INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa · University of Ottawa

NORMALIZATION OF NATURAL KILLER CELL ACTIVITY WITH EFFECTIVE ANTIRETROVIRAL THERAPY COINCIDES WITH RESTORATION OF NATURAL KILLER CELL RECEPTOR EXPRESSSION

BY

KARL G. PARATO

THESIS

Submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry, Microbiology, and Immunology

Faculty of Medicine

University of Ottawa

ABSTRACT

Natural Killer (NK) cell function is likely important in the control of HIV infection. We evaluated the impact of potent antiretroviral therapy (ART) on NK cell number and activity in ART naïve patients over a period of 48 weeks. To address potential mechanisms of restored NK activity with ART a cross-sectional study was undertaken to evaluate surface expression of CD94, CD158a, CD158b, p70 and CD161, receptors known to regulate NK cell cytolytic activity. Median baseline NK activity was 56.09 lytic units for 10% lysis (LU_{10}) and decreased to 28.18 LU_{10} and 18.42 LU_{10} at 8 and 24 weeks respectively, levels similar to HIV seronegative individuals. Expression of CD158a, and CD161 was greater in individuals not receiving ART as compared individuals receiving ART or HIV seronegative individuals. Normalization of NK cytolysis due to ART coincides with restoration of NK receptor expression, indicating a role for NK activity in HIV immunopathogenesis.

ACKNOWLEDGMENTS

The support of many people over the last two years is appreciated greatly, and is paramount to the success of this project. Thanks to Dr. Angel, he has guided me and given the best possible opportunity in scientific research that anyone could to offer, for which I am extremely grateful. As well, I would also like to thank Dr. Angel for all the guidance in my personal development and my friends and family for all their support and encouragement over the past years. The support of my thesis committee members Drs. Karen Copeland and Kathy Wright is much appreciated and also the support of Dr. Ashok Kumar. Moreover, thanks are given to Dr. Lionel Filion for helping me gain entrance into the Master's of Science program at the University of Ottawa.

I gratefully appreciate the support of the Ontario HIV Treatment Network for granting me a studentship to pursue this work. Thanks are also warranted to the Medical research Council of Canada and Roche Laboratories for supporting this work. The kind gift of the EXPFIT program from Dr. Hugh Pross is also appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
ART	Antiretroviral Therapy
CD	Cluster of Differentiation
CO_2	Carbon Dioxide
CTL	Cytotoxic T Lymphocyte
DiO	3,3′-Diooctadecyloxacarbocyanine perchlorate
DMSO	DimethylSulfoxide
DNA	Deoxyribonucleic Acid
FCS	Fetal Bovine Serum
FDC	Follicular Dendritic Cells
HIV	Human Immuno deficiency Virus
HLA	Human Leucocyte Antigen
IFN	Interferon
IL	Interleukin
IQR	Interquartile Range
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motifs
ITAM	Immunoreceptor Tyrosine-based Activation Motifs
KIR	Killer Immunoglobulin Type Receptor
LTR	Long Terminal Repeat

LU_{10}	Lytic Units at 10% Lysis
MHC	Major Histocompatibility Complex
Ml	Millilitre
NASBA	Nucleic Acid Based Amplification System
NK	Natural Killer
NKC	Natural Killer Complex
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
RNA	Ribonucleic Acid
RRE	Rev Response Element
RT	Reverse Transcriptase
SD	Standard Deviation
µl	Microlitres

I. INTRODUCTION

Natural Killer (NK) cells are non-T-cell lymphocytes that can mediate MHC non-restricted cytotoxicity (1). As cellular effectors of innate immunity, they do not require prior antigen exposure to generate rapid cytotoxicity and cytokine responses against virus infected cells (2, 3). NK cell activity has a well established role in the defense against a number of viral infections in humans and impaired NK cell cytotoxicity is associated with increased risk of severe disseminating infections with Herpes simplex virus, Epstein-Barr virus and human cytomegalovirus (4, 5). The role of NK cell function in protection from, or control of HIV infection is however not well established. NK cell defects have been observed in HIV infection, particularly in the setting of advanced disease with marked CD4 T cell depletion (6). Although it is primarily impaired NK cell function that has been observed, decreases in NK cell number in HIV infected individuals have also been demonstrated (7).

The existence of Natural Killer Cell Receptors, which are ligands for polymorphic MHC class I molecules, was predicted based on the observation that NK cells lysed HLA class I-deficient B lymphoblastoid cell lines, but did not lyse these target cells when transfected with certain HLA class I genes (8). Those receptors encoded by the Natural Killer Complex (NKC) are CD161 and CD94. Other molecules represented by the Killer Inhibitory Receptor gene family are CD158a, CD158b and p70. Although, initially described to inhibit cytotoxicity, several NK receptors have demonstrated both positive and negative regulatory effects on NK cell function (2, 5, 9). This function is highly dependent on the cytoplasmic tail structure of the receptor and its

association with certain signal transducing molecules or in the case of CD94 by the receptors association with other molecules at the cell surface, (2, 10). Changes in NK receptor expression are known to modulate NK cell function in murine cytomegalovirus infections (11). There is also evidence for changes in the expression of CD94 on NK cells during HIV infection (12) but associated changes in cellular function have not been evaluated.

With the development of combination antiretroviral regimes that are capable of dramatically suppressing HIV replication, treatment of HIV infected individuals can result in partial restoration of immune function (13-17). The role of NK cells in HIV infection remains poorly understood and few studies have addressed the impact of highly active antiretroviral therapy (ART) on NK cell activity. We, therefore, evaluated NK cell number and function in early infection and changes over the course of effective ART. To address potential mechanisms of restored NK activity with ART a cross-sectional study was undertaken to evaluate NK cell receptor expression, as NK cell mediated lysis is modulated by ligation of these receptors with MHC class I molecules. To do so, the proportions of NK cells expressing CD94, CD158a, CD158b, p70 and CD161 were measured by four-colour flow cytometry. Such information would provide additional insight into the mechanisms of HIV-induced immunodefficiency and potentially identify sites to target with immune based therapies.

A. ROLE OF NK CELLS IN VIRAL INFECTION

NK cells are large granular lymphocytes, which are known to secrete various cytokines and have cytolytic potential. NK cells contain granules that harbour perforin and granzymes that mediate cytotolysis of target cells. They can be identified by the surface expression of CD16 and CD56 and absence of CD3 (18). The immediate response of a host to viral invasion is the induction of cellular pathways that constitute the innate immune defense system. The earliest indication of a role for NK cells in viral infection came from the observations that NK cells are activated by virus induced IFN-α/β to lyse target cells (19). As well, during primary infection these activation mechanisms precede adaptive immune responses (20). Evidence for NK cells playing a role in viral defense is increasing (21). The most notable case in humans is that of one individual who was incapable of producing cells of the NK phenotype. This was discovered after she presented with a severe chicken pox infection and and later developed near fatal infections with human cytomegalovirus and herpes simplex virus (5, 21). Despite this, the role of NK mediated lysis in antiviral defense is poorly understood. During viral infection the *in vivo* mechanisms induced by endogenous production of IFN-α/β cause an enhancement in NK cell lysis in which the molecular cytolytic components are similar to those used by cytotoxic T lymphocytes (CTL), including the production and release perforin (22). However, the role that NK cell cytolysis plays in the defense against viral infections has yet to be defined. This is partially due to the complex control mechanisms which prevent NK cell- mediated

cytolysis of normal or "self" cells. Understanding these mechanisms is paramount to understanding the role of NK cell cytotoxicity in antiviral defense.

NK cell cytolysis, is dependent on ligands of the target cells to which the NK cell has a corresponding receptor. These ligands on the target cell are most often MHC class I molecules and the receptors are specific to NK cells and certain subsets of CD8+ T lymphocytes (23-26). Once triggered, the receptors on NK cells will transduce intracellular signals that will determine the ability of the NK to elicit cytotoxic effects to kill the target cell (27). In some cases after adaptive immunity has induced production of antibodies specific to viral determinants on infected cells, NK cells can act through the Fc class III receptor (CD16), to elicit it's cytotoxic effects. CD16 is well known to activate NK cells and activate the cytotoxic mechanisms in NK cells and NK mediated cytotoxicity is more prominent after adaptive immune responses are in place or during secondary infection (28, 29). This method for induction of cytolysis is known as antibody dependent cell-mediated cytotoxicity or ADCC. While, there is limited *in vivo* evidence of ADCC as an anti-viral defense mechanism, some have found that high ADCC remained significantly associated with a lower risk of death in HIV disease (30). In contrast, other evidence points to ADCC as a cause of CD4+ T cells depletion during HIV disease (29).

NK cell cytotoxic defenses are most likely generated against those viruses that down regulate MHC class I molecules. Many viruses and especially herpes group viruses have evolved strategies to evade recognition by T cells, one of which is to downregulate MHC class I in infected cells (31-35). The numerous NK specific

receptors on NK cells are well known to inhibit NK cell cytotoxicity when in ligation with MHC class I (2, 4, 23-27, 34, 36-48). Thus in situations where virus infected cells downregulate MHC class I and T cell effector mechanisms become ineffective NK cell mediated lysis is likely a prominent mechanism of defense. Despite the fact that much remains to be investigated about the significance of NK cell-positive and -negative receptor signaling for antiviral defense, the ever-increasing evidence indicates that these receptors are critical to NK cell defense systems within the human host (45).

B. NATURAL KILLER CELL RECEPTOR BIOLOGY

As effectors of the innate immune system, natural killer cells lyse cellular targets and can also produce cytokines that modulate acquired immunity (5). Potential targets of NK cells are determined by the presence of ligands on target cells, which can interact with two classes of receptors on NK cells. In humans there are two sets of NK receptors classified by their structural design. The C-type (Ca^{2+}-dependent)-lectin-like receptors are disulfide linked, dimeric type II integral membrane proteins which are primarily found on chromosome 12 within the coding region known as the NK cell complex (NKC) (41, 49). The second set of receptors are usually monomeric type I glycoprotiens related to the immunoglobin superfamily (50) (43, 51-53). Both sets of receptors mediate their intracellular signaling activity through their cytoplasmic sequences termed immunoreceptor tyrosine-based inhibitory motifs (ITIMs). In addition both sets of receptors include related isoforms that express ITAMs, known to have stimulatory potential (47, 54).

1. Receptors of the NK Cell Complex

Humans have an NKC located on chromosome 12 p12.3-p13.1. Within a region that encodes CD161 (NKR-P1A), CD94, and NKG2A, C, D, E (55, 56). CD161 is expressed as a disulfide bonded homodimer on most NK cells and on a subset of "memory" T cells. To date, the ligand for this molecule has not been identified but cross-linking of CD161 with monoclonal antibodies can either inhibit or activate cytotoxicity (55, 57). Interestingly, the expression of CD161 is specifically upregulated by the cytokine IL-12, a cytokine known to enhance NK cell activity. CD161 transcription, mRNA accumulation, and surface expression are all increased by IL-12 (58).

Using subtractive hybridization to isolate genes preferentially transcribed by the human NK cell, the family of NKG2 genes was discovered within the NKC (59, 60). The NKG2 genes illustrate polymorphisms in both the extracellular and cytoplasmic domains indicating heterogeneity in signal transduction and ligand binding. Moreover, NKG2 glycoproteins form disulfide-bonded heterodimers with CD94, and this complex is known to interact with various forms of MHC class I molecules (61-63).

2. Killer Cell Immunoglobulin Type Receptors

Killer cell immunoglobulin type receptors (KIR) were first identified by antibodies to glycoproteins on NK cells that disrupted the interaction between the NK cells and targets laden with HLA-A, B, C (64-67). A family of KIR genes was later

identified on chromosome 19 q13.4, of which there are a possible 25 cDNAs that can encode KIR glycoproteins (67). Among the KIR glycoproteins there is some homogeneity in their extracellular portions while their intracellular portions are more variable. Of the KIR glycoproteins implicated in interacting with MHC class I molecules, three distinct protein isoforms have been described (figure 1). Those glycoproteins, which are, involved in HLA-C recognition, are generally monomeric glycoproteins and contain two immunoglobulin like domains in their extracellular regions, and have the CD158 cluster of differentiation designation. These molecules exists in two distinct forms, p58 (~58 kDa) and p50 (~50kDa) (43, 51, 53). The intracellular portion of the inhibitory p58 molecules contains two characteristic ITIM motifs, which are involved in the transduction of the inhibitory signals (43), whereas the stimulatory p50 molecules lack these ITIM motifs (47). KIR proteins reactive with HLA-B are usually ~70 kDa glycoproteins with three immunoglobulin type domains in their extracellular regions, and are often described as p70 type molecules (50). Sequence analysis of p70 molecules has also indicated the presence or absence of ITIM motifs, in their intracellular regions. Furthermore, a homodimer version of p70 molecules are composed of two disulfide linked ~70 kDa molecules and are known to have specificity to certain HLA-A ligands (43, 51, 53).

