-B*57 genotype. Panel D. Scatter plots show the same as in Panel C for untreated viremic progressors carrying the *h/*y+B*57 versus Bw6 hmz genotypes. For Panels B-D the line through each scatter plot is the median for the group. Mann-Whitney U tests were used to test the significance of between group differences. P-values for comparison are shown over the line linking 2 groups.

Figure 4

721.221 (221) transfectants expressing HLA-B*57:01 and B*27:02 but not B*35:02 suppress 3DL1+NK cell function.

Panel A. Flow cytometry plots of peripheral blood mononuclear cells from a representative individual carrying the 3DL1*h/*y genotype with HLA-B*57 (*h/*y+B57) following stimulation with media alone, the parental 221 cell line and 221 cells transfected with single HLA alleles. The 221 stimulus is indicated over the plots with the parental cell line designated as 221, 221-B*57:01 as B*57, 221-B*27:02 as B*27 and 221-B*35:02 as B*35. NK cells staining positive for the Z27 monoclonal antibody (3DL1+) cells were gated on in the upper panels and Z27- (3DL1-) NK cells in the lower panels. The number in each plot shows the frequency of the IFN-γ+ 3DL1+ (upper) or 3DL1- (lower) response in the boxed area following stimulation. Panels B-D show pooled data from 10 *h/*y+B57 SP. Panels B and C shows the results for the percent contribution of total IFN-γ secretion within the 3DL1+ (Panel B) and 3DL1- (Panel C) compartments, respectively. Mann Whitney U tests assessed the significance of differences in the percent contributions of IFN-γ to the 3DL1+ response and 3DL1- response upon stimulation with 221-B*57:01, 221-B*27:02, 221-B*35:02 transfectants versus the 221 parental cell line. Panel D show results from 10 *h/*y+B57 subject for tri-functional 3DL1+ NK response following culture with the 221 transfectant panel expressed as a percentage of the response to the 221 parental cell line. Paired t-tests were used to test the significance of the frequencies of tri-functional 3DL1+ NK cells upon stimulation with 221-B*57:01 and 221-B*27:02 transfectants versus the 221-B*35:01 transfectant. For Panels B-D, bar and error bar lengths represent the mean and standard error for the group. * = $p<0.05$, ** = $p<0.01$.

Supplementary Figure 1

Gating strategy. Panel A. The live, lymphocytic singlet population was used to gate on NK cells, which were defined as CD3- and CD56+/-CD16+/-. Boolean gating was then used to determine the frequency of the possible combinations of IFN-γ, TNF-α, CD107a and Z27 expression on NK cells. Panel B shows flow cytometry plots using the isotype control for the Z27 monoclonal antibody that was used to set the gate for KIR3DL1+ NK cells (top row). An unstimulated control was used to set the gates for IFN-γ+, TNF-α+ and CD107a+ NK cells (middle row). Panel C shows positive staining for IFN-γ, TNF-α, CD107a and Z27 in a K562 stimulated test sample (bottom row).

Supplementary Figure 2

Flow cytometry plots of the frequency of tri-functional NK cells from a representative *h/*y+B57 and Bw6 individuals.

The frequency of tri-functional NK cells from representative *h/*y+B*57 SP (upper panels) and Bw6 hmz SP (lower panels) are shown. In the left hand plots CD107a positive NK cells are gated on and in the right hand plots the following subsets of NK cells are identified; IFN-γ -TNF-α- NK cells (Quadrant A), IFN-γ +TNF-α- (Quadrant B), IFN-γ+TNF-α+ (Quadrant C), IFN-γ-TNF-α+ (Quadrant D). The frequency each NK cell subset is indicated in the quadrant with tri-functional cells shown in Quadrant C.

Supplementary Figure 3

Comparison of percentages of NK cells and NK subsets among HIV infected SP with different 3DL1/HLA-B combinations.

SP carrying the 3DL1/HLA-B genotypes: *h/*y+B*57, *l/*x+B*57*, h/*y+Other-Bw4 and Bw6 hmz were compared for the percent of CD3-CD56+/-CD16+/- NK cells (A), CD56 bright NK cells (B), CD56 dim NK cells (C) and CD56-CD16+ NK cells (D). Mann-Whitney U tests were used to assess the significance of between-group differences. P-values <0.05 are shown over the line connecting the two groups being compared.

Figure 1

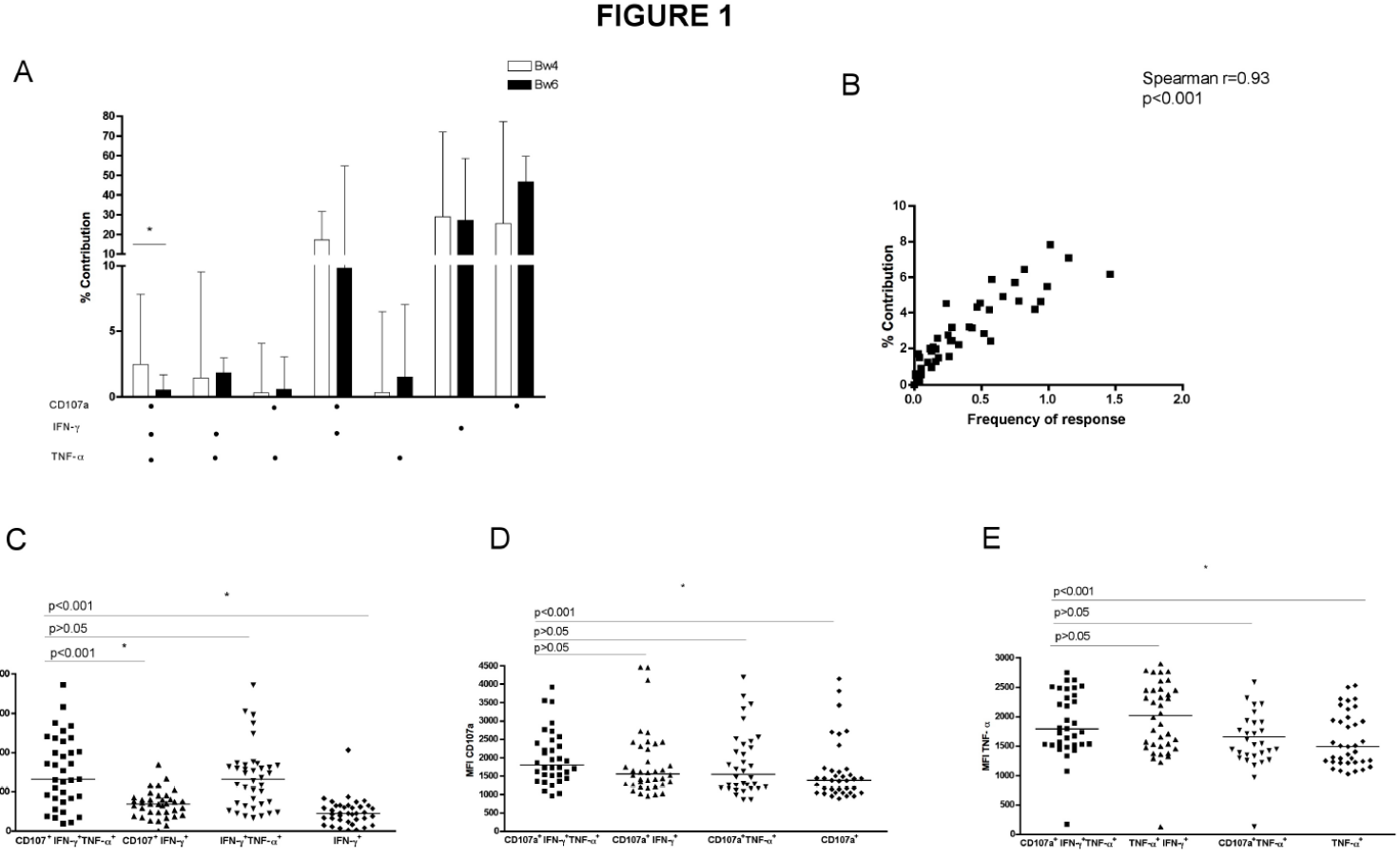


FIGURE 1
A
Bw4
Bw6
% Contribution
CD107a
IFN-γ
TNF-α
B
Spearman r=0.93
p<0.001
% Contribution
Frequency of response
C
p<0.001
p>0.05
p<0.001
MFI IFN-γ
CD107+ IFN-γ+TNF-α+
CD107+ IFN-γ+
IFN-γ+TNF-α+
IFN-γ+
D
p<0.001
p>0.05
p>0.05
MFI CD107a
CD107a+ IFN-γ+TNF-α+
CD107a+ IFN-γ+
CD107a+TNF-α+
CD107a+
E
p<0.001
p>0.05
p>0.05
MFI TNF- α
CD107a+ IFN-γ+TNF-α+
TNF-α+ IFN-γ+
CD107a+TNF-α+
TNF-α+