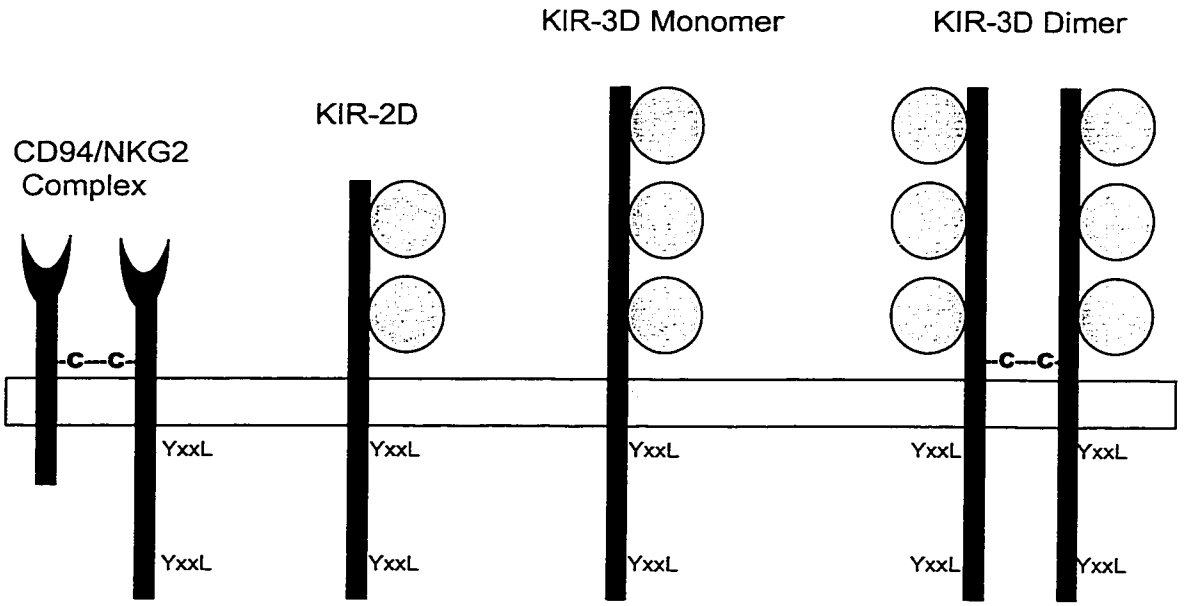

KIR-3D Monomer
KIR-3D Dimer
KIR-2D
CD94/NKG2
Complex
C—C
C—C
YxxL
YxxL
YxxL
YxxL
YxxL
YxxL
YxxL
YxxL
YxxL
YxxL

Figure 1. Diagrammatic representation of the human inhibitory CD94-NKG2A , and KIR molecules. YxxL denotes the ITIM sequence of these receptors located in the cytoplasmic tail portion of the receptor. Disulfide bonds are indicated by C—C. The CD94 are type II proteins of the C-type lectin superfamily and the KIR are type I proteins of the immunoglobulin superfamily (the gray circles represent immunoglobulin motifs).

3. NK Cell Recognition of MHC Class I

The three-immunoglobulin domain KIR designated NKB1 recognizes amino acids 77–83 in the α1 domain of certain HLA-B heavy chains most well described being the HLA-Bw4 motif (figure 2) (68). Most two-immunoglobulin domain KIRs recognize a polymorphism at positions 77 and 80 of the HLA-C heavy chain (42). This has been confirmed by direct binding studies using soluble recombinant two-immunoglobulin domain KIR and HLA-C ligands (53, 65, 69) and further study has illustrated that this receptor-ligand interaction occurs with a 1:1 stochiometry (69).

The role of β2-microglobulin and peptides in KIR recognition is not well substantiated and the bound peptides in the major groove of the MHC class I molecules are ill defined. Mandelboim et al. have suggested that the two-immunoglobulin domain KIR, in the absence of bound peptides, are capable of recognizing HLA-C ligands, whereas studies of a three-immunoglobulin domain KIR demonstrate a necessity for a bound peptide to the HLA-B*2705 class I heavy chain (70). In the latter situation, bound peptide could cause alterations in amino acids at positions 77 and 80 in the HLA-C heavy chain and affect binding between the class I complex and the KIR (71). Most studies demonstrate that KIR do not discriminate between "self" and "foreign" peptides, however it is speculated that different peptides can affect the conformation of the class I molecule and alter the KIR binding site, thus there may be a physiological role for peptides in NK cell function.

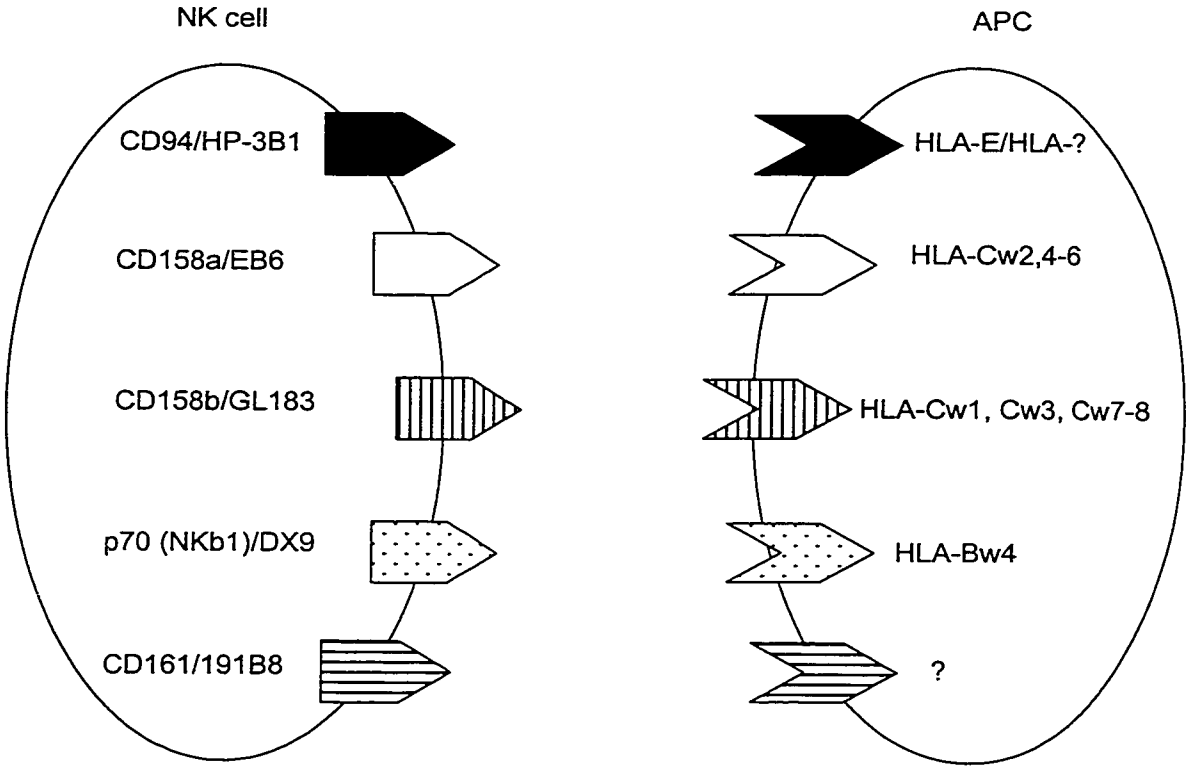

NK cell
CD94/HP-3B1
CD158a/EB6
CD158b/GL183
p70 (NKb1)/DX9
CD161/191B8
APC
HLA-E/HLA-?
HLA-Cw2,4-6
HLA-Cw1, Cw3, Cw7-8
HLA-Bw4
?

Figure 2. Diagrammatic representation of the NK receptors reactive with HLA-A, B, and C ligands. The two-immunoglobulin domain KIR indentified by the antibodies EB6 and GL18 can are known to have some specificity to HLA-C alleles, which contain an asparagine at residue, 77 and a lysine at residue 80 in the HLA-C heavy chain. The three immunoglobulin domain KIR p70 (NKB1) is recognized by the monoclonal antibody DX9 and this KIR binds alleles possessing the Bw4 motif. The C-lectin type receptor CD161 is recognized by the antibody 191B8 and its ligand has yet to be determined.

Unlike KIR, CD94/NKG2 heterodimers do not recognize the polymorphism defined by amino acids 77 and 80 in the HLA-C molecules. Recent evidence demonstrates that the CD94/NKG2 complex has a strong affinity for the non-classical MHC class I molecule, HLA-E. This receptor-ligand interaction, requires that HLA-E be bound with a leader sequence peptide of a classical MHC molecule for its assembly and stabilization (72).

4. Signal Transduction and NK Cell Receptors

Ligation of most cellular receptors elicits intracellular responses that are mediated by either tyrosine kinases that activate or tyrosine phosphatases that inhibit signaling (73). The most investigated NK cell receptor signaling cascades are those of the inhibitory KIRs (74). These receptors appear to have nearly indentical inhibitory signaling cascades irrespective of the type of KIR (74). Upon cross linking, these receptors result in tyrosine phosphorylation of the ITIM (44). Sequence analysis of ITIM motifs indicate a common I/VxYxxL/V region, which is known to bind SHP-1 tyrosine phosphatase and can interfere with positive signals transduced by other membrane receptors (44). The NKG2 family of molecules, which associate with the receptor CD94, also contain two cytoplasmic ITIM sequences and these ITIMs are known to recruit the SHP-1 and inhibit NK cell activity. In contrast, isoforms of KIR and the NKG2 family lack ITIMs in their cytoplasmic domain and these are known to activate rather than inhibit NK activity (75). These isoforms, more than often possess a CxCP motif, which is a motif found in the transmemebrane molecules CD4 and CD8 (76). The mechanism and the molecules involved in mediating the increases in NK cell activity

have not been elucidated and require further investigation. Since these receptors have been clearly demonstrated to influence NK cell activity, the role of these receptors in NK cell activity during viral infections is necessary in understanding the immunopathology of diseases such as HIV.

C. GENETIC ORGANIZATION AND REPLICATION CYCLE OF HIV

The Human Immunodificiency virus (HIV) virus is genetically composed of two identical (+)-strand RNA copies of its viral genome (figure 3). After infection of a susceptible host cell the RNA strands are reverse transcribed to produce the provirus. The 5′ and 3′ ends of the proviral DNA consist of a 634 nucleotide long terminal repeat segment. Here the 5′-LTR controls the initiation of RNA transcription, and the 3′-LTR controls RNA polyadenylation and termination. The structural components of the virus are encoded by *gag*, which produces a protein, that makes up the viral core proteins and *env*, which produces the protein components necessary for receptor binding and membrane fusion for the purposes of viral entry. The gene that encodes viral protease, reverse transcriptase, ribonuclease H activity and integrase is the *pol* segment. The proteins that control viral replication are encoded by *tat* a transcriptional activator protein and *rev* a post-transcriptional trans activator protein. *Tat* influences control of transcription through the Tat-response element and *rev* enhances structural gene expression by promoting transfer on incompletely spliced viral RNAs from the nucleus to the cytoplasm. This latter activity is regulated by the Rev response element (RRE).

Other proteins involved in the various functions of the HIV life cycle are *vif, vpr, vpu* and *nef*. Vif enhances infectivity of virus particles, the function of Vpr is not fully described, Vpu is necessary for effective virus budding, and Nef has many diverse effects and is suspected to influence viral replication, T cell activation, MHC downregulation and, CD4 downregulation (77).

The components of the HIV genome all work in concert to expidite infectivity, viral replication, and survival of the virus within the host. HIV entry into the host cell is mediated through CD4 and a co-receptor (78). These co-receptors are known to be certain chemokine receptors, but only some have been identified are known to play a role in viral entry (79, 80). After viral fusion the virus capsid is partially uncoated and then forms a ribonucleoprotein complex that has reverse transcriptase activity. The HIV RNA genome is then reverse transcribed by reverse transcriptase to produce proviral DNA, and a mediated (81). The products from reverse transcription eventually produce linear double stranded DNA, the Gag matrix protein, the accessory Vpr protein, and viral integrase (82). These products together are known as the pre-integration complex, and this complex is transported to the nucleus by targeting mechanisms inherent to the Gag matrix and the Vpr proteins (83-86).

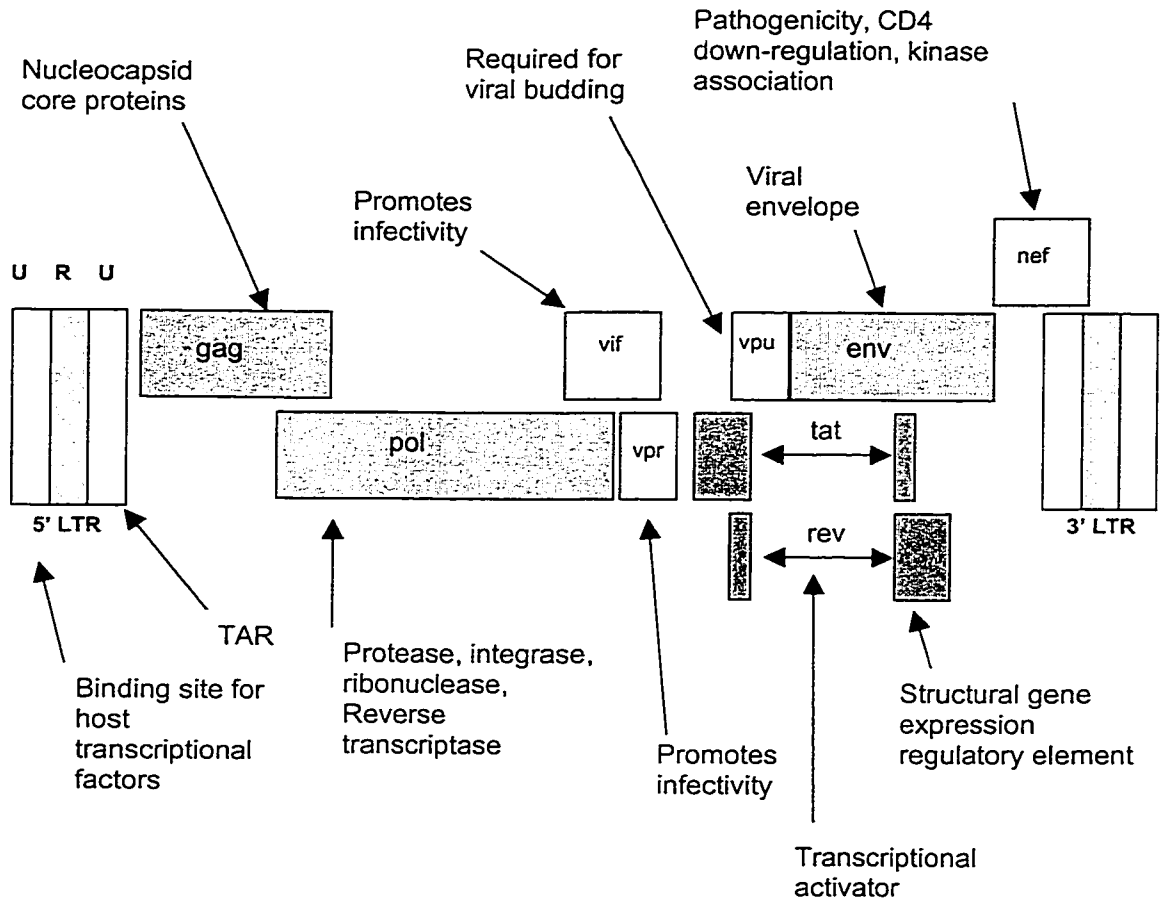

Nucleocapsid core proteins
Required for viral budding
Pathogenicity, CD4 down-regulation, kinase association
Promotes infectivity
Viral envelope
U R U
gag
vif
vpu
env
nef
pol
vpr
tat
rev
5' LTR
3' LTR
TAR
Binding site for host transcriptional factors
Protease, integrase, ribonuclease, Reverse transcriptase
Promotes infectivity
Transcriptional activator
Structural gene expression regulatory element

Figure 3. HIV-1 genome. The genetic components of the HIV-1 virus and the possible functions of each gene.

In the nucleus, viral integrase initiates a series of enzymatic steps, including exonuclease trimming of the linear double-stranded DNA, endonucleolytic cleavage of host chromosomal DNA, and ligation of viral DNA (87). This integration process is preferentially directed to areas of open chromatin structure. In some cases the provirus does not produce viral products and is considered latent (88). Upon activation of the cell, conditions can be induced so that transcription of the viral genome begins (89, 90). In this instance, the viral transcriptional trans activator protein, Tat, which binds to the TAR element in the viral RNA, can upregulate viral transcription (91, 92). As well, HIV expression can be induced by transactivator proteins from other viruses such as human T-cell leukemia virus type 1, herpes viruses, adenoviruses, hepadnaviruses, and papoviruses (93). Once viral RNA is produced, a series of events occur leading to the assembly of viable virus. In viral assembly, RNAs are located to the plasma membrane to be incorporated into a virus particle (94, 95). As well, some RNA is spliced and transported to the cytoplasm to serve as the mRNA for synthesis precursor proteins such as Gag-Pol. Splicing variants include segments ranging from 4.5-5.5kb, which contain mRNAs for Vpr, Vif, and/or a bicistronic mRNA for Vpu and Env (96). Moreover, RNA splicing products ranging from 1.8-2.2kb contain Tat, Rev, and Nef, the degree of splicing is often being increased by the presence of Rev. The Gag and Gag-Pol precursor protein are assembled into virus particles at the plasma membrane and envelope precursor protein is proteolytically cleaved in the host cells secretory pathways (97). Subsequently, all of the viral components including Gag, Gag-Pol precursor protein, envelope proteins viral RNA, and the Vpr protein aggregate at the cell surface and bud releasing a noninfectious immature virus particle. This particle is then made infectious

through the action of the viral protease (Pr), which results in the processing of Gag and Gag-Pol precursor proteins (98).

D. THE IMMUNOPATHOLOGY OF HIV INFECTION

HIV belongs to the lentivirus family and a hallmark of this virus family is a chronic and persistent infection, which leads to the development of various pathological conditions. Products of persistent viral replication can be detected in the plasma and lymphoid tissue in chronically infected individuals (99). In most cases the infection is never eliminated despite the efforts of the host immune response (34, 100-102). Subsequently, this chronic infection leads to development of symptoms that are associated with HIV. In HIV infection, these include the development of opportunistic infections and malignancies that are a result of generalized immune dysfunction (99).

The natural cycle of the immune system upon presentation of foreign material during any foreign invasion is to recognize foreign antigens, mount a specific response to the invading organism or agent, eliminate them and then return to a steady state in order to respond effectively to different invading organisms and or to a secondary infection of the same organism (anamnestic response). In the case of HIV infection the constant presence of viral replication is reciprocated by a constant activation of the immune system. This constant state of immune activation is somewhat advantageous to the production of virus since many host mechanisms the virus uses for replication are heightened during immune activation (103-105). Quiescent CD4+ T cells can be infected with HIV but reverse transcription, integration, and virus spread are more

efficient in activated T cells. Moreover, much of the underlying pathologies of HIV infection is a result of prolonged immune activation. Immune activation often results in hyperactivation of B cells leading to hypergammaglobulinemia; aberrant lymphocyte proliferation; aberrant activation of monocytes; lymphoid hyperplasia; increased secretion of proinflammatory cytokines; elevated levels neopterin; β2 microglobulin, interferon, soluble IL-2 receptors as well as various autoimmue phenomena (103, 104). As well, some studies have demonstrated that immune activation induces expression of virus in latently infected CD4+ T cells(93).

During HIV infection, as opposed to many other viral infections, no immune response has been indicated to be completely protective against HIV. This inability for the host to develop a protective response is in part due to the inherent characteristics of HIV. These characteristics are the overwhelming replication kinetics of virus (100-102)., the error rate of the HIV reverse transcriptase (106, 107), and the ability of HIV to downregulate MHC molecules (34). One of the main indicators of a hosts protection to viral infection is virus specific CTL. In HIV infected individuals there is detectable HIV specific CTL from peripheral blood isolation of CD8+ T cells (108). This may be a disadvantage because it appears that the HIV specific CTL are concentrated to the peripheral blood as opposed to within lymphoid tissue, which is the most prominent site of viral replication (109). This failure to prevent viral replication in lymphoid tissue is another manner by which HIV can evade host mechanisms.

There are several other immune defects associated with HIV infection. Dysfunction is present in many facets of the immune system, including in T- and B-lymphocytes, antigen presenting cells, NK cells and neutrophils. Immunodeficiency is especially prevalent in the later stages of the disease when the adaptive immune response diminishes as a result of ongoing viral replication and CD4+ T cell depletion. For this reason the CD4+ T cell counts of HIV seropositive individuals is a good indicator of the extent of immune suppression (110, 111). Studies have demonstrated that almost 90% of individuals with a CD4+ T cell count of less than 200 cells/μl will experience an AIDS defining illness within 4 years(112).

Numerous mechanisms attributed to HIV infection of CD4+ T cells are likely responsible for the observed defect in T cell phenotype and function. These would include defects in the distribution of T cell subset populations, altered expression of IL-2 receptors and decreased IL-2 production (113). As well, interference with CD4 expression by HIV gp120, Nef, and Vpu, may hamper the capacity of CD4+ T cells to interact with MHC class II molecules(114-116). CD4+CD45RO+ memory T cells seem to be especially susceptible to infection with HIV or the deleterious effects of the virus (117). As well, circulating gp120 molecules may dysregulate the expression of costimulatory molecules in uninfected CD4+ T cells and induce anergy(118-120).

Recent investigation of the immunopathogenesis of HIV infection indicate that in the late stages of HIV infection there are perturbations of the CD4+ T cell receptor Vβ repertoire(109, 121-124). These investigations employed the use of polymerase chain

reaction (PCR) over the CDR3 region with primers specific for the T-cell receptor Vβ families. The products of these PCR reactions provide insight into the recombination of variable, diversity and junctional genes segments. What was discovered is that there was a lack of certain recombination products in the later stages of HIV infection, thus indicating perturbations in the Vβ repertoire. This was particularily evident when the CD4+ T cell counts were below 200 cells/μl (125). Moreover, increases in CD4+ T cell counts due to anti-retroviral therapy were associated with a partial restoration of the Vβ repertoires, to its previous state (125). However, it is possible that with prolonged viral suppression and maintenance of CD4+ T cell counts the repertoire may be restored.

Other significant changes in HIV seropositive individuals that reflect their immune competence is their lymphoid tissue architecture. In the later stages of HIV disease there is marked disruption of the lymphoid tissue architecture (126, 127). Lymph nodes from HIV seropositive individuals eventually develop follicular involution, hypervascularity, and fibrosis (128-130). Follicular involution indicates loss of follicular dendritic cells (FDCs), and as a result the ability of the seropostive individual to mount an immune response to new antigens is severely impaired (128). Loss of FDCs and lymphoid architecture results in loss of control of viral replication and enhanced susceptibility to opportunistic infections (130).

E. HIV INFECTION AND NK CELLS

Various NK cell deficiencies have been reported in HIV infected individuals and are most notable with severe immunodeficiency (131-133). As of yet there is no *in vivo* evidence indicating infection of NK cells by HIV. The resultant deficiencies in NK cell function are likely an indirect effect perhaps due to generalized immune activation(134). Non-productive infections of NK cells have been reported *in vitro* with HIV-1 isolates (135). Although, NK cells do not normally express CD4 they can be induced to express CD4 at the cell surface by infection with human herpesvirus 6, which could possibly lead to an *in vivo* scenario where NK cells could be productively infected with HIV.

The current studies describing NK cell activity and phenotype in HIV infection are considerably limited. The most prominent of these studies is by Ullum *et al.* (136). Their group described reduced NK cell function during the symptomatic stages of AIDS, which has been confirmed by other studies, as well (132, 137) (137). It is proposed that defective NK activity is in part due to the lack of the appropriate cytokines for normal function, in vitro studies with NK cells from HIV infected individuals demonstrate that addition of either IL-2, IL-12 or IFN-α can restore defective NK cell function (138). In terms of NK cell number many studies found no relative difference in the percentages of NK cells (CD16+, CD56+, and CD16+CD56+) between HIV seropostive and seronegative individuals, but differences in the absolute number of CD16+CD56- lymphocytes were present (138, 139). The significance of this decrease in absolute numbers of NK cells has yet to be determined (139).

Some groups have evaluated NK receptor expression on NK cells and T cells in HIV seropositive individuals. Knowing that NK receptors are critical to the functional state of NK cells it is important to understand their role in NK cell dysfunction during HIV infection. Andre *et al.*, evaluated NK receptor expression on NK and T cells in HIV seropositive and seronegative individuals, in association with clinical status, absolute CD4+ T cell number and viral load (12). Through the use of two-colour flow cytometry, they found that the percentages of NK cells, which express CD94 and NKG2A, increased with the progression of infection. This study does not provide insight into the functional relevance of CD94 but it does conclude that HIV can alter CD94 expression on NK cells and this in turn may have affect on NK functionality. However, an analysis of NK receptors has yet to be investigated in HIV seropositive individuals undergoing ART.

F. ANTIRETROVIRAL THERAPY AND IMMUNE RECONSTITUTION

HIV infected individuals now have numerous treatment options because of a greater understanding of the pathogenesis of HIV infection. The most recent advance in HIV antiretroviral therapy is the development of pharmaceuticals that inhibit HIV-1 protease(15-17, 140-142). Reverse transcriptase (RT) inhibitors were the first pharmaceuticals developed as treatments for HIV infection, and are still widely used in combination with HIV-1 protease inhibitors(143, 144). The majority of RT inhibitors are nucleoside analogs and inhibit reverse transcription because their structures lack a 3' hydroxyl moiety required for chain elongation. The main disadvantage of RT inhibitors is the various neurological and hematological side effects, the occurrence of RT inhibitor

resistant virus, and their ineffectiveness on cells already infected with HIV-1(145). The HIV-1 protease functions by cleaving the Gag precursor polyprotein into the p24 and p17 virion components, which are required for viral assembly. Protease inhibitors lead to production of noninfectious virions from cells already infected with HIV, and because of this, protease inhibitors in combination with RT inhibitors is now a widely used treatment regimen for individuals infected with HIV (146). Combination therapy is a well established approach to suppress viral production in infected individuals for prolonged periods of time (17).

The ability of combination therapy to reduce viral replication to an undetectable level often results in partial restoration of immune function (15, 17, 140). This is primarily evident in the fact that patients who respond to combination ART are at decreased risk of developing HIV associated opportunistic infections (15, 147, 148). *In vitro* indicators of immune function also reflect this immune competence. After initiation of ART many groups have reported presumably beneficial phenotypic changes in the T cell population including: rises in the memory T cell population, a rise in naïve CD4+ lymphocytes paralleled by a decline in the CD8+ T cell population, and a reduction in T cell activation markers (15). As well, our group has established that some of these phenotypic changes are often associated with improvements in many facets of adaptive immunity. These improvements include enhancement of lymphoproliferative responses to mitogen, recall and/or HIV antigens, and increases in IL-2 and IL-12 production from peripheral blood mononuclear cells (PBMC) (14). However, few studies have investigated changes in NK cell phenotype or function in HIV infected individuals treated with ART.

II. RATIONALE

The deleterious effects of HIV on the immune system are known to initiate the progression to AIDS. Investigating the mechanisms that propogate the immunodeficiencies in HIV infected individuals is paramount to development of therapies that can restore immune competence and eradicate virus from the host. Current antiretroviral therapy shows some promise in the restoration of immune competence in HIV infected individuals, however the potential extent of this immune restoration is not fully understood and there is limited information on the impact of HIV and ART on NK cell function and phenotype. We, therefore, evaluated NK cell number and function in early infection and changes over the course of effective ART. To address potential mechanisms of changes in NK activity with ART a cross-sectional study was undertaken to evaluate NK cell receptor expression. This was achieved by determining the proportions of NK cells expressing CD94, CD158a, CD158b, p70 and CD161 by four-colour flow cytometry. Such information would provide additional insight into the mechanisms of HIV-induced immunodeficiency and potentially identify sites to target with immune based therapies.

III. PURPOSE

To determine the impact of effective ART on NK cell function and to determine if changes in NK phenotype may explain possible changes in function in HIV infected individuals. This will provide further insight into the immunopathogenesis of HIV infection and the restorative impact of ART.