Figure 2

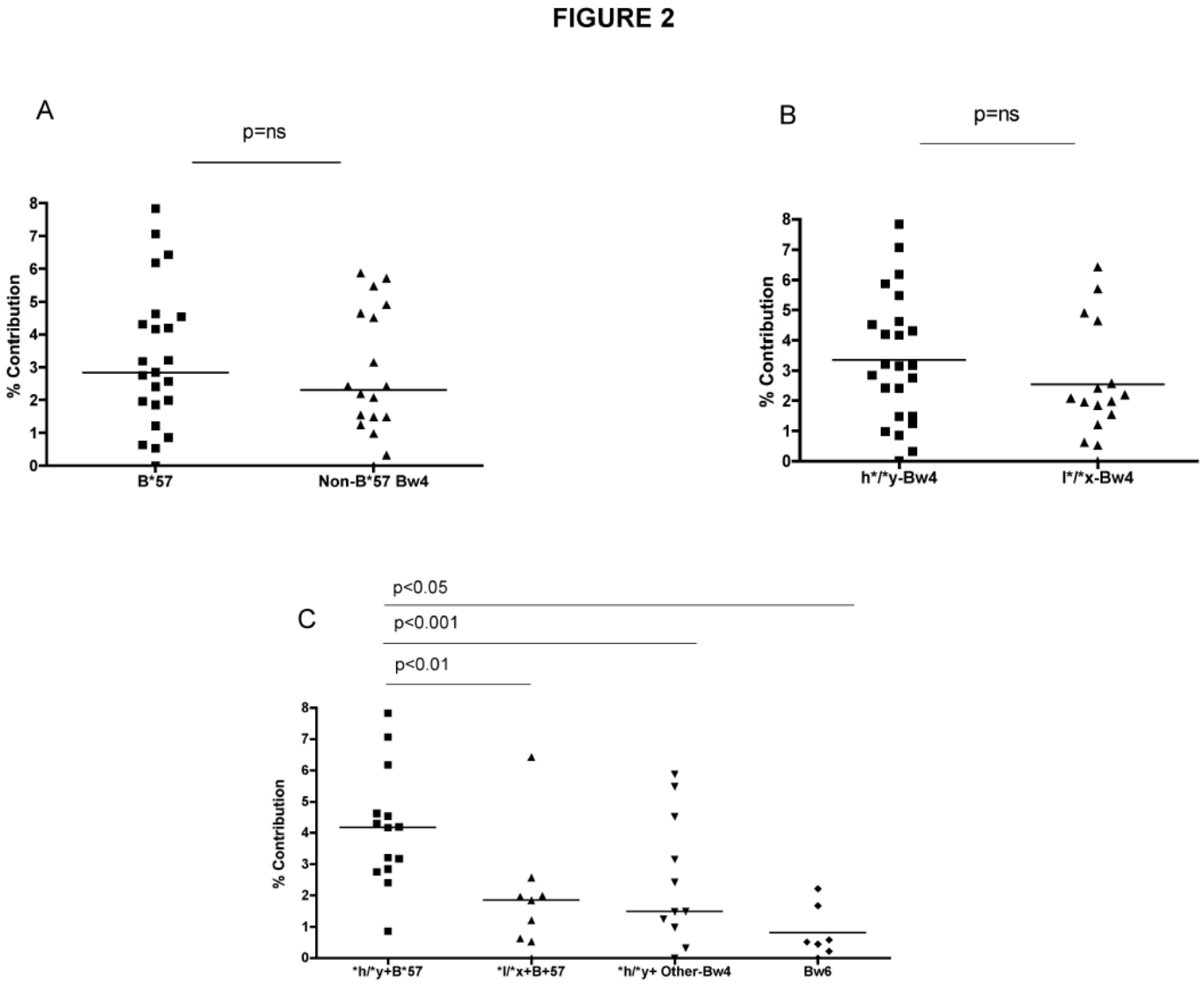


FIGURE 2
A
p=ns
% Contribution
B*57
Non-B*57 Bw4
B
p=ns
% Contribution
h*/*y-Bw4
l*/*x-Bw4
C
p<0.05
p<0.001
p<0.01
% Contribution
*h/*y+B*57
*l/*x+B+57
*h/*y+ Other-Bw4
Bw6

Figure 3

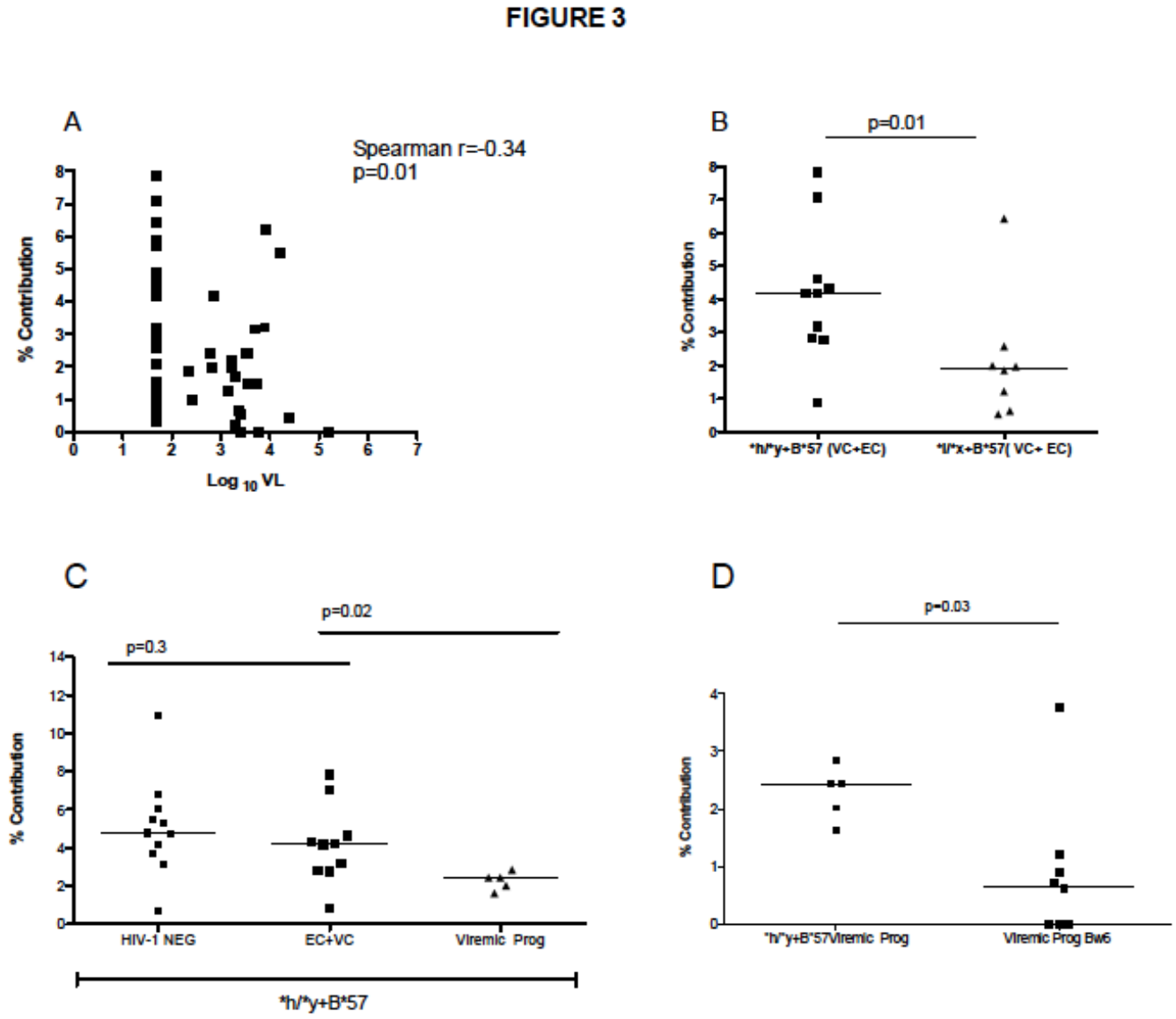


FIGURE 3
A
Spearman r=-0.34
p=0.01
% Contribution
Log 10 VL
B
p=0.01
% Contribution
*h/*y+B*57 (VC+EC)
*I/*x+B*57(VC+ EC)
C
p=0.02
p=0.3
% Contribution
HIV-1 NEG
EC+VC
Viremic Prog
*h/*y+B*57
D
p=0.03
% Contribution
*h/*y+B*57Viremic Prog
Viremic Prog Bw6

Figure 4

A

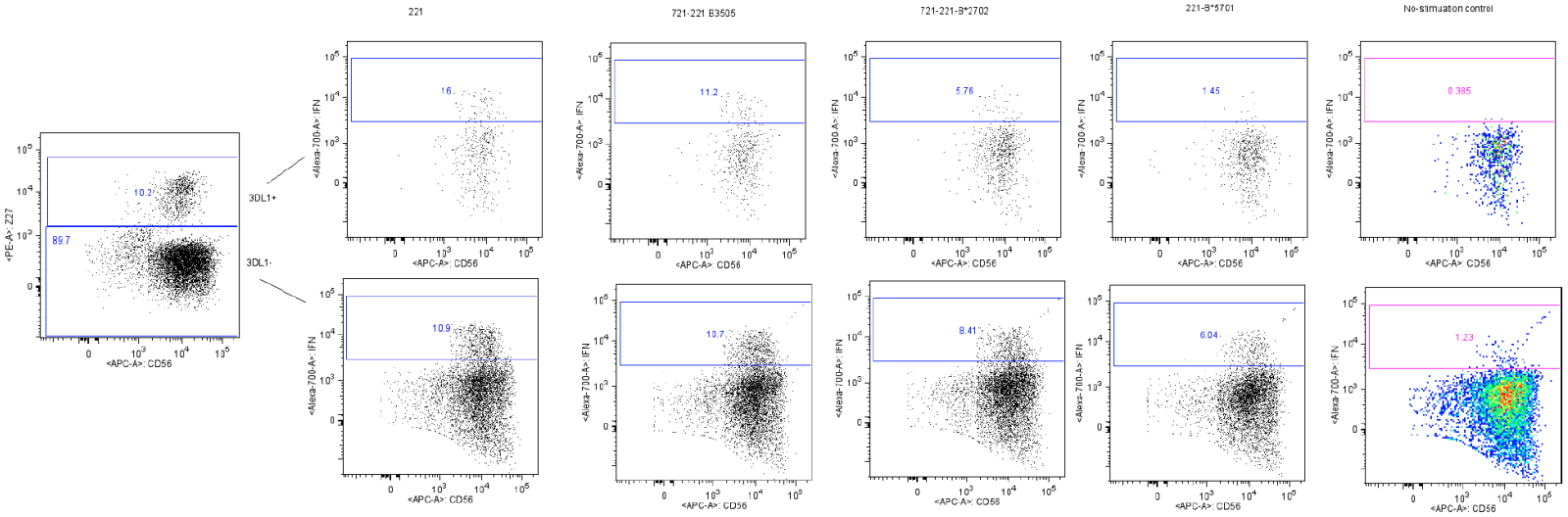

221
721-221 B3505
721-221-B*2702
221-B*5701
No-stimulation control
3DL1+
3DL1-
10.2
89.7
16
11.2
5.76
1.45
0.385
10.9
10.7
8.41
6.04
1.23
<PE-A>: 227
<APC-A>: CD56
<Alexa-700-A>: IFN