IV. HYPOTHESIS

NK cell function and phenotype in HIV infected individuals are partially restored to levels observed in HIV seronegative individuals after the initiation of ART. The working hypotheses are: 1) HIV infected individuals receiving ART will have a normalization of NK cell activity to levels that are observed in HIV seronegative individuals; 2) the observed decreases in NK cytolysis coincide with changes in the expression of NK receptors, and these changes will result in NK cell phenotype more closely resembling HIV seronegative individuals.

V. OBJECTIVES

The following objectives can elucidate if NK cell function and phenotype in HIV infected individuals are partially restored to leveis observed in HIV seronegative individuals after the implementation of ART.

1) Measurement of the viral load and absolute CD4+ T cell count in HIV infected individuals over the duration of combination anti-retroviral therapy that consists of Nelfainavir, Saquinavir, and two reverse transcriptase inhibitors.

2) Determination of NK cell cytolytic activity and NK cell percentages in HIV infected individuals before ART and over the duration of therapy and compare to values obtained from HIV seronegative individuals.

3) Measurement of NK receptors expression on NK cell from three populations; HIV seronegative individuals, untreated HIV seropostive individuals and HIV seropositive individuals receiving ART.

VI. MATERIALS AND METHODS

A. Patient Populations

The populations assessed for NK activity were patients involved in an antiretroviral clinical trial at the Immunodeficiency Clinic of the Ottawa Hospital-General Campus. Requirements for enrollment were age between 18-60 years, a positive serological test for HIV-1 and a baseline HIV RNA plasma viral load of greater than or equal to 500 copies/ml. Patients were excluded if they had received reverse transcriptase inhibitors for more than 6 months or any previous treatment with HIV protease inhibitors. After obtaining informed consent, patients were enrolled in the study at which time they received saquinavir (Invirase) 600 mg t.i.d., and after 4 days Nelfinavir 750 mg t.i.d. was added. Eight days after starting saquinavir, two reverse transcriptase inhibitors were added.

In a cross sectional analyses of NK specific receptor expression three cohorts were analyzed. These consisted of HIV-1 seronegative volunteers (Group A), HIV-1 seropositive individuals receiving ART achieving HIV-1 RNA levels below the limit of detection (<50 copies/ml) for greater than 1 year (Group B) and untreated HIV-1 seropositive patients (Group C).

B. Measurement of lymphocyte subsets and viral load

CD4+ T cell counts for all patients were measured using standardized techniques at an independent flow cytometry facility that participates in federal quality assurance programs. HIV-1 RNA levels in plasma for patients enrolled in the prospective clinical trial were quantified using a commercially available NASBA (Nucleic Acid Sequenced Based Amplification System) and electro-chemiluminescence detection system (NUCLISENS™ HIV-1 QT; Organon Teknika Corporation, Durham, North Carolina, USA). This assay had a quantification limit of 2.70 $_{log10}$ copies/ml of liquid. Plasma HIV-1 RNA levels for patients enrolled in the cross sectional study were measured by the Chiron 3.0 bDNA assay (1.7 $\log_{10}$ copies /ml).

C. Preparation of NK Effector and Target Cells

At baseline, week 8, 24, and 48 peripheral venous blood was obtained from each patient into sodium heparin vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) were then separated from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (Amersham-Pharmacia Biotech, Inc., Baie d'Urfé, Québec). These cells were used as effector cells. Isolated PBMC were washed with RPMI 1640/10 (RPMI 1640 and 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin; Life Technologies, Burlington, Ontario, Canada) and resuspended at a density of $3x10^6$ cells/ml in cryopreservation

media (80% FBS/20% DMSO). Cells were aliquoted into cryovials and preserved in liquid nitrogen until further use.

The target cells, a K562 human erythroleukemic cell line (American Type Culture Collection, Rockville, MA) were maintained in vented cap 75 cm^2 tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) with RPMI/10 medium at 37°C in a humidified 5% CO_2 incubator. Cells were monitored daily and maintained at a density of no more than $3x10^5$ cells/ml to ensure cells were in log growth phase.

D. Determination of NK Activity

Cryopreserved PBMC to be used as effector cells, were removed from liquid nitrogen storage and placed in a 37°C water bath for 4 minutes. The cell suspension was added to a 15 ml conical centrifuge tube (Becton Dickinson, Franklin Lakes, NJ) followed by 5 ml of RPMI/10. The cells were then centrifuged at 400xg for 10 minutes to pellet the cells. The supernatant was decanted and the cell pellet was then resuspended to a concentration of $1x10^6$ cells/ml in RPMI/10. The yield of viable PBMC after crypreservation was between 80% and 95%. In order to decrease interassay variability, cryopreserved cells from all time points for each patient were thawed and assayed for NK activity on the same day. The cells were then incubated for 5 hrs at 37°C and 5% CO_2, after which time they were used as effectors in cytoxicity assays.

To determine NK activity, as measured by percent specific lysis of target cells, a flow cytometric method that relies on the staining characteristics of 3,3'-Dioctadecyloxacarbocyanine perchlorate (DiO; Molecular Probes, Eugene, OR) was utilized. This method for determination of NK activity has been described elsewhere and is at least as sensitive with less variability than conventional chromium release assays (149). The K562 target cell line was stained by incubation with 10µl of 30mM DiO per $1x10^6$ cells/ml for 20 minutes at 37°C, 5% CO_2 followed by washing in PBS and resuspension at $1x10^6$cells/ml in phenol red free RPMI/10. Target cells ($1x10^5$ cells) were then be added to effector cells to create effector to target (E:T) ratios ranging from 1:50 to 1:3.125 in a final volume of 1 ml in phenol red free RPMI/10. To ensure effector-target contact the cells were centrifuged at 1000 x g for 10 seconds and incubated for 2 hours at 37°C, 5% CO_2 in 1 ml of RPMI/10, with the addition of 5 mg/ml of propidium iodide (PI) for the last 15 minutes of incubation.

After incubation, the cells were analyzed by flow cytometry. The percentage of lysed target cells was calculated by dividing the number of cells lysed (those with positive fluorescence for both DiO and PI) by the total number of target cells (those with positive DiO fluorescence). The percentage of cells lysed in the absence of effector cells (background) was then subtracted to determine the percent specific lysis. Lytic units at 10% lysis (LU_{10}) were calculated as described by Pross *et al.* (150).

E. Immunophenotyping

Whole blood samples were drawn into sodium heparin vacutainer tubes. One hundered microllitres of blood was incubated with the appropriate antibody combination for 30 minutes at ambient temperature in the dark in 12mm x 75mm polystyrene tubes (Becton Dickinson). Subsequently, the sample tubes were processed by the Q-Prep/ImmunoPrep lysing system (Beckman Coulter) followed by fluorescence analysis performed on a Beckman Coulter ALTRA flow cytometer. Resulting profiles were analyzed with the EXPO version 2.0 software package. Samples analyzed for NK activity were also analyzed for NK cell percentages relative to total lymphocyte population (determined by light scatter analysis) by use of the Simultest™ CD3/CD16+CD56+ Reagent (Becton Dickinson). The antibodies used for four colour analysis of NK receptor expression on NK cells were CD3-ECD, CD16-FITC, CD56-PC5, in addition to one of CD94-PE, CD158a-PE, CD158b-PE (Beckman Coulter), NKB1(p70)-PE, or CD161-PE (Pharmingen, San Diego, CA). Although additional NK receptors have been described (41), these NK receptors were evaluated because of the availability of specific antibodies and these NK receptors have been well characterized.

E. Data Analysis

Absolute CD4+ T cell count, viral load, and NK Receptor expression were reported as mean ± standard deviation. NK cell percentage and NK cell activity were reported using the median and interquartile range (IQR) since their values were not

normally distributed. NK cell percentage and NK cell activity over time were compared using a Kruskel-Wallis ANOVA on Ranks normalized to baseline values with Dunnett's Test for pairwise comparisons. NK cell percentage and NK cell activity at each time point was compared to that found in an HIV seronegative cohort with Mann-Whitney Ranked Sum Test. A t-test was used to compare the levels of expression of each NK receptor between patient groups.

VII. RESULTS

A. Plasma HIV-1 RNA levels, CD4+ T cell counts and NK cell percentages.

Of the ten patients enrolled in the ART clinical trial nine were male, the median age was 40 years (range 35-55) and all patients were ART naive at study entry. The mean baseline plasma HIV-1 RNA concentration was 4.93 $\log_{10}$ copies/ml. After 8 weeks of therapy eight patients had plasma HIV-1 RNA levels below the limit of detection (<2.70 $\log_{10}$ copies/ml). Two patients had HIV RNA levels of 2.73 $\log_{10}$ and 3.23 $\log_{10}$ copies /ml. At 24 and 48 weeks of therapy all ten patients had levels below the limit of detection (Table 1).

The mean CD4+ T cell count at baseline was 552 cells/ml. This progressively increased to 557, 594 and 661 cells/μL at 8, 24 and 48 weeks, respectively (Table 1.). At baseline 3.10% (2.20-3.90) [median, interquartile range (IQR)] of lymphocytes were NK cells and this did not change significantly over the course of therapy (Table 1). This was

somewhat lower than that observed in HIV-1 seronegative donors, 5.65 % (3.30-7.00)(n=10), but this did not reach statistical significance.

Table 1. *Plasma HIV-1 RNA concentration, Absolute CD4+ T cell count and NK cell (CD3-CD56+CD16+) percentage in HIV-1 seropositive patients on ART.*

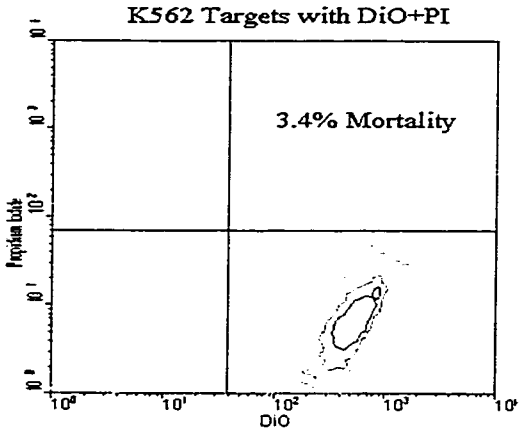
	Baseline	Week 8	Week 24	Week 48	HIV seronegative donors
HIV-1 RNA conc. (log_{10} copies/ml)[a)]	4.84 ±0.73	2.76 ±0.55	2.70 ±0.00	2.70 ±0.00	
Number of individuals with < 2.70 log_{10} copies/ml of HIV-1 RNA	0/10	8/10	10/10	10/10	
Absolute CD4+ T cell count (cells/μL)[a)]	552 ±330	557 ±293	594 ±254	661 ±215	
NK cell percentage[b)]	3.10 (2.20-3.90)	3.53 (1.30-5.50)	3.75 (1.80-7.50)	4.45 (2.60-6.80)	5.65 (3.30-7.00)

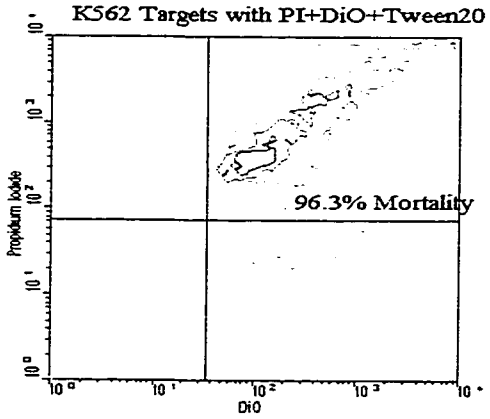
a) Mean ±SD, viral load values < 2.70 log_{10} copies/ml of HIV-1 RNA were assigned a value of 2.70 for the purpose of calculations.

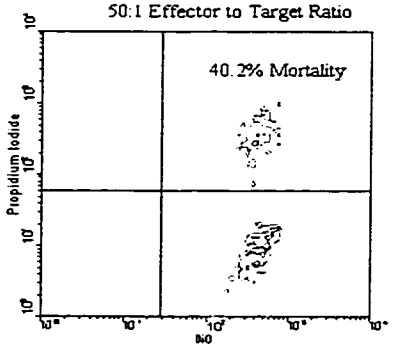
b) Median, IQR

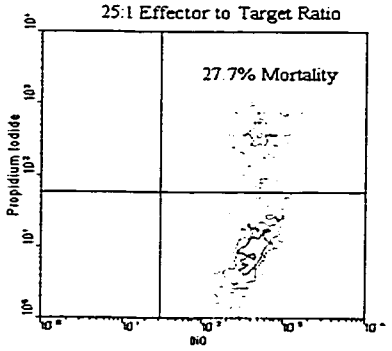
\B. Determination of NK activity

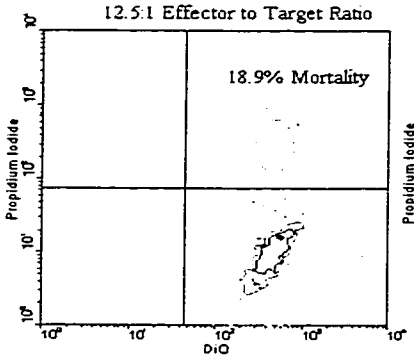
An example of the flow cytometric analysis of NK cell activity is illustrated in figure 4. Lytic units at 10% lysis (LU_{10}) were calculated as described by Pross *et al.*. NK cell activity was greater in HIV-1 infected patients at baseline compared to HIV-1 seronegative controls, median 56.09 units vs. 10.90 units ($p<0.001$ by Mann Whitney Ranked Sum test; figure 5). With the initiation of therapy there was a significant decrease in NK cell activity by week 8 (56.09 units vs. 28.18 units $p=0.011$ by Kruskal-Wallis ANOVA on Ranks and $p<0.05$ by Dunnett's test for pairwise comparison). Beyond week 8, the level of NK activity remained stable. The level of NK activity at 8, 24, and 48 weeks of therapy (28.18, 18.42, and 19.13 units respectively) was not significantly different then that observed in HIV seronegative controls.