Figure 4

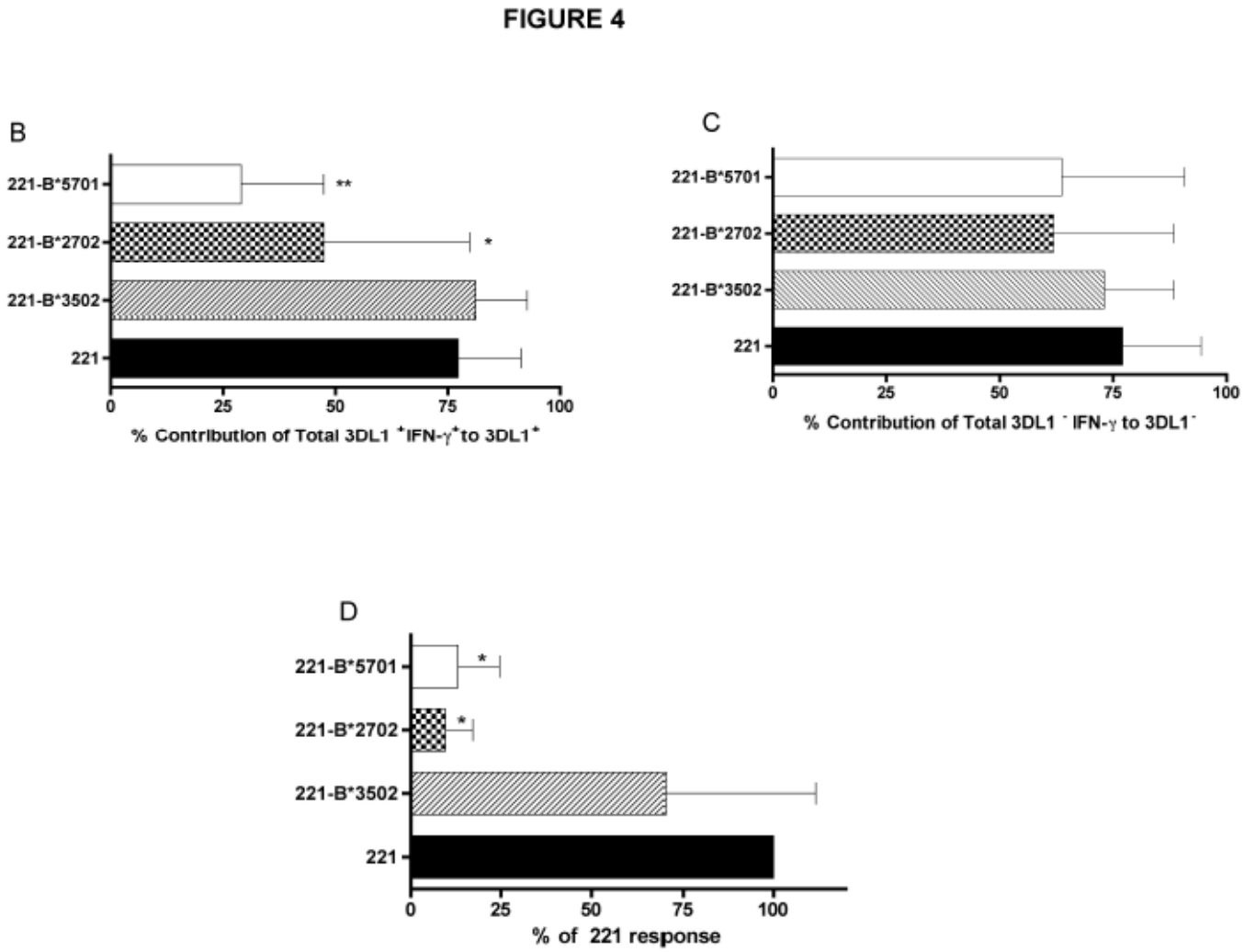
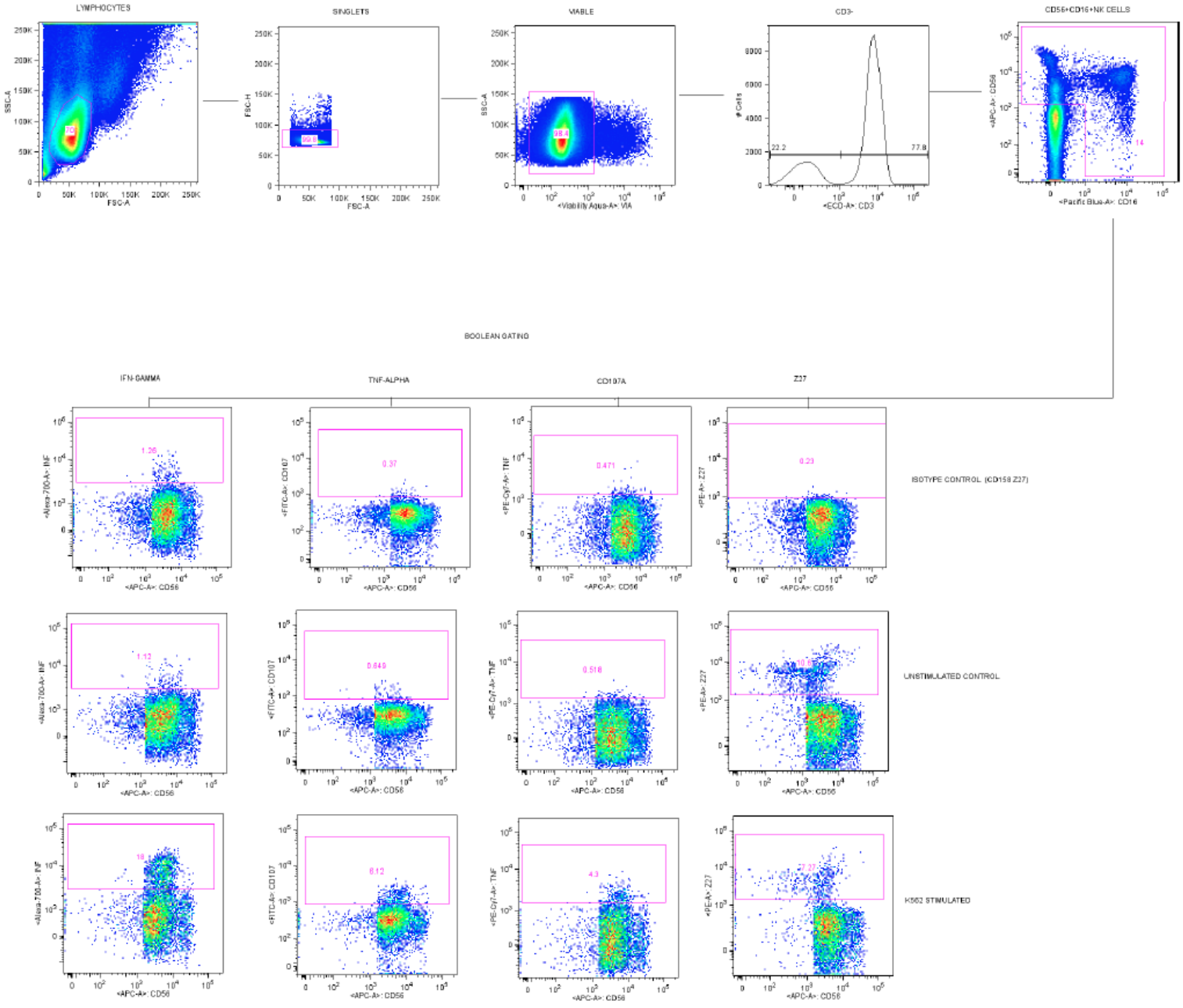
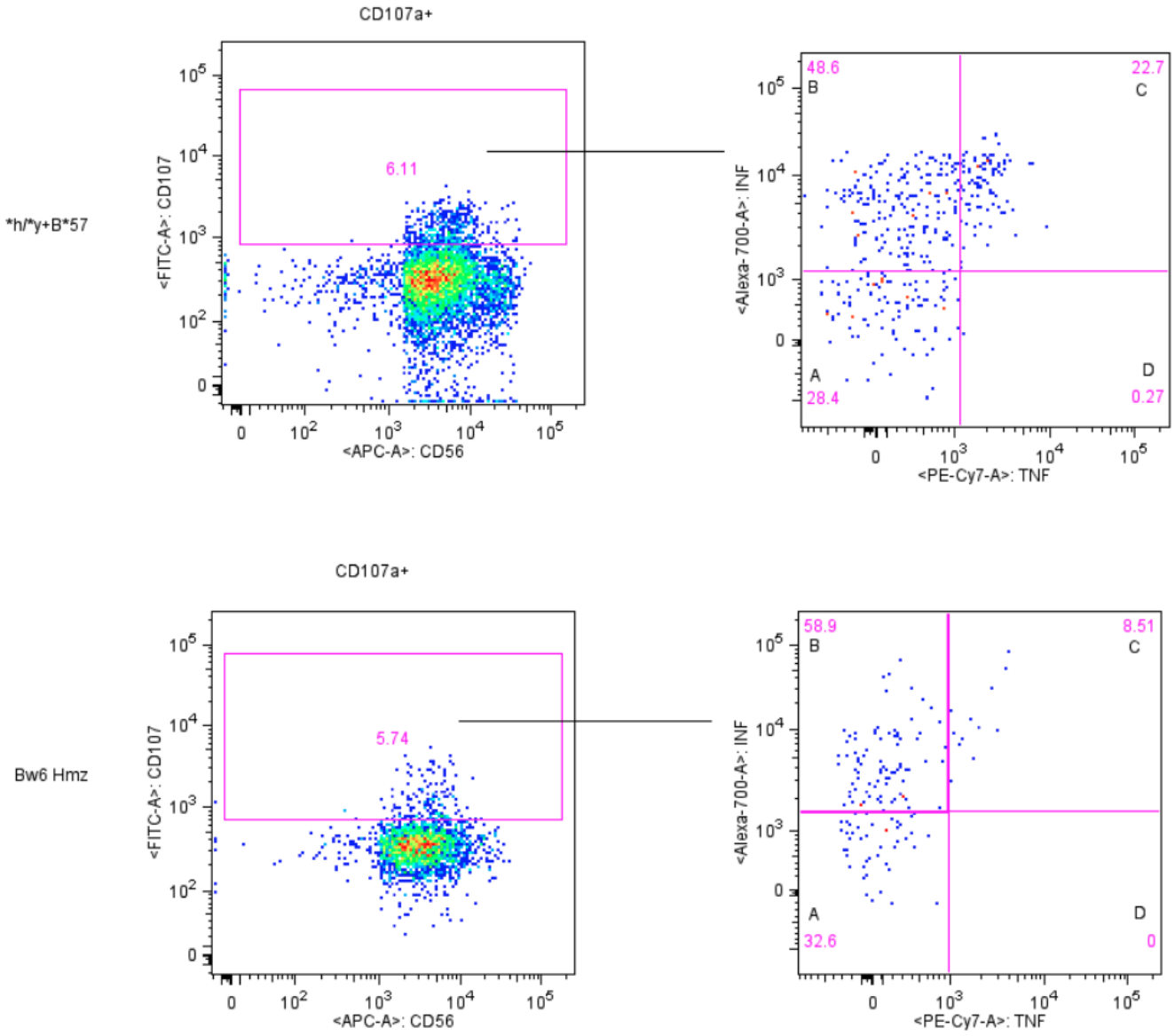


FIGURE 4
B
221-B*5701
221-B*2702
221-B*3502
221
**
*
0
25
50
75
100
% Contribution of Total 3DL1 +IFN-γ+ to 3DL1+
C
221-B*5701
221-B*2702
221-B*3502
221
0
25
50
75
100
% Contribution of Total 3DL1 - IFN-γ to 3DL1-
D
221-B*5701
221-B*2702
221-B*3502
221
*
*
0
25
50
75
100
% of 221 response

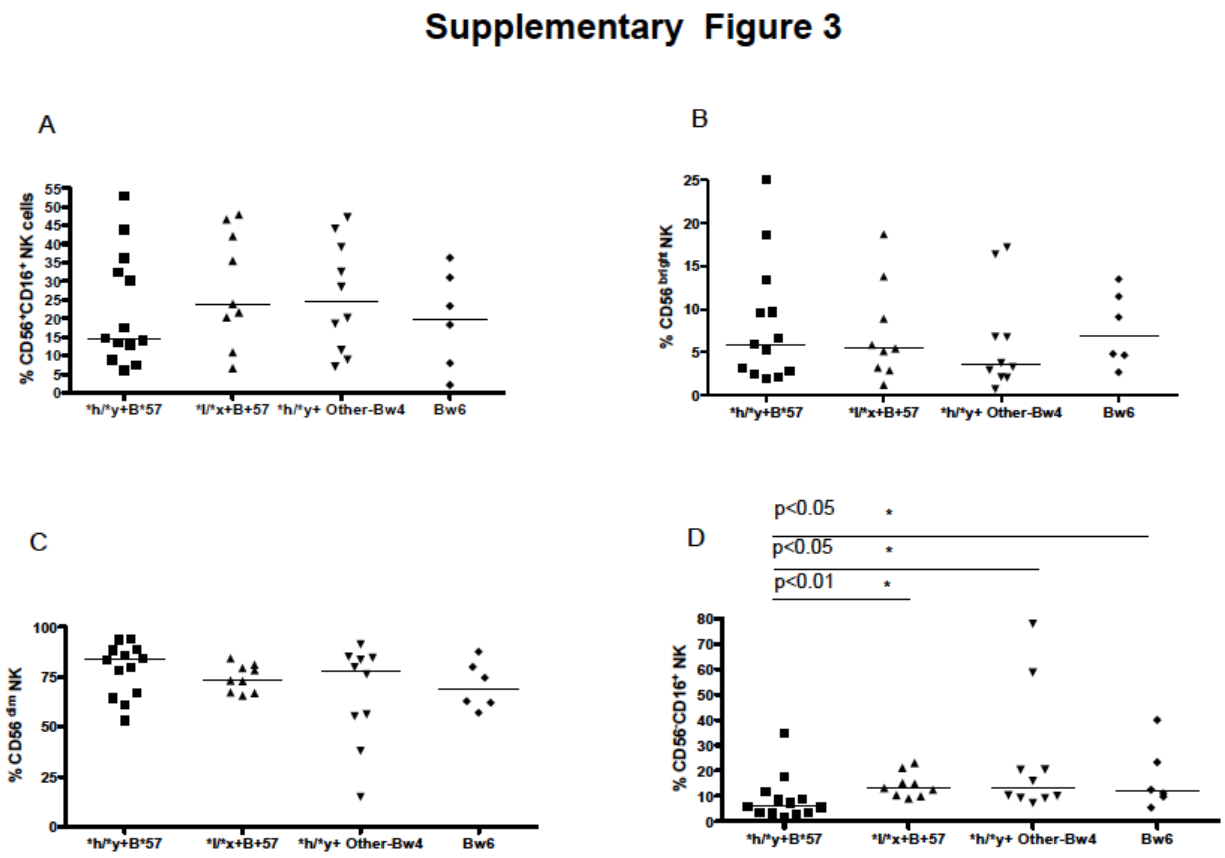
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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CHAPTER THREE AND FOUR LINKER

In the study presented in Chapter three of this thesis I noted some variability in the percent contribution of stimulated tri-functional cells within groups of SP carrying similar KIR3DL1/HLA-B combinations. I questioned whether the contribution of KIR/HLA combinations other than KIR3DL1/HLA-B to NK functional potential contributed to variability. To address this I compared the tri-functional potential of NK cells from HIV-1 infected slow progressors (SP) categorized according to the number of their S-iKIR in Chapter four.

CHAPTER FOUR

Quantitative influence of inhibitory Killer-Immunoglobulin-like Receptors to self HLA-B and HLA-C ligands on NK cell poly-functional potential

Quantitative influence of inhibitory Killer-Immunoglobulin-like Receptors to self HLA-B and HLA-C ligands on NK cell poly-functional potential

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Running head: KIR to self HLA influences NK function

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ABSTRACT

Inhibitory Killer Immunoglobulin-like Receptors (iKIR) interact with HLA ligands to license natural killer (NK) cells for functional competence. Previous studies stimulating peripheral blood mononuclear cells with the HLA devoid K562 cell line revealed that NK cells from individuals with an iKIR encoded by the KIR3DL1 locus to self HLA ligands encoded by the HLA-B locus were more functional than those from individuals who were homozygous for HLA-Bw6 with no ligands for these iKIR. For these studies the NK functional subset characterized by expression of the degranulation marker CD107a and secretion of IFN-γ and TNF-α differentiated subjects with this iKIR to self HLA (S-iKIR) from Bw6 homozygotes. We questioned whether other iKIR to self HLA (S-iKIR) could contribute to NK cell tri-functional potential. We grouped HIV-infected slow progressors according to whether they expressed 1, 2 or 3 S-iKIR. Following stimulation, the percent contribution of NK cells expressing CD107a and secreting IFN-γ and TNF-α to the total NK response was assessed. This measure increased with increasing numbers of S-iKIR carried. These observations support a quantitative model for NK cell licensing.

INTRODUCTION

Natural Killer (NK) cells are important in innate immune defenses to transformed and virus infected cells [508]. NK cell activation is governed by the integration of signals transmitted through a stochastically expressed repertoire of germ-line encoded activating and inhibitory receptors, which trigger cytotoxicity and cytokine/chemokine secretion without prior exposure to antigen. Dominant control of NK cell activation is mediated by inhibitory NK cell receptors (iNKR) that bind to HLA alleles as markers of self on normal cells [551]. An important set of HLA-specific iNKR in humans are encoded by the polygenic and polymorphic Killer Immunoglobulin-like Receptor (KIR) gene family [509].

NK cells gain functional competence through an HLA-dependent process termed licensing [483, 484, 544]. Licensing ensures tolerance to self by endowing NK cells that express inhibitory KIR (iKIR) to self HLA alleles (S-iKIR) with functional competency, while those lacking iNKR to self HLA remain unlicensed and hyporesponsive [483, 484, 544]. The rheostat model of NK licensing postulates that NK cell education is a quantitative and dynamic process whereby the strength of the signals delivered by major histocompatibility complex (MHC) class I molecules is proportional to a an NK cell activation threshold [487]. Inhibitory and activating receptor inputs quantitatively tune the NK cell activation pathways and translate into corresponding response levels [544] [490].

Recent data from studies in humans support this quantitative model of NK cell licensing by reporting higher levels of potency among NK cells expressing two or more versus one S-iKIR [488, 489].

Epidemiological studies have linked the carriage of certain KIR/HLA receptor-ligand combinations to slower time to AIDS and lower VL [516]. Compared to Bw6 homozygotes (hmz) with no ligands for KIR3DL1 NK receptors, carriage of KIR3DL1*h/*y and HLA-B57 (*h/*y+B*57) is the KIR/HLA receptor ligand combination most strongly associated with slow time to AIDS and VL control [516]. NK cells from carriers of *h/*y+B*57 have a higher percent contribution to the total response of tri-functional cells characterized by CD107a expression as a marker for degranulation and secretion of IFN-γ and TNF-α than carriers of the NK receptor or HLA ligand alone or Bw6 hmz with no alleles able to interact with KIR3DL1 alleles [523]. The higher functional potential of NK cells from *h/*y+B*57 carriers may play a role in their HIV disease course. We noted some variability in the percent contribution of stimulated tri-functional NK cells from individuals carrying similar KIR3DL1/HLA-Bw4 combinations. We questioned whether the contribution of KIR/HLA combinations other than KIR3DL1/HLA-Bw4 to NK functional potential contributed to this variability. To address this we compared the tri-functional potential of NK cells from HIV infected slow progressors (SP) categorized according to the number of their S-iKIR. We report here that K562 stimulated NK cells from SP exhibit an increasing tri-functional potential with increasing S-iKIRs.

MATERIALS AND METHODS

Study population

The study population included 45 HIV-infected SP. The SP designation included treatment naïve individuals maintaining CD4 counts >400 cells/mm^3 for >7 years (n=10), Elite Controllers (EC) followed for >1 year with VL <50 copies/ml of plasma (n=24) and Viral Controllers (VC) followed for >1 year with VL <3000 copies/ml of plasma (n=11). Median and range Log_{10} VL, CD4 counts, age and duration of infection for SP with 1, 2 or 3 S-iKIR are shown in Table 1.

MHC and KIR genotyping

All subjects were HLA typed as previously described [523]. KIR region genotyping was performed using LIFECODES KIR SSO typing kits (Gen-Probe, Inc) according to the manufacturer's directions. Results were read on a Bio-Plex 200 instrument (Bio-Rad). The following iKIR-HLA receptor ligand pairs were considered: 1) KIR2DL1 with HLA-C2 2) KIR2DL2/3 with HLA-C1 and 3) KIR3DL1 with HLA-Bw4.

Assessment of NK cell functional potential

The tri-functional potential of $CD3^-CD56^{+/-}CD16^{+/-}$ NK cells stimulated with K562 cells was assessed as previously described using the same gating strategy [523]. The percent contribution of tri-functional ($CD107a^+$ IFN-γ^+ TNF-α^+) NK cells to the total NK response was calculated after correcting for unstimulated cell background.