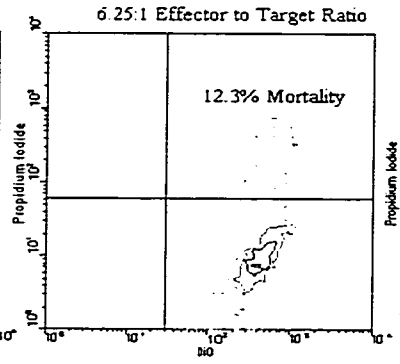

K562 Targets with DiO+PI
3.4% Mortality
Propidium Iodide
DiO


K562 Targets with PI+DiO+Tween20
96.3% Mortality
Propidium Iodide
DiO


50:1 Effector to Target Ratio
40.2% Mortality
Propidium Iodide
DiO


25:1 Effector to Target Ratio
27.7% Mortality
Propidium Iodide
DiO


12.5:1 Effector to Target Ratio
18.9% Mortality
Propidium Iodide
DiO


6.25:1 Effector to Target Ratio
12.3% Mortality
Propidium Iodide
DiO

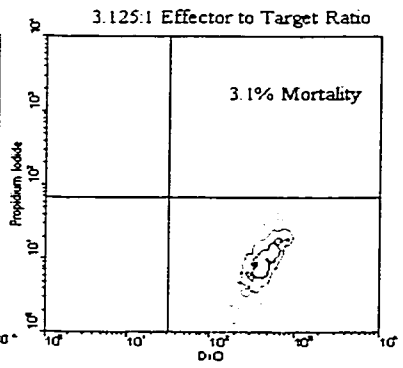

3.125:1 Effector to Target Ratio
3.1% Mortality
Propidium Iodide
DiO

Figure 4. Representative contour plot for determination of NK cytolyitc activity by flow cytometry. Utilizing effector to target ratios of 50:1 to 3.125:1, NK lytic activity from PBMC was measured by PI uptake in the DiO stained K562 target cell line by PI uptake. Percent mortality is determined by the DiO and PI positive events, which were acquired 3.5 hours after effector and target incubation.

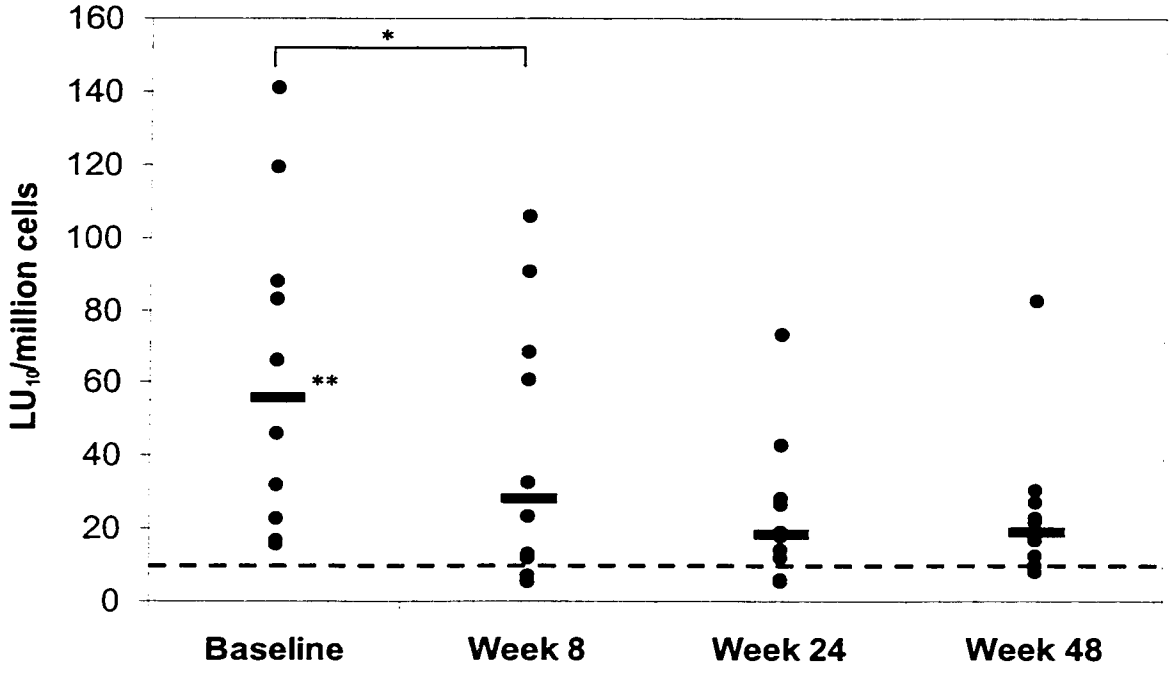

160
140
120
100
80
60
40
20
0
LU10/million cells
*
**
Baseline
Week 8
Week 24
Week 48

Figure 5. NK cytolytic activity expressed in lytic units at 10% lysis (LU_{10}) for ten ART naïve patients at baseline, weeks 8, 24, and 48 of therapy. The median LU_{10} at each time point is represented by the solid bar (—) and the median LU_{10} for HIV seronegative individuals (n=10) is represented by the dashed line(----) (*p=0.011 baseline vs. week 8; ** p<0.001 baseline vs HIV seronegative controls)

C. NK specific receptor expression

The determination of NK specific receptors (CD94, CD158a, CD158b, NKB1, and CD161) was undertaken to address possible mechanism involved in the ART induced changes in NK cytolytic activity, since ligation of these receptors is known to modulate NK function. In this cross-sectional analysis four-colour flow cytometry was used to determine the expression of NK receptors on CD3-CD16+CD56+ lymphocytes.

In a cross sectional study, the mean viral load and CD4+ T cell count for the untreated HIV-1 seropositive cohort (Group C; n=10) were 4.90 $\log_{10}$ copies/ml and 207 cells/uL, respectively (Table 2.). For the group of patients on ART with suppressed HIV-1 RNA levels (<50 copies/ml of plasma) for greater than 1 year (Group B; n=9) mean CD4 T cell count was 525 cells/uL. The mean HIV-1 RNA level and CD4 + T cell count prior to initiation of ART was 4.75 $\log_{10}$ copies/ml and 250 cells/μl. Prior to initiation of ART The patients with suppressed plasma viremia, were similar with respect to CD4+ T cell count and viral load to the untreated group. This suggests that the comparison of these two populations may accurately reflect the elevation of NK receptor expression before and after effective ART within the same study population.

The percentage of NK cells expressing CD94, CD158b and p70 was not significantly different between the two HIV seropositive populations (Groups B and C) or between either of these groups and the HIV seronegative population (Group A; n=6)(figure 6). NK cells from Group C expressed significantly more CD158a (8.18 ±

5.72%) than either Group B (2.66 ± 5.46%; p=0.046) or Group A (0.92 ± 1.00%; p=0.009. This same pattern was observed for expression of CD161 with 24.72 ± 18.07% of NK cells from Group C expressing CD161 compared to 11.20% ± 4.56 (p=0.04) and 6.97% ± 2.69 (p=0.02) in Groups B and A, respectively. There was no significant difference between Groups A and B in the percentage of cells expressing CD158a or CD161.

Table 2. *Characteristics of individuals for the cross-sectional study*

	Group A	Group B[a)]	Group C
Group size	6	9	10
CD4+ T cell count (cells/μL) [a)]		525 ±171	207 ±161
Baseline CD4+ T cell count (cells/μL) [a,b)]		250 ±164	
HIV-1 RNA conc. (log_{10} copies/ml) [a)]		1.70 ±0.00	4.90 ±0.36
Baseline HIV-1 RNA conc. (log_{10} copies/ml) [a,b)]		4.75 ±0.55	

Group A - HIV seronegative
Group B - HIV seropositive, viral load <50 log_{10} copies/ml for greater than one year
Group C – HIV seropositive, untreated

a) Mean ±SD
b) Baseline values represent values prior to the initiation of ART

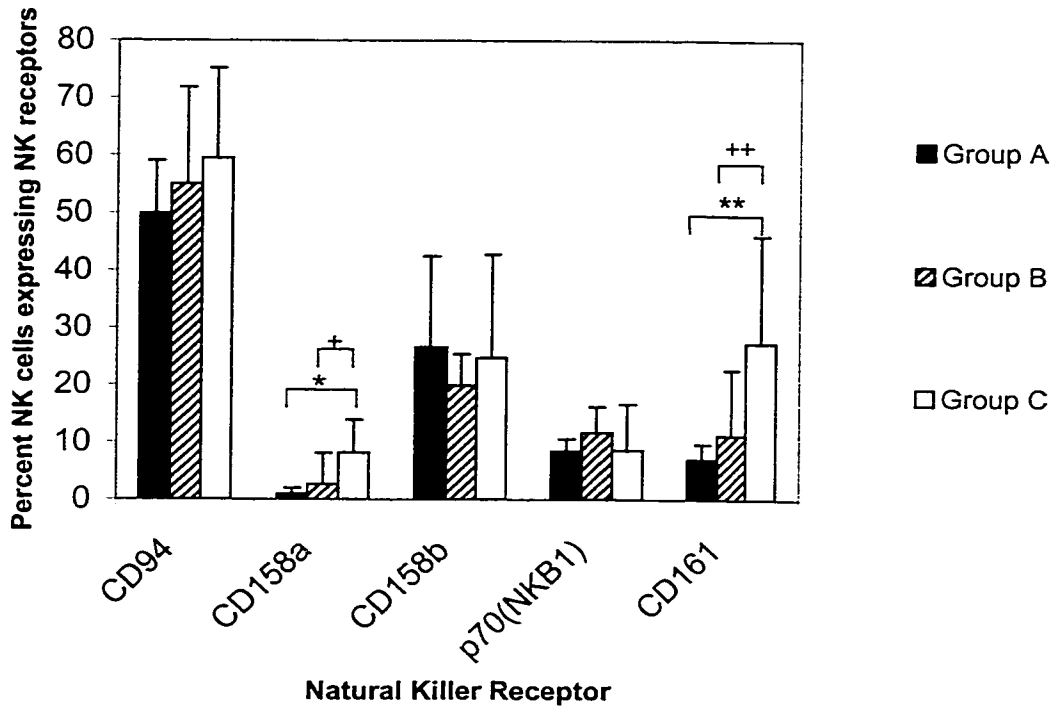

Percent NK cells expressing NK receptors
80
70
60
50
40
30
20
10
0
CD94
CD158a
CD158b
p70(NKB1)
CD161
Natural Killer Receptor
Group A
Group B
Group C
+
*
++
**

Figure 6. Percentage of NK cells expressing cell surface receptors CD94, CD158a, CD158b, p70 and CD161. Whole blood cells from HIV seronegative controls (Group A), patients with HIV-1 plasma RNA levels <50 copies for greater than 1 year (Group B) and untreated HIV-1 seropositive patients (Group C) were stained for four colour flow cytometric analysis.(+ p=0.046, * p=0.009, ++ p=0.04, ** p=0.02)

VIII. DISCUSSION

In order to better understand the role of NK cells in HIV infection we evaluated the impact of ART on NK cell number, phenotype, and activity. To address possible mechanisms of altered NK activity in HIV infection, the impact of viral suppression with ART on the expression of NK receptors was also evaluated. Consistent with other reports, NK cell percentage was not significantly different in HIV infected individuals compared to HIV seronegative controls (6, 138). Some studies have found decreases in NK cell number in HIV infected individuals but this has only been observed in patients with severe immunodeficiency and absolute lymphopenia (137). The similarity of NK cell percentage between HIV-1 seropositive and seronegative individuals, that was observed here, could possibly be attributed to the fact that NK cells are rarely infected with HIV and when they are infected they appear to be relatively resistant to the cytopathic effects of HIV infection (135). We observed no significant change in NK cell percentage with effective ART. This is consistent with that observed by Ledermann *et al.* who evaluated NK cell number in a group of patients treated with a protease inhibitor based regime (13). Another report describes an increase in NK cell number with ART (139). However, in that report absolute NK cell number per millilitre of blood was evaluated as opposed to relative percentages, and the rise in the total T lymphocyte population with ART likely explains their observation (139). The lack of difference in NK cell percentage between HIV infected and uninfected individuals does not preclude a difference in NK activity nor does the absence of change in the relative number of NK cells in response to ART, preclude changes in NK cell function.

A flow cytometric method to evaluate NK activity was employed because such methods are inherently more precise and accurate compared to conventional chromium release assays (149). The evaluation of NK cell activity here was in a group of patients that were early in the course of infection (mean baseline CD4=552 cells/μL). The observation of high NK activity in the patient population at baseline is not an unexpected observation. In other viral infections in humans and in mouse models peak NK cell activity occurs early in the course of infection (20, 151). The associated decline in NK cell activity with the suppression of plasma viremia is a pattern similar to that observed in other viral infections where control or clearance of viremia is associated with a decline in NK cell activity (152). This observed "normalization" of NK cell activity compliments the improvements in other aspects of immune function that occur with the use of protease inhibitor-based therapy (14-17, 139).

Interpretation of the observed decrease in NK cell function associated with effective ART must take into consideration the assay used for this evaluation. PBMC mediated lysis of K562 cells may not be an accurate measure of *in vivo* killing, despite being the standard and a well-accepted method of determining NK cell activity,. The extent of NK activity measured depends upon both the effector cell and target cell; the latter of which (K562 cells) is unchanged over the course of these investigations. This may, therefore, not accurately reflect the potential impact of ART and suppression of viral replication on potential target cells *in vivo*. In addition, the observed enhanced NK activity prior to therapy may reflect an *in vivo* phenomenon that may be attributed to cytolytic cells other than typical NK cells. The presence of CD8+ T cell MHC

unrestricted killing has been observed in HIV infection (153) and may contribute to the NK activity observed in untreated HIV infected patients. This promiscuous cytotoxic T cell lysis may then be downregulated with suppression of viral replication.

The changes in NK cell activity in response to ART may be due in part to changes in the expression of NK specific receptors. We observed, in a cross-sectional analysis, that the proportions of NK cells expressing CD158a and CD161 were increased in untreated HIV seropositive individuals compared to those individuals with suppressed viral replication or seronegative individuals. The detection of the extracellular moieties of these receptors by antibody does not allow for the explicit determination of the receptor activity as stimulatory or inhibitory, since this activity is defined in the intracellular portion of these receptors (27, 42-45, 47, 48, 53), or in the case of CD94, by its association with a set of co-receptors which are not fully described (46). The changes in both NK receptor expression and NK lytic activity with suppression of viral replication does however suggest a functional significance to the observed alteration in NK receptor expression.

This association between phenotypic changes (NK receptor expression) and functional changes (NK lytic activity) in response to suppression of viral replication appear to be the first such observation. Multiple phenotypic changes in CD4+ and CD8+ T cells in response to ART have been consistently observed. Decreases in the percentage of cells expressing the activation markers CD38 and HLA-DR, as well as increases in the percentage of cells of naïve phenotype (CD45RA+and CD62L+) have been reported by several investigators (140, 141) but these phenotypic changes have not been

associated with any changes in specific cellular function. The expression of CD28, a co-stimulatory molecule necessary for optimal T cell function, increases on CD8+ T cells with effective ART (14) but no specific functional improvement in CD8+ T cells has been attributed to this increase in expression. Functional changes have included enhanced proliferative responses, cytokine and chemokine production(14, 140, 142, 154, 155) but none has been attributed to specific phenotypic changes.

Interestingly, recent evidence demonstrates that HIV-1 selectively downregulates HLA-A and B but does not significantly effect the surface expression of HLA-C and E in a lymphoblastoid cell line(34). The proposed advantage for HIV in this selective downregulation is that *in vivo*, HIV infected cells could potentially avoid CTL responses by the reducing antigen specific presentation due to decreases in the surface expression of HLA-A and B and at the same time escape NK cell mediated lysis by maintenance of expression of HLA-C and E. The premise is that HLA-C and E are effective at attenuating NK cell mediated lysis through their cognate inhibitory NK receptors and that these HLA molecules are not effective at mediating HIV specific CTL because of their low expression at the cell surface (156, 157) or, in the case of HLA-E, its preference for presentation of signal-sequence derived peptides from MHC class I molecules (158). As CD161 is well established in mouse and rat systems in inducing NK cell cytotoxicity upon cross linking (159-161) its upregulation in patients with untreated HIV infection may contribute to the enhanced NK cell cytoxicity observed in this cohort. This up-regulation of CD161 during uncontrolled viral replication may be an immediate response by which cytotolytic function is generated to eliminate virally

infected cells until the more mature specific cytolytic function of CTL can take effect but may also persist if specific CTL effector function does not develop or is inefficient.

Though the expression of CD94 was not significantly greater in untreated individuals there was a trend for greater surface expression in patients with uncontrolled viral replication. This is similar to reports that demonstrated the percentage of CD56+ cells expressing CD94 was significantly greater in HIV infected individuals (12, 39). The potential significance of this is that CD94 paired with certain NKG2 molecules is known to have a strong association with HLA-E (24). The coincidental rise of CD158a and CD94 for the respective ligands, HLA-C and HLA-E, suggests specificity in the increased expression of these NK receptors during HIV infection. Moreover, in other viral infections, a similar pattern of altered NK receptor expression is not evident (162, 163), suggesting specificity of the regulation of CD94 and CD158a during HIV infection. This in part may be a NK cell compensatory response to the downregulation of specific MHC class I molecules. The resultant alterations in NK cell receptor expression early in the course of infection and during ongoing viral replication could be a defense mechanism in controlling viral infections.

As it appears that NK cells can be infected with HIV (135); this may result in alterations in NK cell receptor expression and ultimately effect NK cell activity. NK cell infection with HIV has, however, only been described as an *in vitro* phenomena (135) and even if infection can be established *in vivo* it is likely a rare event and cannot likely account for changes in NK cell phenotype or activity. It is possible that the alteration in NK cell receptor expression is the result of generalized immune activation and due to the myriad of paracrine influences present in an activated immune system.

However, in other settings of immune activation in which changes of NK receptors have been described, the pattern of altered NK receptor expression is different (164) suggesting that this is not entirely a non-specific phenomenon.

In the present study, NK receptor expression and NK lytic activity in patients on effective ART approached that observed in seronegative donors. This suggests an association between NK receptor expression and NK cell function and that these are influenced by the ongoing viral replication. The presence of this association supports the concept that NK cell activity is an important component of the host response to infection with HIV. As this family of NK receptors plays a role in additional aspects of cellular cytotoxicity, including CD8+ T cell mediated CTL, regulation of these molecules in HIV infection likely has important implications in HIV immunopathogenesis and may potentially serve as targets of immune based therapy.

IX. SUMMARY

At present there has been no comprehensive study of NK cell number, function and the expression of NK receptors in the setting of HIV and during the course of effective antiretroviral therapy. We therefore studied the impact of ART on NK cell number and activity in ART naïve patients over a period of 48 weeks. To address potential mechanisms of the impact of ART on NK activity, a cross sectional study was undertaken to evaluate NK cell receptor expression. The proportions of NK cells expressing CD94, CD158a, CD158b, NKB1 (p70), and CD161 was evaluated in untreated HIV positive patients, patients with HIV-1 RNA levels <50 copies/ml for

greater than one year and HIV seronegative individuals. This study revealed the following:

1. The percentage of NK cells was not significantly different between HIV seronegative individuals and HIV infected patients either prior to or while receiving ART.
2. Effective ART was associated with a decrease in NK activity to levels similar to HIV seronegative individuals by 8 weeks and remained so over the 48 week duration of the study.
3. In a cross sectional study, expression of CD158a and CD161 on NK cells was elevated in patients with uncontrolled viral replication
4. NK receptor expression in patients with suppressed viral loads was comparable to that in seronegative donors.

X. CONCLUSIONS

The use of effective ART with suppression of viral replication results in decreases in NK cytolysis, which coincides with changes in the expression of NK receptors. These changes are characterized by a NK cell phenotype more closely resembling that seen in HIV seronegative individuals. Normalization of NK receptor expression may, therefore, be a manner by which therapy with potent antiretroviral agents results in restored immune function.

XI. REFERENCES

1. Lanier, L.L. 2000. Turning on natural killer cells. *J Exp Med.* 191:1259-62.

2. Lanier, L.L. 1998. NK cell receptors. *Annu Rev Immunol.* 16:359-93.

3. Tay, C.H., E. Szomolanyi-Tsuda, and R.M. Welsh. 1998. Control of infections by NK cells. *Curr Top Microbiol Immunol.* 230:193-220.

4. Biron, C.A., H.C. Su, and J.S. Orange. 1996. Function and Regulation of Natural Killer (NK) Cells during Viral Infections: Characterization of Responses in Vivo. *Methods.* 9:379-93.

5. Biron, C.A., K.B. Nguyen, G.C. Pien, L.P. Cousens, and T.P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol.* 17:189-220.

6. Brenner, B.G., C. Gryllis, M. Gornitsky, and M.A. Wainberg. 1993. Changes in natural immunity during the course of HIV-1 infection. *Clin Exp Immunol.* 93:142-8.

7. Bruunsgaard, H., C. Pedersen, P. Skinhoj, and B.K. Pedersen. 1997. Clinical progression of HIV infection: role of NK cells. *Scand J Immunol.* 46:91-5.

8. Lanier, L.L. 1998. Follow the leader: NK cell receptors for classical and nonclassical MHC class I. *Cell.* 92:705-7.

9. Moretta, A., S. Sivori, M. Ponte, M.C. Mingari, and L. Moretta. 1998. Stimulatory receptors in NK and T cells. *Curr Top Microbiol Immunol.* 230:15-23.

10. Lanier, L.L. 1997. Natural killer cells: from no receptors to too many. *Immunity.* 6:371-8.

11. Depatie, C., A. Chalifour, C. Pare, S.H. Lee, S.M. Vidal, and S. Lemieux. 1999. Assessment of Cmv1 candidates by genetic mapping and in vivo antibody depletion of NK cell subsets. *Int Immunol.* 11:1541-51.

12. Andre, P., C. Brunet, S. Guia, H. Gallais, J. Sampol, E. Vivier, and F. Dignat-George. 1999. Differential regulation of killer cell Ig-like receptors and CD94 lectin-like dimers on NK and T lymphocytes from HIV-1-infected individuals. *Eur J Immunol.* 29:1076-85.

13. Lederman, M.M., E. Connick, A. Landay, D.R. Kuritzkes, J. Spritzler, M. St. Clair, B.L. Kotzin, L. Fox, M.H. Chiozzi, J.M. Leonard, F. Rousseau, M. Wade, J.D. Roe, A. Martinez, and H. Kessler. 1998. Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: results of AIDS Clinical Trials Group Protocol 315. *J Infect Dis.* 178:70-9.

14. Angel, J.B., A. Kumar, K. Parato, L.G. Filion, F. Diaz-Mitoma, P. Daftarian, B. Pham, E. Sun, J.M. Leonard, and D.W. Cameron. 1998. Improvement in cell-mediated immune function during potent anti-human immunodeficiency virus therapy with ritonavir plus saquinavir. *J Infect Dis.* 177:898-904.

15. Autran, B., G. Carcelaint, T.S. Li, G. Gorochov, C. Blanc, M. Renaud, M. Durali, D. Mathez, V. Calvez, J. Leibowitch, C. Katlama, and P. Debre. 1999. Restoration of the immune system with anti-retroviral therapy. *Immunol Lett.* 66:207-11.

16. Pontesilli, O., S. Kerkhof-Garde, D.W. Notermans, N.A. Foudraine, M.T. Roos, M.R. Klein, S.A. Danner, J.M. Lange, and F. Miedema. 1999. Functional T cell reconstitution and human immunodeficiency virus-1- specific cell-mediated immunity during highly active antiretroviral therapy. *J Infect Dis.* 180:76-86.

17. Wendland, T., H. Furrer, P.L. Vernazza, K. Frutig, A. Christen, L. Matter, R. Malinverni, and W.J. Pichler. 1999. HAART in HIV-infected patients: restoration of antigen-specific CD4 T- cell responses in vitro is correlated with CD4 memory T-cell reconstitution, whereas improvement in delayed type hypersensitivity is related to a decrease in viraemia. *AIDS.* 13:1857-62.

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

19. Bancroft, G.J., G.R. Shellam, and J.E. Chalmer. 1981. Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. *J Immunol.* 126:988-94.

20. Grundy, J.E., J. Trapman, J.E. Allan, G.R. Shellam, and C.J. Melief. 1982. Evidence for a protective role of interferon in resistance to murine cytomegalovirus and its control by non-H-2-linked genes. *Infect Immun.* 37:143-50.

21. Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med.* 320:1731-5.

22. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature.* 369:31-7.

23. Vales-Gomez, M., H.T. Reyburn, R.A. Erskine, M. Lopez-Botet, and J.L. Strominger. 1999. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *Embo J.* 18:4250-60.

24. Vales-Gomez, M., H. Reyburn, and J. Strominger. 2000. Molecular analyses of the interactions between human NK receptors and their HLA ligands. *Hum Immunol.* 61:28-38.

25. De Maria, A., and L. Moretta. 2000. HLA-class I-specific inhibitory receptors in HIV-1 infection. *Hum Immunol.* 61:74-81.

26. Bakker, A.B., J. Wu, J.H. Phillips, and L.L. Lanier. 2000. NK cell activation: distinct stimulatory pathways counterbalancing inhibitory signals. *Hum Immunol.* 61:18-27.

27. Blery, M., L. Olcese, and E. Vivier. 2000. Early signaling via inhibitory and activating NK receptors. *Hum Immunol.* 61:51-64.

28. Giavedoni, L.D., M.C. Velasquillo, L.M. Parodi, G.B. Hubbard, and V.L. Hodara. 2000. Cytokine expression, natural killer cell activation, and phenotypic changes in lymphoid cells from rhesus macaques during acute infection with pathogenic simian immunodeficiency virus. *J Virol.* 74:1648-57.

29. Skowron, G., B.F. Cole, D. Zheng, G. Accetta, and B. Yen-Lieberman. 1997. gp120-directed antibody-dependent cellular cytotoxicity as a major determinant of the rate of decline in CD4 percentage in HIV-1 disease. *AIDS.* 11:1807-14.

30. Forthal, D.N., G. Landucci, R. Haubrich, B. Keenan, B.D. Kuppermann, J.G. Tilles, and J. Kaplan. 1999. Antibody-dependent cellular cytotoxicity independently predicts survival in severely immunocompromised human immunodeficiency virus-infected patients. *J Infect Dis.* 180:1338-41.

31. Campbell, A.E., and J.S. Slater. 1994. Down-regulation of major histocompatibility complex class I synthesis by murine cytomegalovirus early gene expression. *J Virol.* 68:1805-11.

32. del Val, M., H. Hengel, H. Hacker, U. Hartlaub, T. Ruppert, P. Lucin, and U.H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J Exp Med.* 176:729-38.

33. Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature.* 375:411-5.

34. Cohen, G.B., R.T. Gandhi, D.M. Davis, O. Mandelboim, B.K. Chen, J.L. Strominger, and D. Baltimore. 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity.* 10:661-71.

35. Jones, T.R., L.K. Hanson, L. Sun, J.S. Slater, R.M. Stenberg, and A.E. Campbell. 1995. Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J Virol.* 69:4830-41.

36. Wang, L.L., D.T. Chu, A.O. Dokun, and W.M. Yokoyama. 2000. Inducible expression of the gp49B inhibitory receptor on NK cells. *J Immunol.* 164:5215-20.