Statistical analysis

GraphPad Instat 3.05 and GraphPad Prism 4.01 (GraphPad Software) were used for statistical analyses and graphical presentations. A Pearson correlation test was used to test the significance of the trend for increasing percent contribution of tri-functional NK cells to the total NK cell response with increasing S-iKIR number. A Kruskall-Wallis test was used to assess the significance of between-group differences in VL. P-values <0.05 were considered significant.

RESULTS

Nine SP had 1 S-iKIR, 22 had 2 and 14 had 3 S-iKIR. The median (range) percent contribution of tri-functional NK cells to the total response was 0.23 (0.0-5.11), 1.4 (0.0-4.9), and 3.5 (0.0-7.8) from SP with 1, 2 and 3 S-iKIR, respectively. The tri-functional potential of NK cells increased with increasing numbers of S-iKIR carried ($r=0.38$, $p=0.01$; Pearson correlation test) (Figure 1).

Previous studies have reported a dis-regulation in NK cell function with increasing VL in HIV-infected subjects [1]. Since our study population included some SP with detectable VL we compared between-group VL levels to address whether differences in NK cell tri-functional potential could be accounted for by differences in VL. No significant differences were detected ($p=0.14$; Kruskal-Wallis test).

DISCUSSION

According to the rheostat NK education model, the strength of the inhibitory input received by NK cells determines the threshold of activation that is set in each NK cell both at the level of number of responding NK cells and number of effector functions [487, 490]. Here, we showed that NK cells from SP had higher levels of tri-functional potential with carriage of increasing numbers of S-iKIR and that this could not be accounted for by VL alone. These results are consistent with the interpretation that the NK cell licensing process rather than VL alone accounts for the higher percentage of stimulated tri-functional NK cells seen in SP with higher numbers of S-iKIR.

In this report, functional potential was defined as the percent contribution of tri-functional cells to the total response, a measure highly correlated with the frequency of stimulated tri-functional NK cells [523]. Either way of reporting results generated similar conclusions. We focused on tri-functional NK cells because this functional subset was the only one that contributed more to total NK function when NK cells were from carriers of the KIR3DL1/HLA-Bw4 S-iKIR compared to carriers of no receptor-ligand pair for this combined genotype. Furthermore, tri-functional NK cells may have more effective anti-viral function on a per cell basis as each of the functions elicited by these cells is more potent than the same function in mono-functional NK cells. (Kamya et al, submitted).

We attribute increasing levels of NK cell tri-functionality with increasing numbers of S-iKIR to higher levels of inhibitory input during NK cell education [487]. Whether this finding translates into SP with more S-iKIR having an overall more potent

missing self-response to virally infected cells and not just stimulation with an HLA-devoid cell line warrants further investigation.

In this study we did not consider the impact of other iNKRs to self HLA. We reasoned that the influence on NK education of iNKR such as NKG2A:CD94 and LILRB1, which have broad range HLA recognition would be comparable from one person to another, as has been reported [489] [552]. Therefore, these iNKRs would be expected to contribute minimally to between-subject variability in NK functional potential. We did not consider the influence of activating NK receptors on functional potential as their role in tuning NK functional potential is only starting to be addressed [509] [553].

The cumulative strength of inhibitory input received by an NK cell has aspects that are both quantitative (ie: the number of iNKR-HLA interactions) and qualitative in terms of which KIR and HLA alleles are expressed and interact as NK receptor-ligand pairs. Variability in NK functional potential may also arise from the specific receptors, ligands and their copy number, all of which may influence the potency of the interaction between particular receptors and their ligands [544] [490] [523].

In this study we used HLA-deficient K562 cells as a stimulus. A more comprehensive examination of the link between the strength of the inhibitory input received by NK cells during their education and NK cell functionality will be possible by using antibody panels detecting functional NK subsets based on which iNKR they express. Data generated using such an approach can provide information on which NK subsets are responding, their relative contribution to the total response and the functional potential of each NK cell subset.

Epidemiological studies showing associations between carriage of certain KIR/HLA genotypes with HIV susceptibility and disease outcome highlight the importance of NK cell education in the context of viral infection. The data presented here add to a growing body of evidence illustrating that the functional responsiveness of NK cells is proportional to the strength of the inhibitory input received by NK cells during their education in support of a quantitative model for NK cell licensing. By demonstrating that the number of S-iKIR has an additive effect on NK cell tri-functional potential ours is the first study to our knowledge to address the quantitative nature of KIR/HLA combined genotypes within the context of HIV infection.

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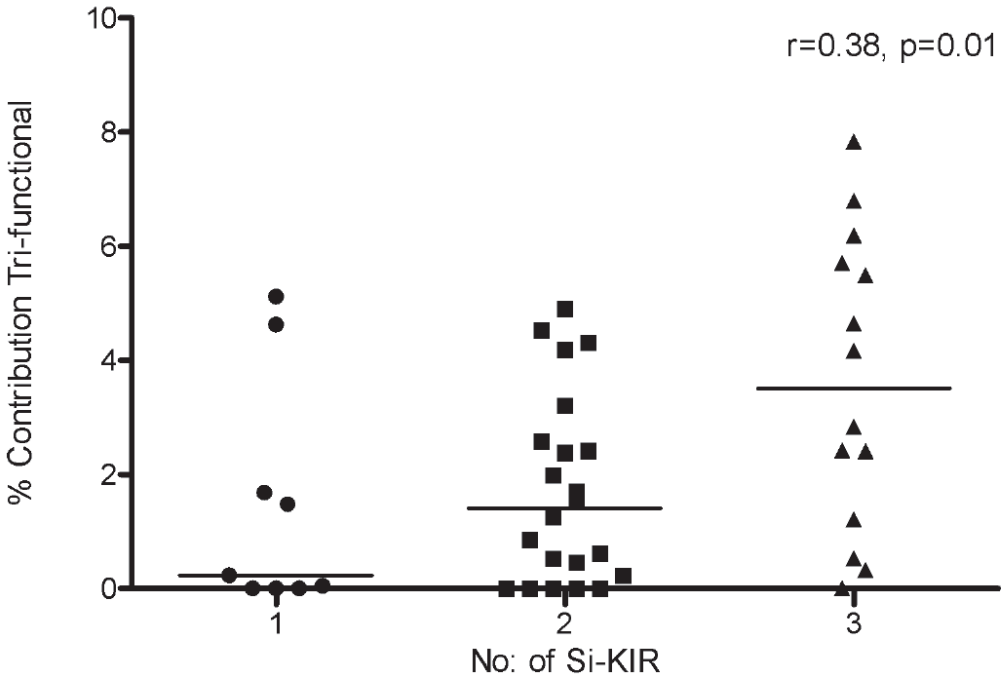
CHAPTER FOUR LEGEND

Figure 1

Higher percent contribution of tri-functional NK cells to the total NK cell response in HIV-1 infected slow progressors (SP) with increasing numbers of inhibitory Killer Immunoglobulin-like Receptors (KIR) to self HLA alleles (S-iKIR).

Scatter plots show the percent contribution of tri-functional NK cells to the total K562 stimulated NK response for SP with 1, 2 or 3 S-iKIR. The following iKIR-HLA receptor ligand pairs were considered: 1) KIR2DL1 with HLA-C2 2) KIR2DL2/3 with HLA-C1 and 3) KIR3DL1 with HLA-Bw4. HLA-C1 alleles have an Asparagine at position 80 and include HLA-Cw1, Cw3, Cw7, Cw8, Cw13, Cw14 and Cw16. HLA-C2 alleles have a Lysine at position 80 and include HLA-Cw2, Cw4, Cw5, Cw6, Cw17 and Cw18. Each data point represents results from a single individual. The line through each scatter plot is the median for the group. A Pearson correlation test was used to test the significance of the trend for increasing NK tri-functional potential with increasing numbers of S-iKIR.

Figure 1



* p<0.05

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CHAPTER FIVE
ORIGINAL SCHOLARSHIP

CHAPTER TWO

1. The study presented in Chapter two of this thesis is the first to examine a link between levels of T-cell immune activation and longitudinal changes in absolute CD4 count in a setting of controlled viremia.
2. We are the first to associate heterogeneity in T-cell immune activation levels and expression of protective HLA and KIR/HLA combined genotypes.
3. The data presented is the first to suggest that elevated levels of T-cell immune activation among EC who express protective HLA and KIR/HLA genotypes reflects the mounting of a beneficial rather than detrimental antiviral response.

CHAPTER THREE

1. The study presented in Chapter three of this thesis is the first to suggest a new mechanism underlying the protective role of HLA-B*57 in the context of HIV infection.
2. We are the first to provide functional data in support of epidemiological studies that associate the expression of HLA-B*57 and the ***h**/*y KIR3DL1 receptor-grouping with superior viral control and slower time to AIDS in HIV-infected SP.
3. We are the first to demonstrate that NK cells from SP carrying genotypes encoding this NK receptor ligand pair have higher poly-functional potential than

NK cells from carriers of the either receptor or the ligand alone or neither in support of NK cell licensing theory.

4. We are the first to suggest that HLA-B*57 may mediate it protective effects on HIV infection through NK cells as well as through $CD8^+$ T cells.

CHAPTER FOUR

1. The study presented in Chapter four of this thesis is the first to examine a quantitative relationship between the number of S-iKR expressed by SP and the NK cell functional potential.
2. We are the first to demonstrate that in HIV the cumulative strength of inhibitory input received by NK cells during development influences functional responsiveness.
3. We are the first to suggest that the expression of more S-iKIR translates into increased NK cell potency under conditions other than steady-state which lead to NK cell stimulation.
4. We are the first to present functional data in support of a quantitative model of NK cell licensing in humans within the context of HIV infection.

THESIS DISCUSSION

In the absence of ARV therapy, the majority of HIV infected persons exhibit detectable VL with high levels of viral replication and declining $CD4^+$ T-cell counts. A subset of HIV-infected individuals (approximately 5%) display a more benign disease course by either sustaining their $CD4^+$ T-cell levels and/or maintaining VL levels below the limit of detection of current assays. Intense interest has focused on identifying the specific mechanisms underlying their benign disease course that can be targeted by novel immune-based therapies and ultimately aid in the design of an effective vaccine for HIV.

Chapter two of this thesis examines whether $CD8^+$ T cell immune activation drives $CD4^+$ T-cell decline within the context of superior control of HIV viral replication. From a cohort of longitudinally followed EC, I investigated whether EC with declining, stable, or rising $CD4^+$ T-cell counts differed in their levels of T-cell immune activation. Given the association between immune activation and disease progression, I hypothesized that EC with stable or rising $CD4^+$ T-cell counts would have lower immune activation levels in comparison to those with declining $CD4^+$ T-cell counts. Additionally, I assessed whether the expression of HLA alleles or KIR/HLA combined genotypes associated with favorable HIV disease outcomes could account for variations in T-cell immune activation levels among EC. I found that elevated immune activation in ECs is not associated with a faster rate of CD4 decline but may reflect the ability to mount immune responses able to control viremia in those with protective HLA and KIR/HLA genotypes.

Chapters three and four of this thesis report evidence in support of the rheostat model of licensing. The work described in these chapters provides a functional basis for

epidemiological observations linking carriage of certain KIR/HLA compound genotypes with favorable HIV disease outcomes. In chapter three I examine whether SP with different KIR/HLA compound genotypes vary in their NK cell functional potential. I hypothesized that NK poly-functional potential would be higher in NK cells from SP carrying the *h/*y+B57 combined genotype that is associated with slower time to AIDS and lower VL setpoint versus SP carrying either the KIR3DL1*h/*y receptor grouping or the HLA-B57 ligand alone. NK cells from subjects carrying the *h/*y+B57 genotype exhibited the highest tri-functional potential (positive for three markers of anti-viral function) which was dependent on co-carriage of the (*h/**y) NK receptor grouping and the HLA-B*57 ligand. Tri-functional cells secreted more of each function tested on a per-cell basis than each corresponding mono-functional NK subsets. Although VL influenced NK functionality, individuals with defined KIR/HLA genotypes exhibited differences in NK cell poly-functionality that could not be accounted for by VL alone. Therefore, the protective effect of HLA-B*57 on slow progression to AIDS and low VL may be mediated in part through its interaction with KIR3DL1 alleles to educate NK cells for potent activity upon stimulation.

Chapter four of this thesis assesses whether a quantitative relationship exists between the number of iKIR to self-HLA (S-iKIR) carried by a SP and the NK cell tri-functional potential of their NK cells following stimulation. I hypothesized that stimulated NK cells from SP with three S-iKIR would exhibit higher levels of tri-functionality that those from SP with two or one S-iKIR. Following stimulation, the percent contribution of tri-functional NK cells the total NK response increased with

increasing numbers of S-iKIR carried. Collectively these observations support a quantitative model for NK cell licensing within the context of HIV infection.

Does T-cell immune activation contribute to $CD4^+$ T–cell depletion in a setting of controlled viremia?

Untreated HIV infection is characterized by uncontrolled viral replication and severe immune dysregulation. This immune dysregulation begins during the earliest stages of acute infection and fundamentally compromises the capacity of the host to control viral replication [122, 199-201, 360, 362]. The establishment of a state of chronic immune activation is associated with a poor prognosis for HIV. The increased expression of activation markers such as CD38 and HLA-DR on both $CD4^+$ and $CD8^+$ T-cells has strong prognostic value for disease progression that is independent of plasma VL and $CD4^+$ T-cell count [348, 554]. Importantly, generalized immune activation rather than the direct cytopathic effects of the virus on $CD4^+$ T cells is thought to significantly contribute to $CD4^+$ T-cell decline [357, 555-558].

The relative contributions of immune activation on disease progression independent of viremia are difficult to ascertain because of the strong correlation between HIV VL and immune activation. Studies in EC with undetectable VL continue to enhance our understanding of how T-cell activation causes immunodeficiency during HIV infection. Although EC cannot be distinguished from each other based on VL, some exhibit evidence of HIV disease progression with low or declining $CD4^+$ counts [199-

201, 203, 360, 362]. An elevated level of CD38 and HLA-DR expression on $CD8^+$ T-cell reflects progressive disease in most viremic HIV infected individuals [348, 554]. Several studies including that which is presented in chapter two also report that EC maintain abnormally high levels of T-cell activation as defined by frequency of $CD8^+$ $CD38^+$HLA-DR^+ T-cells when compared to HIV-uninfected persons [199, 348].

Even though the levels of T-cell activation among EC are lower than in patients with progressive disease, EC with declining $CD4^+$ T-cell counts may be experiencing the pathological consequences of heightened levels of T-cell activation. By assessing T-cell immune activation levels in a longitudinally followed cohort of EC, the study could specifically address whether the abnormally high T-cell activation levels seen in EC contributes to $CD4^+$ T-cell depletion. The results presented in chapter 2 conclude that this is not the case since I found that the level of activated $CD38^+DR^+CD8^+$ cells was not higher in individuals with declining CD4 counts than that in individuals with stable or increasing CD4 counts. This contrasts with results from Hunt et al. who found significant correlations between the percent of $CD38^+DR^+CD4^+$ and $CD38^+DR^+CD8^+$ T cells and concurrent absolute CD4 count in 30 VL controllers [199]. Using a single CD4 count as a surrogate marker for disease progression is a weakness of the study by Hunt et al. In HIV infection CD4 counts decline with time, their slope can change over different periods of infection and biological fluctuations in CD4 count may occur, all of which may influence the strength of this cross-sectionally determined marker as a surrogate for disease progression. My access to multiple longitudinal CD4 counts, which allowed me to calculate the annual slope of CD4 change for each subject provided a more definitive measure of disease progression in EC. In fact, chapter 2 describes one of the larger EC

groups with longitudinal follow up reported on to date. Although I was not the first to observe declining CD4 counts in EC, this groups was large enough to conclude that this phenomenon is fairly frequent among EC as it was seen in 7 of 25 (28%) of this population. The study population numbers allowed me to question whether there was a mechanistic link between chronic T-cell activation and progressive immunodeficiency within the context of controlled viremia. Although it is possible that the study population size did not have the power to detect differences in immune activation levels between EC with CD4 count slope that do or do not decline the absence of a any hint of a trend argues against this.

An alternate approach to investigating whether T cell activation is a cause rather than a consequence of $CD4^+$ T-cell depletion in EC would be to expand the markers of T-cell activation I measured to include other immunophenotypic and serum markers that have been used to quantify the level of T-cell immune activation in HIV. These could include markers such as the MFI of CD38 on $CD8^+$ T-cells, and the expression of CD25, CD69, CD70 on $CD4^+$ and $CD8^+$ T-cells as well as serum levels of neopterin, TNFRII and β2-microglobulin [354-356, 558]. In doing so, such an analysis could examine whether EC with stable/or increasing $CD4^+$ T-cells can be distinguished from those with declining $CD4^+$ counts using these other markers of immune activation.

During the first 8-12 weeks of HIV infection, $CD4^+$ and $CD8^+$ T cells exhibit extreme changes and variability in immune activation levels longitudinally. After the 12^{th} week, a steady state of T-cell immune activation within the CD4 and CD8 compartments is attained, called an "immune activation setpoint" [357]. The immune activation setpoint varies widely between individuals but remains relatively stable within a given patient

once a steady level is reached. Importantly, the $CD8^+$ T-cell activation setpoint is a strong independent predictor of the rate of $CD4^+$ T-cell decline [357]. The data presented in chapter two confirms the notion of an immune activation setpoint in HIV-infection proposed by Deeks et al by demonstrating that the variability in the percent of $CD38^+DR^+CD8^+$ T cells from the same EC measured at 6 time points was less that the variability observed among unrelated HIV infected EC or progressors [357].

Even though EC could not be distinguished based upon the expression of T-cell markers of immune activation, their abnormally high levels compared to uninfected individuals may reflect other aspects of the immune response that are dysregulated enough to significantly contribute to declining $CD4^+$ counts in some EC. Accordingly, recent data from Hunt et al. report lower frequencies of regulatory T-cells (Tregs) in EC when compared to uninfected persons, HIV infected progressors and successfully treated patients [559]**.** Tregs ordinarily down-modulate antiviral immune responses such that the negative aspects of an uncontrolled inflammatory response to infection are avoided [560-562]. On the other hand a low Treg response might increase HIV-specific responses contributing to the clearance of HIV-infected cells and the superior control of viral replication. However a low Treg response may also allow for an increased level of generalized immune activation, contributing to $CD4^+$ T cell depletion in a setting of controlled viremia [559].

Several functional immunologic and host genetic studies suggest that high levels of HIV-specific $CD4^+$ and $CD8^+$ T cells with preserved function are likely to play a significant role in the suppression of viral replication in EC [227, 228, 252, 275, 289, 291, 531, 563-569]. Data from Hunt et al. suggests that EC are capable of maintaining

these strikingly high frequencies of functional HIV-specific $CD4^+$ and $CD8^+$ T-cells because these responses are sustained in an environment of weak immunoregulation by Tregs [559]**.** Assessing whether there are differences in the frequencies of HIV-specific Tregs between EC with stable, increasing or declining $CD4^+$ T-cells, could further elucidate whether inappropriate immunoregulation contributes to $CD4^+$ T-cell decline.

Multiple mechanisms have been proposed to explain how EC maintain undetectable VL. Defects in viral replication due to gross deletions and/or deleterious mutations can explain viral control in only a fraction of the EC because most EC harbor replication competent virus [174, 208, 564]**.** The question of whether $CD4^+$ T cells from EC are as easily infected with HIV after ex vivo stimulation as cells from HIV-uninfected persons is controversial and a current area of investigation [566, 570]**.**

EC are enriched for protective class I alleles such as HLA-B*5701 and polymorphisms associated with HLA-C expression, which implicate a fundamental role for HIV-specific T-cells in the control of viral replication [252, 566-568]. As variations in their T-cell immune activation levels could not account for differences in absolute $CD4^+$ count change among EC, I investigated whether the expression of protective HLA alleles and KIR/HLA combined genotypes could. Chapter two reports that the percent of $CD38^+HLA\text{-}DR^+CD8^+$ T cells was higher in EC expressing the protective HLA-B*57 allele and the KIR3DS1/HLA-Bw4*80I compound genotype associated with slower time to AIDS. Therefore, it appears that the elevated $CD8^+$ T-cell immune activation levels from EC that express protective HLA and KIR/HLA combined genotypes may not reflect a negative inflammatory consequence but the mounting of a beneficial immune response. Work by Hunt et al. supports this concept by reporting a strong relationship between

adaptive HIV-specific immune responses and generalized T cell activation and among EC [571]. Higher frequencies of $CD4^+$ T cells producing both IL-2 and IFN-γ in response to stimulation with HIV Gag peptides were positively associated with higher frequencies of activated $CD4^+$ T-cells [559]**.**

Despite potent HIV-specific T-cell responses and undetectable viremia, why EC do not completely eradicate the virus remains a key unanswered question. It is plausible that the immune response critical for the immunological control of viral replication paradoxically promotes HIV persistence. The preferential expansion and activation of HIV-specific $CD4^+$ T-cells at sites of viral replication may create a pool of susceptible targets for direct HIV infection [199, 289, 563, 565, 572]**.** Recent data from Hunt et al. report a significant positive correlation between activated HIV-specific T cell responses and cell-associated HIV RNA in EC that was independent of potential confounders such as HLA, $CD4^+$ T-cell count age and sex. Therefore although increased levels of immune activation could potentially support an effective HIV-specific immune response, activated HIV specific $CD4^+$ T-cells might also serve as targets for HIV and continually replenish the reservoir of latently infected cells [571]. Interestingly, the study also reports a positive correlation between cell-associated viral burden and activated $CD8^+$ T-cell frequencies but not HIV-specific $CD8^+$ T cells per se [571]. This is consistent with the concept that EC are incapable of completely eradicating HIV infection because within an environment of generalized heightened immune activation, activated HIV-specific $CD4^+$ T-cells may continue to replenish the latent reservoir.