37. Huard, B., and K. Fruh. 2000. A role for MHC class I down-regulation in NK cell lysis of herpes virus- infected cells. *Eur J Immunol.* 30:509-15.

38. Bezouska, K., C.T. Yuen, J. O'Brien, R.A. Childs, W. Chai, A.M. Lawson, K. Drbal, A. Fiserova, M. Pospisil, and T. Feizi. 1994. Oligosaccharide ligands for NKR-P1 protein activate NK cells and cytotoxicity. *Nature.* 372:150-7.

39. Galiani, M.D., E. Aguado, R. Tarazona, P. Romero, I. Molina, M. Santamaria, R. Solana, and J. Pena. 1999. Expression of killer inhibitory receptors on cytotoxic cells from HIV-1- infected individuals. *Clin Exp Immunol.* 115:472-6.

40. Poccia, F., B. Cipriani, S. Vendetti, V. Colizzi, Y. Poquet, L. Battistini, M. Lopez-Botet, J.J. Fournie, and M.L. Gougeon. 1997. CD94/NKG2 inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive V gamma 9V delta 2 T lymphocytes. *J Immunol.* 159:6009-17.

41. Ryan, J.C., and W.E. Seaman. 1997. Divergent functions of lectin-like receptors on NK cells. *Immunol Rev.* 155:79-89.

42. Colonna, M., G. Borsellino, M. Falco, G.B. Ferrara, and J.L. Strominger. 1993. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A.* 90:12000-4.

43. D'Andrea, A., C. Chang, K. Franz-Bacon, T. McClanahan, J.H. Phillips, and L.L. Lanier. 1995. Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. *J Immunol.* 155:2306-10.

44. Binstadt, B.A., K.M. Brumbaugh, C.J. Dick, A.M. Scharenberg, B.L. Williams, M. Colonna, L.L. Lanier, J.P. Kinet, R.T. Abraham, and P.J. Leibson. 1996. Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation. *Immunity.* 5:629-38.

45. Binstadt, B.A., K.M. Brumbaugh, and P.J. Leibson. 1997. Signal transduction by human NK cell MHC-recognizing receptors. *Immunol Rev.* 155:197-203.

46. Lopez-Botet, M., T. Bellon, M. Llano, F. Navarro, P. Garcia, and M. de Miguel. 2000. Paired inhibitory and triggering NK cell receptors for HLA class I molecules. *Hum Immunol.* 61:7-17.

47. Lanier, L.L., B.C. Corliss, J. Wu, C. Leong, and J.H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature.* 391:703-7.

48. Vely, F., L. Olcese, M. Blery, and E. Vivier. 1996. Function of killer cell inhibitory receptors for MHC class I molecules. *Immunol Lett.* 54:145-50.

49. Yokoyama, W.M., and W.E. Seaman. 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annu Rev Immunol.* 11:613-35.

50. Peruzzi, M., N. Wagtmann, and E.O. Long. 1996. A p70 killer cell inhibitory receptor specific for several HLA-B allotypes discriminates among peptides bound to HLA-B*2705. *J Exp Med.* 184:1585-90.

51. Colonna, M., and J. Samaridis. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science.* 268:405-8.

52. Long, E.O., M. Colonna, and L.L. Lanier. 1996. Inhibitory MHC class I receptors on NK and T cells: a standard nomenclature. *Immunol Today.* 17:100.

53. Wagtmann, N., R. Biassoni, C. Cantoni, S. Verdiani, M.S. Malnati, M. Vitale, C. Bottino, L. Moretta, A. Moretta, and E.O. Long. 1995. Molecular clones of the p58 NK cell receptor reveal immunoglobulin- related molecules with diversity in both the extra- and intracellular domains. *Immunity.* 2:439-49.

54. Lanier, L.L., B. Corliss, J. Wu, and J.H. Phillips. 1998. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity*. 8:693-701.

55. Lanier, L.L., C. Chang, and J.H. Phillips. 1994. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *J Immunol*. 153:2417-28.

56. Adamkiewicz, T.V., C. McSherry, F.H. Bach, and J.P. Houchins. 1994. Natural killer lectin-like receptors have divergent carboxy-termini, distinct from C-type lectins *Immunogenetics*. 39:218.

57. Poggi, A., P. Costa, L. Morelli, C. Cantoni, N. Pella, F. Spada, R. Biassoni, L. Nanni, V. Revello, E. Tomasello, M.C. Mingari, A. Moretta, and L. Moretta. 1996. Expression of human NKRP1A by CD34+ immature thymocytes: NKRP1A- mediated regulation of proliferation and cytolytic activity. *Eur J Immunol*. 26:1266-72.

58. Azzoni, L., O. Zatsepina, B. Abebe, I.M. Bennett, P. Kanakaraj, and B. Perussia. 1998. Differential transcriptional regulation of CD161 and a novel gene, 197/15a, by IL-2, IL-15, and IL-12 in NK and T cells. *J Immunol*. 161:3493-500.

59. Houchins, J.P., T. Yabe, C. McSherry, and F.H. Bach. 1991. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J Exp Med*. 173:1017-20.

60. Yabe, T., C. McSherry, F.H. Bach, P. Fisch, R.P. Schall, P.M. Sondel, and J.P. Houchins. 1993. A multigene family on human chromosome 12 encodes natural killer-cell lectins. *Immunogenetics*. 37:455-60.

61. Brooks, A.G., P.E. Posch, C.J. Scorzelli, F. Borrego, and J.E. Coligan. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J Exp Med.* 185:795-800.

62. Carretero, M., C. Cantoni, T. Bellon, C. Bottino, R. Biassoni, A. Rodriguez, J.J. Perez-Villar, L. Moretta, A. Moretta, and M. Lopez-Botet. 1997. The CD94 and NKG2-A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. *Eur J Immunol.* 27:563-7.

63. Lazetic, S., C. Chang, J.P. Houchins, L.L. Lanier, and J.H. Phillips. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J Immunol.* 157:4741-5.

64. Dohring, C., D. Scheidegger, J. Samaridis, M. Cella, and M. Colonna. 1996. A human killer inhibitory receptor specific for HLA-A1,2. *J Immunol.* 156:3098-101.

65. Dohring, C., and M. Colonna. 1996. Human natural killer cell inhibitory receptors bind to HLA class I molecules. *Eur J Immunol.* 26:365-9.

66. Litwin, V., J. Gumperz, P. Parham, J.H. Phillips, and L.L. Lanier. 1994. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J Exp Med.* 180:537-43.

67. Moretta, A., M. Vitale, C. Bottino, A.M. Orengo, L. Morelli, R. Augugliaro, M. Barbaresi, E. Ciccone, and L. Moretta. 1993. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med.* 178:597-604.

68. Gumperz, J.E., V. Litwin, J.H. Phillips, L.L. Lanier, and P. Parham. 1995. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med.* 181:1133-44.

69. Fan, Q.R., D.N. Garboczi, C.C. Winter, N. Wagtmann, E.O. Long, and D.C. Wiley. 1996. Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. *Proc Natl Acad Sci U S A.* 93:7178-83.

70. Malnati, M.S., M. Peruzzi, K.C. Parker, W.E. Biddison, E. Ciccone, A. Moretta, and E.O. Long. 1995. Peptide specificity in the recognition of MHC class I by natural killer cell clones. *Science.* 267:1016-8.

71. Peruzzi, M., K.C. Parker, E.O. Long, and M.S. Malnati. 1996. Peptide sequence requirements for the recognition of HLA-B*2705 by specific natural killer cells. *J Immunol.* 157:3350-6.

72. Braud, V.M., D.S. Allan, C.A. O'Callaghan, K. Soderstrom, A. D'Andrea, G.S. Ogg, S. Lazetic, N.T. Young, J.I. Bell, J.H. Phillips, L.L. Lanier, and A.J. McMichael. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature.* 391:795-9.

73. Thomas, M.L. 1995. Of ITAMs and ITIMs: turning on and off the B cell antigen receptor. *J Exp Med.* 181:1953-6.

74. Leibson, P.J. 1997. Signal transduction during natural killer cell activation: inside the mind of a killer. *Immunity.* 6:655-61.

75. Mandelboim, O., D.M. Davis, H.T. Reyburn, M. Vales-Gomez, E.G. Sheu, L. Pazmany, and J.L. Strominger. 1996. Enhancement of class II-restricted T cell

responses by costimulatory NK receptors for class I MHC proteins. *Science.* 274:2097-100.

76. Campbell, K.S., and R. Giorda. 1997. The cytoplasmic domain of rat NKR-P1 receptor interacts with the N- terminal domain of p56(lck) via cysteine residues. *Eur J Immunol.* 27:72-7.

77. Frankel, A.D., and J.A. Young. 1998. HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem.* 67:1-25.

78. Maddon, P.J., A.G. Dalgleish, J.S. McDougal, P.R. Clapham, R.A. Weiss, and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell.* 47:333-48.

79. Moore, J.P., A. Trkola, and T. Dragic. 1997. Co-receptors for HIV-1 entry. *Curr Opin Immunol.* 9:551-62.

80. Virelizier, J.L. 1999. Blocking HIV co-receptors by chemokines. *Dev Biol Stand.* 97:105-9.

81. Trono, D. 1992. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. *J Virol.* 66:4893-900.

82. Heinzinger, N.K., M.I. Bukinsky, S.A. Haggerty, A.M. Ragland, V. Kewalramani, M.A. Lee, H.E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman. 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci U S A.* 91:7311-5.

83. Sherman, M.P., C.M. de Noronha, D. Pearce, and W.C. Greene. 2000. Human immunodeficiency virus type 1 vpr contains two leucine-rich helices that

mediate glucocorticoid receptor coactivation independently of its effects on G(2) cell cycle arrest. *J Virol.* 74:8159-65.

84. Yao, S., A.M. Torres, A.A. Azad, I.G. Macreadie, and R.S. Norton. 1998. Solution structure of peptides from HIV-1 Vpr protein that cause membrane permeabilization and growth arrest. *J Pept Sci.* 4:426-35.

85. Jenkins, Y., M. McEntee, K. Weis, and W.C. Greene. 1998. Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways. *J Cell Biol.* 143:875-85.

86. Zhou, W., and M.D. Resh. 1996. Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. *J Virol.* 70:8540-8.

87. Katz, R.A., and A.M. Skalka. 1994. The retroviral enzymes. *Annu Rev Biochem.* 63:133-73.

88. Cooper, D.A., and S. Emery. 1999. Latent reservoirs of HIV infection: flushing with IL-2?. *Nat Med.* 5:611-2.

89. Stein, B., H.J. Rahmsdorf, A. Steffen, M. Litfin, and P. Herrlich. 1989. UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-fos, and metallothionein. *Mol Cell Biol.* 9:5169-81.

90. Stein, B., M. Kramer, H.J. Rahmsdorf, H. Ponta, and P. Herrlich. 1989. UV-induced transcription from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat and UV-induced secretion of an extracellular factor that induces HIV-1 transcription in nonirradiated cells. *J Virol.* 63:4540-4.

91. Zhang, J., N. Tamilarasu, S. Hwang, M.E. Garber, I. Huq, K.A. Jones, and T.M. Rana. 2000. HIV-1 TAR RNA enhances the interaction between Tat and cyclin T1. *J Biol Chem.*

92. Yamamoto, R., M. Katahira, S. Nishikawa, T. Baba, K. Taira, and P.K. Kumar. 2000. A novel RNA motif that binds efficiently and specifically to the Ttat protein of HIV and inhibits the trans-activation by Tat of transcription in vitro and in vivo. *Genes Cells.* 5:371-88.

93. Zack, J.A., A.J. Cann, J.P. Lugo, and I.S. Chen. 1988. HIV-1 production from infected peripheral blood T cells after HTLV-I induced mitogenic stimulation. *Science.* 240:1026-9.

94. Tang, H., K.L. Kuhen, and F. Wong-Staal. 1999. Lentivirus replication and regulation. *Annu Rev Genet.* 33:133-70.

95. Weiss, R.A. 2000. Getting to know HIV. *Trop Med Int Health.* 5:A10-5.

96. Schwartz, S., B.K. Felber, D.M. Benko, E.M. Fenyo, and G.N. Pavlakis. 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J Virol.* 64:2519-29.

97. Ratner, L. 1992. Glucosidase inhibitors for treatment of HIV-1 infection. *AIDS Res Hum Retroviruses.* 8:165-73.

98. Wills, J.W., and R.C. Craven. 1991. Form, function, and use of retroviral gag proteins. *AIDS.* 5:639-54.

99. Lemp, G.F., S.F. Payne, G.W. Rutherford, N.A. Hessol, W. Winkelstein, Jr., J.A. Wiley, A.R. Moss, R.E. Chaisson, R.T. Chen, D.W. Feigal, Jr., and et al. 1990. Projections of AIDS morbidity and mortality in San Francisco [see comments]. *Jama.* 263:1497-501.

100. Wei, X., S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, B.H. Hahn, and et al. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature*. 373:117-22.

101. Perelson, A.S., P. Essunger, Y. Cao, M. Vesanen, A. Hurley, K. Saksela, M. Markowitz, and D.D. Ho. 1997. Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature*. 387:188-91.

102. Perelson, A.S., P. Essunger, and D.D. Ho. 1997. Dynamics of HIV-1 and CD4+ lymphocytes in vivo. *AIDS*. 11:S17-24.

103. Ascher, M.S., and H.W. Sheppard. 1988. AIDS as immune system activation: a model for pathogenesis. *Clin Exp Immunol*. 73:165-7.

104. Ascher, M.S., and H.W. Sheppard. 1990. AIDS as immune system activation. II. The panergic imnesia hypothesis. *J Acquir Immune Defic Syndr*. 3:177-91.

105. Fauci, A.S. 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. *Science*. 239:617-22.

106. Roberts, J.D., K. Bebenek, and T.A. Kunkel. 1988. The accuracy of reverse transcriptase from HIV-1. *Science*. 242:1171-3.