The study presented in chapter two adds to a growing body of evidence demonstrating that despite undetectable virema, EC are not a homogenous subset [361,

573, 574]. It has become increasingly important to acknowledge heterogeneity among EC as it may reflect differences in the mechanisms underlying their control of viral replication and furthermore indicate instances of disease progression within the context of viral control. Exploring immunological differences among EC such as variations in the levels of T-cell activation and Tregs frequency, will continue to enhance our understanding of the unique immunological environment that allows for their superior control of viral replication. This knowledge will hopefully allow us to gain insights into how this can be recapitulated in a vaccine setting.

A wealth of data suggests that most EC maintain the control of viral replication at least in part through potent HIV-specific T cell responses. The enrichment of protective HLA alleles such as HLA-B*57 and HLA-B*27 in cohorts of EC strongly supports a role for CTL mediated immune pressure in the control of viral replication. The presentation of critical viral peptides to HIV specific $CD8^+$ T cells by protective HLA alleles is thought to lead to a distinct immune response such that viral escape from these responses comes at a significant fitness cost. Furthermore, SNPs associated with HLA-B*57 and upstream of HLA-C have also been associated with viral control [252, 255]. However, the ultra-deep sequencing data examining the viral sequences from EC demonstrates that certain EC still maintain viral control even after documented escape from HLA-restricted epitopes. Additionally, many EC express no protective HLA alleles indicating that associations between enhanced viral control and HLA expression may involve immunologic mechanisms outside of T-cell mediated immunity [227, 228, 575].

Licensed to protect?

NK cells are vital mediators of the antiviral innate immune response that also strongly influence the quality of the adaptive immune response to HIV [423]. HIV infection is associated with the severe functional impairment of NK cells that is evident very early after infection and escalates during disease progression [1, 499, 576-578].

NK cells vary in the number and specificity of the MHC class I specific inhibitory receptors they express [446]. Host MHC class I molecules secure NK cell self-tolerance by conveying functional competence only to NK cells that express self-specific inhibitory receptors during a maturation process termed licensing [483, 491, 579, 580]. Epidemiologic correlations linking certain KIR/HLA genotypes with variations in HIV disease outcome constitute an area in which NK cell education is of key importance [522, 581]. Therefore, understanding the basics of NK cell education and its consequences will aid in providing a functional basis for the role HLA/KIR combinations play in HIV pathology.

Several models have been proposed to explain the educational effects of MHC class I molecules on NK cell function. Kim and colleagues in the arming model of NK cell education posit that NK cells are initially hyporesponsive and become 'licensed' or 'armed' when their NK cell inhibitory receptors engage self-MHC class I during maturation [483, 582]**.** The alternative disarming model stipulates that NK cells are responsive by default and become hyporesponsive or 'disarmed' in the absence of inhibitory input [491, 583]**.**

Individuals MHC class I alleles are not equally efficient at educating NK cells for the capacity to reject MHC-deficient cells *in vivo*. Some MHC class I alleles have a higher 'educating impact' in comparison to others [485]. The successive introduction of MHC class I alleles into the mouse host genome leads to a gradual increase in the efficiency of missing self-rejection. NK cell responsiveness was higher in NK cells expressing multiple inhibitory self-receptors than in NK cells expressing only one self-receptor in the presence of a given MHC class I repertoire. Increases in the efficiency of missing self-rejection were also seen when MHC class I alleles with strong educating impact were introduced to the mouse host genome. At least two quantitative aspects contributed to the increased NK cell functionality; a gradual increase in the number of NK cells in the responsive pool and an increase in the functional efficiency of individual NK cells within the responsive pool. To reconcile these observations with the arming and disarming models of NK cell education, Brodin et al. liken NK cell education to a rheostat in which NK cell responsiveness is tuned along a continuum determined by the strength of inhibitory input received by the individual NK cell during NK cell education. The overall strength of inhibitory input has two components; (1) a qualitative component involving the binding strength of each the MHC class I/ inhibitory receptor interaction and (2) a quantitative aspect encompassing the number of MHC class I ligand/inhibitory receptor pairs.

The data presented in chapters three and four collectively support a quantitative model for NK cell licensing. In chapter three, I report that upon stimulation with MHC class I devoid targets, the highest frequencies of tri-functional NK cells come from KIR3DL1 hmz SP who co-carried at least copy of an HLA-Bw4 allele when compared to

those from HLA-Bw6 hmz. Among HLA-Bw4 carriers, those who express HLA-B*57 and the KIR3DL1 *h/*y receptor grouping exhibit the highest level of tri-functionality that was dependent upon the carriage of both the *h/*y receptor grouping and the HLA-B*57 ligand. The finding that NK cells from 3DL1*h/*y+B*57 carriers have higher tri-functional potential than those from carriers of 3DL1*h/*y with Bw4 alleles other than HLA-B*57 is consistent with reports indicating that NK cell responsiveness is influenced by the strength of the MHC class I/inhibitory receptor interaction and in line with rheostat model of licensing theory [490, 513, 540, 584]. The data presented in chapter four corroborates data in support of the concept that NK cells vary in their responsiveness to stimulatory engagement depending on the number of self-MHC specific receptors expressed by demonstrating that K562 stimulated NK cells from SP with increasing numbers of S-iKIRs have increasing levels of tri-functional potential [488, 490, 584].

Whereas both chapters three and four are consistent with predictions of the rheostat model of licensing in identifying a potential relationship between NK cell poly-functionality and the strength of inhibitory input received during NK education, both studies have certain limitations. One major limitation is the selection bias associated with working with SP. The over-representation of SP alleles such as HLA-B*57 and B*27 with a Bw4 motif and the rarity of Bw6 alleles in the study population limits our capacity to rigorously assess whether carriage of increasing S-iKIR numbers influences NK poly-functionality. Bw6 hmz do not have an S-iKIR for the KIR3DL1/Bw4 NK receptor ligand pair but would express 1 or 2 S-iKIR for KIR2D/HLA-C receptor-ligand pairs. The low number of Bw6 hmz in the study population included in chapter 4 precludes being able to address the contribution of KIR2D/HLA-C carriage to NK cell functional

potential when not co-carried by a KIR3DL1 HLA-Bw4 combination. By increasing the number of subjects homozygous for Bw6 alleles in chapter 4, a clearer assessment of the effect KIR2D/HLA-C combined genotypes have on NK polyfunctionality without KIR3DL1/HLA-Bw4 interactions would be possible and the question as to whether a quantitative relationship exists between the number of S-iKIR and polyfunctionality could be better addressed.

In both studies HLA-deficient K562 cells were used as stimuli. These cells interrupt multiple inhibitory signals mediated by iNKR recognizing HLA alleles as ligand and preclude isolating the influence of individual S-iKIR. Using the 721.221 HLA devoid EBV transformed cell line and a panel of 721.221 transfectants expressing single HLA alleles I was able to confirm that the activation seen upon stimulation with the K562 cells was at least in part due to disruptions of inhibitory signals mediated by the inhibitory 3DL1 receptors because only the 721.221 cells expressing a ligand for 3DL1, the Bw4*80I alleles B*57:01 and B*27:02, suppressed the function of $3DL1^{+}$ NK cells from *h/*y+B57 carriers. However, because the majority of the experiments were carried out using K562 cells as stimuli, to rule out the possibility that the variations in poly-functionality were K562 specific and not based on KIR/HLA genotype all experiments should have included 721.221 as a stimulation condition. In this regard, for the study subjects whose cells were stimulated with both K562 and 721.221 an examination of how results were correlated for the contribution of tri-functional, bi-functional and mono-functional responses to the total NK response was carried out. Positive correlations for all three NK cell functional profiles were detected. Therefore, although 721.221 and K562 did not stimulate identical results both stimulated NK cells in such a manner that the

functional profile they induced in these cells was correlated and stimulation with K562 or 721.221 cells should produce results leading to similar conclusions.

A more comprehensive examination of the link between the strength of the inhibitory input received by NK cells during their education and NK cell poly-functionality should include antibody panels that detect functional NK subsets based on which iNKR they express. Expanding the antibody panel to detect NK subsets expressing single or defined combinations of other NK receptors, use of blocking antibodies for NK receptors and a broader array of 721.221 transfectants will further aid in dissecting the contribution of other KIR/HLA to stimulated NK function.

Data generated using such an approach can provide information on which NK subsets are responding, their relative contribution to the total response and the functional potential of each NK cell subset. Furthermore, such data can address additional fundamental concepts of the rheostat model of licensing such as whether increases in inhibitory strength received during NK cell education translate into increases in the number of NK cells that contribute to the responsive pool.

The impact of other iNKRs to self-HLA on NK cell poly-functionality should have been considered even though these iNKRs are expected to contribute minimally to between subject-variability in NK functional potential. The NKG2A:CD94 recognizes the leader sequence of several HLA alleles complexed to HLA-E. Since all individuals would be expected to possess HLA alleles having the leader epitope restricted by HLA-E licensing through NKG2A:CD94 would be expected to be similar from one person to another and therefore contribute minimally to between subject variability. This has been shown to be the case experimentally [489, 552]. The iNKR LILRB1 also has broad range

HLA recognition and stimulated NK cells bearing this receptor as their only iNKR are hyporesponsive suggesting the educational impact of signaling through this receptor may be minimal [489, 552]. The iKIR KIR3DL2, which binds HLA-A*03/*11 in the presence of a restricted set of peptides may also contribute minimally to NK education [489]. It also remains possible that variability in NK cell poly-functionality within groups constituted based on the number of S-iKIR or KIR3DL/HLA-B combined genotypes arises from the influence of activating receptors on NK education. Although the ligands for many activating NK receptors are unknown, their role in NK cell education and tuning activation thresholds is starting to be addressed [426, 553]. Experimental tools and approaches able to take all iNKR-ligand combinations and activating NKR into consideration in the types of functional studies reported in chapters 3 and 4 would be an interesting future direction to pursue.

According to the rheostat model of NK cell education, NK cells that receive the strongest inhibitory input display a more efficient response per cell, reflected by an increased likelihood to deliver more effector functions. Data presented in chapter three are consistent with this concept because tri-functional cells secreted more of each function tested on a per-cell basis than each corresponding mono-functional NK subset. This finding parallels what has been reported for T cells where multi-functional T cells exhibit more potent individual functions than corresponding mono-functional T cells [165, 291, 535]. In a vaccine study, the presence of multi-functional T-cell responses correlated with protection against a *Leishmania major* challenge [535]. LTNP who spontaneously control HIV infection posses a greater proportion of HIV-specific $CD8^+$ T-cells capable of performing five functions in comparison to progressors. In this

setting as well, antigen-specific $CD8^+$ T cells produce more cytokine on a per cell basis than mono-functional $CD8^+$ T cells [291]**.** Additionally, HIV-specific $CD8^+$ T cells from LTNP exhibit greater proliferative capacity and a higher degree of cytotoxicity against autologous HIV-infected $CD4^+$ T cells targets than those from progressors[531, 585, 586]. Although it is tempting to attribute causal relationships between poly-functionality and controlled viremia, this is a still a matter of intense debate. The ability of poly-functional HIV-specific $CD8^+$ T cells to suppress HIV appears to be related to high antigen sensitivity [587]**.** The finding that multi-functional NK cells have increased functional potency is novel. Whether this translates to a superior ability of these cells to mediate anti-viral function will need to be formally tested in NK cells using HIV-infected targets. Epidemiological studies from our group showed that carriers of certain KIR/HLA types including *h/*y+B*57 were associated with a reduced risk of infection. It may be that certain KIR/HLA combinations that allow the development of potent multi-functional NK cells may mediate anti-viral effects at crucial steps in HIV transmission before the virus disseminates through the body and becomes difficult to eradicate [259, 267, 588].

Several studies report that during the early phases of HIV-1 infection the interaction of virus with NK cells leads to the pathogenic redistribution of NK cells in the peripheral blood of HIV infected patients [1, 499, 576]. In chapter three I report that *h/*y+B57 SP had lower frequencies of anergic $CD56^-CD16^+$ NK cells. To rule out the possibility that this could account for their increased poly-functional potential, a sub-analysis was performed that excluded the $CD56^-CD16^+$ anergic subpopulation. Similar results were obtained when $CD56^-CD16^+$ cells were removed in that a significantly

higher percent contribution of tri-functional NK cells to the total NK response was observed when NK cells were from carriers of *h/*y+B57 in comparison to *l/*x+B57, *h/*y+Bw4 alleles other than B*57 and Bw6 hmz genotypes.

In Chapter three I examine the relationship between VL and tri-functional potential in SP and detect a weak negative correlation between the NK cell tri-functional potential and VL. As no between-group differences were found in KIR3DL1 hmz SP categorized according to their KIR3DL1/HLA-B combined genotypes, I remain confident that KIR/HLA accounts for part of the variation seen in their NK cell poly-functional potential. Furthermore, upon limiting the analysis to EC+VC, *h/*y+B57 still maintained higher levels of poly-functional NK cells when compared to *l/*x+B57. However, my results do show that VL contributes to some of the variation in NK cell poly-functional responses because SP and progressors matched for the *h/*y+B57 genotype do not exhibit similar levels of poly-functionality. In order to fully address the relationship between NK cell poly-functionality and VL, an examination of the effect of ARV therapy on NK cell poly-functionality in KIR/HLA matched patients should be performed. Such an experiment will require access to successfully treated individuals having the correct KIR/HLA combinations. I was unable to identify enough people with this profile to address this question experimentally.

An inverse correlation between viremia and NK cell mediated suppression of HIV replication has recently been attributed to several mechanisms other than the peripheral expansion of anergic $CD56^{-}CD16^{+}$ NK cells, such alterations in the phenotype and function NK cells in HIV infected patients. These include, decreases in the intracellular stores of perforin and granzyme B, and changes in the surface expression of NCRs that

are critical for NK cell mediated cytotoxicity [577, 578, 589]. Interestingly, the relatively high killing capacity of LTNP $CD8^+$ T cells is thought to be the result of their ability to dramatically upregulate granzyme B and perforin production [287, 531]. Given that decreases in the intracellular stores of perforin and granzyme A in NK cells have been associated with increased viremia, and recent data report the importance of perforin upregulation as an important correlate of T cell functionality, investigations as to whether variations in perforin upregulation are associated with KIR/HLA combined genotype are of key interest.

Additionally data from Fogli et al. demonstrates that peripheral NK cells from HIV viremic patients display a phenotype that is consistent with incomplete activation that may be due to chronic stimulation resulting in NK cell exhaustion and anergy [576] Given the relationship between functional exhaustion and stunted differentiation in T-cells [590, 591], an examination of markers of activation on NK cells within the context of KIR/HLA combined genotypes could yield further insights as to whether a similar phenomenon may be occurring in NK cells.

Several questions regarding fundamental processes governing NK cell education remain unanswered. Recent data from studies in mice have led to a reappraisal of licensing. Lanier and colleagues provide seemingly paradoxical evidence that NK cells devoid of inhibitory receptors for self-MHC (unlicensed NK cells) play a predominant role in certain NK cell mediated responses [493]. Contrary to the licensing theory, the authors demonstrate that unlicensed NK cells were the main mediators of NK-cell mediated control of mouse CMV infection *in vivo*. Depletion of unlicensed NK cells impaired control of viral titers whereas the depletion of licensed NK cells did. The

adoptive transfer of unlicensed NK cells was more protective than licensed NK cells. Collectively these results indicate that unlicensed NK cells are critical for protection against certain viral infections [493]. Furthermore, Elliot et al. demonstrate that unlicensed mature MHC-class I deficient NK cells show 'gain of function' and acquire a licensed phenotype after adoptive transfer into wild-type (WT) hosts. This suggests that neither exposure to MHC class I ligands during NK cell development nor endogenous MHC class I expression by NK cells are absolute requirements for licensing [592]. Additionally, Jonker et al report that the basal responsiveness of NK cells may not be set during the NK cell development but alternatively readjusted when fully mature NK cells are exposed to a changing MHC environment [593].

Collectively, these data suggest that the developmental programming of NK cells is not fixed and that perturbations to the peripheral environment such as viral infection have the potential to greatly influence the capacity of mature NK cells to mount an effector response. The concept of NK cell 're-education,' gaining or losing functional capacity as dictated by changes in the MHC environment, is especially relevant to HIV because of the downregulation of HLA A and B alleles by Nef.

This functional plasticity of NK cells partly explains why NK cells lacking inhibitory receptors for self-MHC class I molecules are allowed to seed the periphery and are not deleted by processes similar to positive and negative selection of T and B-cells. If unlicensed NK cells are indeed capable of breaking anergy under certain inflammatory circumstances, it is possible that evolutionary pressures have allowed for the selection and maintenance of this 'anergic' subset to specifically counter infections pathogens. It has become imminently clear that a complete understanding of NK cell education and re-

education is needed before it will be possible to realize the immunotherapeutic manipulation of NK cells within an HIV vaccine setting.

Accumulating data continue to highlight a role for NK cells at the important interface between innate/adaptive immunity. Whereas mucosal integrity was previously thought to be governed by T-cells, it is becoming increasingly evident that innate immune mechanisms are intimately involved defenses at the level of the GI tract. Recently, the identification of NK-like cells (NCR22) that reside in the mucosae and programmed for IL-22 production presents a novel cellular immune defense mechanism in the gut. Given the integral role the mucosal barrier plays in HIV infection, the importance of understanding the interactions between HIV and this unique subset of NK cells is imperative [594]. Furthermore, MHC class I alleles serve as both ligands for NK cell receptors and present peptides to T-cells. Although rapid CTL escape from immunodominant HLA-B*57 epitopes occurs very early in acute infection, the capacity of some patients to maintain control their viremia may involve NK cells. Fadda et al report that common CTL escape variants modulate the binding of KIR3DL1 to HLA-Bw4 ligands suggesting the NK cells may be able to sense the presence of CTL variants and confer an extra level of immune protection in the face of a failing adaptive immune response [595].

Following HIV infection, a state of chronic immune activation ensues. Although HIV is considered to be a chronic condition, severe immune dysregulation begins early in infection and escalates as the disease progresses. Understanding how HIV perturbs important mediators of the innate immune response is imperative to understanding what the overall consequences of early immune dysregulation are and how they contribute to

HIV-induced pathology. The research described in this thesis aims to understand how SP turn HIV infection from a death sentence into a manageable chronic disease, so as to identify potential correlates of immune correlation as well as gain insights as to new targets for vaccine design.

Overall significance and future directions

The severe immune dysregulation that occurs during the earliest phases of HIV of infection underscores the importance of understanding the effect viremia has on innate immune cells with central roles in shaping the quality of the antiviral immune response. The projects described in this thesis highlight a role for NK cells in HIV pathogenesis by providing functional evidence of heterogeneity among SP that can be attributed to the carriage of KIR/HLA combined genotypes epidemiologically linked with favorable HIV outcomes. However, in addition to their own antiviral functions, NK cells regulate antiviral immunity through interactions with DCs and other cells of the innate immune response. Whereas genetic and functional studies suggest a protective effect of certain KIR/HLA combined genotypes, a complete understanding of the mechanisms underlying protection will have to include rigorous investigations into the effects of HIV infection on cross talk between NK cells, DCs and other innate immune cells. In doing so, information obtained should inform interventions aimed at harnessing innate immunity within the context of an HIV vaccine and the types of immunity a vaccine should emulate.

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