107. Ajito, K., S. Atsumi, D. Ikeda, S. Kondo, T. Takeuchi, and K. Umezawa. 1989. Inhibition of human immunodeficiency virus-associated reverse transcriptase by 14-O-acyladriamycins. *J Antibiot (Tokyo)*. 42:611-9.

108. Walker, B.D., S. Chakrabarti, B. Moss, T.J. Paradis, T. Flynn, A.G. Durno, R.S. Blumberg, J.C. Kaplan, M.S. Hirsch, and R.T. Schooley. 1987. HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature*. 328:345-8.

109. Pantaleo, G., H. Soudeyns, J.F. Demarest, M. Vaccarezza, C. Graziosi, S. Paolucci, M. Daucher, O.J. Cohen, F. Denis, W.E. Biddison, R.P. Sekaly, and A.S.

Fauci. 1997. Evidence for rapid disappearance of initially expanded HIV-specific CD8+ T cell clones during primary HIV infection. *Proc Natl Acad Sci U S A*. 94:9848-53.

110. Taylor, J.M., J.L. Fahey, R. Detels, and J.V. Giorgi. 1989. CD4 percentage, CD4 number, and CD4:CD8 ratio in HIV infection: which to choose and how to use. *J Acquir Immune Defic Syndr*. 2:114-24.

111. Stein, D.S., J.A. Korvick, and S.H. Vermund. 1992. CD4+ lymphocyte cell enumeration for prediction of clinical course of human immunodeficiency virus disease: a review. *J Infect Dis*. 165:352-63.

112. Fahey, J.L., J.M. Taylor, R. Detels, B. Hofmann, R. Melmed, P. Nishanian, and J.V. Giorgi. 1990. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N Engl J Med*. 322:166-72.

113. Pantaleo, G., and A.S. Fauci. 1996. Immunopathogenesis of HIV infection. *Annu Rev Microbiol*. 50:825-54.

114. Stevenson, M., C. Meier, A.M. Mann, N. Chapman, and A. Wasiak. 1988. Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4+ cells: mechanism for persistence in AIDS. *Cell*. 53:483-96.

115. Willey, R.L., F. Maldarelli, M.A. Martin, and K. Strebel. 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *J Virol*. 66:7193-200.

116. Garcia, J.V., and A.D. Miller. 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature*. 350:508-11.

117. Schnittman, S.M., H.C. Lane, J. Greenhouse, J.S. Justement, M. Baseler, and A.S. Fauci. 1990. Preferential infection of CD4+ memory T cells by human

immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. *Proc Natl Acad Sci U S A.* 87:6058-62.

118. Chirmule, N., T.W. McCloskey, R. Hu, V.S. Kalyanaraman, and S. Pahwa. 1995. HIV gp120 inhibits T cell activation by interfering with expression of costimulatory molecules CD40 ligand and CD80 (B71). *J Immunol.* 155:917-24.

119. Liegler, T.J., and D.P. Stites. 1994. HIV-1 gp120 and anti-gp120 induce reversible unresponsiveness in peripheral CD4 T lymphocytes. *J Acquir Immune Defic Syndr.* 7:340-8.

120. Schols, D., and E. De Clercq. 1996. Human immunodeficiency virus type 1 gp120 induces anergy in human peripheral blood lymphocytes by inducing interleukin-10 production. *J Virol.* 70:4953-60.

121. Lane, H.C. 1995. The generation of CD4 T lymphocytes in patients with HIV infection. *J Biol Regul Homeost Agents.* 9:107-9.

122. Ciurli, C., R.P. Sekaly, and H. Soudeyns. 1996. Study of the T cell receptor repertoire in viral immunodeficiency disease. *Springer Semin Immunopathol.* 17:319-32.

123. Carbonari, M., M. Cibati, A.M. Pesce, L. Dell'Anna, G. D'Offizi, A. Angelici, S. Uccini, and M. Fiorilli. 1996. Comparison of the Vbeta repertoire in peripheral blood and in lymph nodes of HIV-infected subjects reveals skewed usage predominantly in CD8+ T cells. *Clin Immunol Immunopathol.* 81:200-9.

124. Boldt-Houle, D.M., B.D. Jamieson, G.M. Aldrovandi, C.R. Rinaldo, Jr., G.D. Ehrlich, and J.A. Zack. 1997. Loss of T cell receptor Vbeta repertoires in HIV type 1-infected SCID- hu mice. *AIDS Res Hum Retroviruses.* 13:125-34.

125. Connors, M., J.A. Kovacs, S. Krevat, J.C. Gea-Banacloche, M.C. Sneller, M. Flanigan, J.A. Metcalf, R.E. Walker, J. Falloon, M. Baseler, I. Feuerstein, H. Masur, and H.C. Lane. 1997. HIV infection induces changes in CD4+ T-cell phenotype and depletions within the CD4+ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. *Nat Med.* 3:533-40.

126. Ioachim, H.L., C.W. Lerner, and M.L. Tapper. 1983. The lymphoid lesions associated with the acquired immunodeficiency syndrome. *Am J Surg Pathol.* 7:543-53.

127. Janossy, G., A.J. Pinching, M. Bofill, J. Weber, J.E. McLaughlin, M. Ornstein, K. Ivory, J.R. Harris, M. Favrot, and D.C. Macdonald-Burns. 1985. An immunohistological approach to persistent lymphadenopathy and its relevance to AIDS. *Clin Exp Immunol.* 59:257-66.

128. Masuda, A., G.F. Burton, B.A. Fuchs, A.K. Szakal, and J.G. Tew. 1993. Destruction of follicular dendritic cells in murine acquired immunodeficiency syndrome (MAIDS). *Adv Exp Med Biol.* 329:411-6.

129. Tew, J.G., G.F. Burton, L.I. Kupp, and A. Szakal. 1993. Follicular dendritic cells in germinal center reactions. *Adv Exp Med Biol.* 329:461-5.

130. Zannini, C., A. Favre, E. Ciccone, A. Plebani, M. Duse, C.E. Grossi, and C. Pesce. 2000. A video densitometric analysis of viral burden and follicular dendritic cell damage in lymph nodes in the latency phase of HIV infection. *Cytometry.* 40:209-13.

131. Bonavida, B., J. Katz, and M. Gottlieb. 1986. Mechanism of defective NK cell activity in patients with acquired immunodeficiency syndrome (AIDS) and

AIDS-related complex. I. Defective trigger on NK cells for NKCF production by target cells, and partial restoration by IL 2. *J Immunol.* 137:1157-63.

132. Plaeger-Marshall, S., C.A. Spina, J.V. Giorgi, R. Mitsuyasu, P. Wolfe, M. Gottlieb, and G. Beall. 1987. Alterations in cytotoxic and phenotypic subsets of natural killer cells in acquired immune deficiency syndrome (AIDS). *J Clin Immunol.* 7:16-23.

133. Voth, R., S. Rossol, E. Graff, H.P. Laubenstein, H.C. Schroder, W.E. Muller, K.H. Meyer zum Buschenfelde, and G. Hess. 1988. Natural killer cell activity as a prognostic parameter in the progression to AIDS. *J Infect Dis.* 157:851-2.

134. Ortona, L., E. Tamburrini, M. Tumbarello, G. Ventura, and R. Cauda. 1988. [Natural killer (NK) activity in patients with HIV infection]. *Boll Ist Sieroter Milan.* 67:135-41.

135. Valentin, A., D. Patenaude, and G.N. Pavlakis. 2000. Infection of Natural Killer Cells by R5 and X4 HIV-1 Strains. *7th Conference on Retroviruses and Opportunistic Infections.*

136. Ullum, H., A. Cozzi Lepri, H. Aladdin, T. Katzenstein, J. Victor, A.N. Phillips, J. Gerstoft, P. Skinhoj, and B. Klarlund Pedersen. 1999. Natural immunity and HIV disease progression. *AIDS.* 13:557-63.

137. Brenner, B.G., A. Dascal, R.G. Margolese, and M.A. Wainberg. 1989. Natural killer cell function in patients with acquired immunodeficiency syndrome and related diseases. *J Leukoc Biol.* 46:75-83.

138. Ullum, H., P.C. Gotzsche, J. Victor, E. Dickmeiss, P. Skinhoj, and B.K. Pedersen. 1995. Defective natural immunity: an early manifestation of human immunodeficiency virus infection. *J Exp Med.* 182:789-99.

139. Villanueva, J.L., J. Caballero, M. Del Nozal, B. Sanchez, P. Viciana, A. Alarcon, and J. Pena. 2000. Peripheral blood natural killer cell reconstitution after highly active antiretroviral therapy. *AIDS*. 14:473-4.

140. Autran, B., G. Carcelain, T.S. Li, C. Blanc, D. Mathez, R. Tubiana, C. Katlama, P. Debre, and J. Leibowitch. 1997. Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease. *Science*. 277:112-6.

141. Bisset, L.R., R.W. Cone, W. Huber, M. Battegay, P.L. Vernazza, R. Weber, P.J. Grob, and M. Opravil. 1998. Highly active antiretroviral therapy during early HIV infection reverses T-cell activation and maturation abnormalities. Swiss HIV Cohort Study. *AIDS*. 12:2115-23.

142. Sondergaard, S.R., H. Aladdin, H. Ullum, J. Gerstoft, P. Skinhoj, and B.K. Pedersen. 1999. Immune function and phenotype before and after highly active antiretroviral therapy. *J Acquir Immune Defic Syndr*. 21:376-83.

143. Eron, J.J., S.L. Benoit, J. Jemsek, R.D. MacArthur, J. Santana, J.B. Quinn, D.R. Kuritzkes, M.A. Fallon, and M. Rubin. 1995. Treatment with lamivudine, zidovudine, or both in HIV-positive patients with 200 to 500 CD4+ cells per cubic millimeter. North American HIV Working Party. *N Engl J Med*. 333:1662-9.

144. Jonckheere, H., J. Anne, and E. De Clercq. 2000. The HIV-1 reverse transcription (RT) process as target for RT inhibitors. *Med Res Rev*. 20:129-54.

145. Temesgen, Z., and A.J. Wright. 1999. Antiretrovirals. *Mayo Clin Proc*. 74:1284-301.

146. Welles, S.L., J.B. Jackson, B. Yen-Lieberman, L. Demeter, A.J. Japour, L.M. Smeaton, V.A. Johnson, D.R. Kuritzkes, R.T. D'Aquila, P.A. Reichelderfer, D.D.

Richman, R. Reichman, M. Fischl, R. Dolin, R.W. Coombs, J.O. Kahn, C. McLaren, J. Todd, S. Kwok, and C.S. Crumpacker. 1996. Prognostic value of plasma human immunodeficiency virus type 1 (HIV-1) RNA levels in patients with advanced HIV-1 disease and with little or no prior zidovudine therapy. AIDS Clinical Trials Group Protocol 116A/116B/117 Team. *J Infect Dis.* 174:696-703.

147. Schmidt-Westhausen, A.M., F. Priepke, F.J. Bergmann, and P.A. Reichart. 2000. Decline in the rate of oral opportunistic infections following introduction of highly active antiretroviral therapy. *J Oral Pathol Med.* 29:336-41.

148. Sepkowitz, K.A. 1998. Effect of HAART on natural history of AIDS-related opportunistic disorders. *Lancet.* 351:228-30.

149. Chang, L., G.A. Gusewitch, D.B. Chritton, J.C. Folz, L.K. Lebeck, and S.L. Nehlsen-Cannarella. 1993. Rapid flow cytometric assay for the assessment of natural killer cell activity. *J Immunol Methods.* 166:45-54.

150. Pross, H.F., and J.A. Maroun. 1984. The standardization of NK cell assays for use in studies of biological response modifiers. *J Immunol Methods.* 68:235-49.

151. Orange, J.S., and C.A. Biron. 1996. Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J Immunol.* 156:4746-56.

152. Janeway, C.A., and P. Travers. 1994. Immunobiology: The immune system in health and disease. . M. Robertson, editor. Garland Publishing. 9:21-9:23.

153. Threlkeld, S.C., P.A. Wentworth, S.A. Kalams, B.M. Wilkes, D.J. Ruhl, E. Keogh, J. Sidney, S. Southwood, B.D. Walker, and A. Sette. 1997. Degenerate and

promiscuous recognition by CTL of peptides presented by the MHC class I A3-like superfamily: implications for vaccine development. *J Immunol.* 159:1648-57.

154. Gorochov, G., A.U. Neumann, A. Kereveur, C. Parizot, T. Li, C. Katlama, M. Karmochkine, G. Raguin, B. Autran, and P. Debre. 1998. Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. *Nat Med.* 4:215-21.

155. Arno, A., L. Ruiz, M. Juan, M.K. Zayat, T. Puig, M. Balague, J. Romeu, R. Pujol, W.A. O'Brien, and B. Clotet. 1998. Impact on the immune system of undetectable plasma HIV-1 RNA for more than 2 years. *AIDS.* 12:697-704.

156. Zemmour, J., A.M. Little, D.J. Schendel, and P. Parham. 1992. The HLA-A,B "negative" mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. *J Immunol.* 148:1941-8.

157. Lawlor, D.A., J. Zemmour, P.D. Ennis, and P. Parham. 1990. Evolution of class-I MHC genes and proteins: from natural selection to thymic selection. *Annu Rev Immunol.* 8:23-63.

158. Braud, V.M., D.S. Allan, D. Wilson, and A.J. McMichael. 1998. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr Biol.* 8:1-10.

159. Arase, N., H. Arase, S.Y. Park, H. Ohno, C. Ra, and T. Saito. 1997. Association with FcRgamma is essential for activation signal through NKR-P1 (CD161) in natural killer (NK) cells and NK1.1+ T cells. *J Exp Med.* 186:1957-63.

160. Ryan, J.C., E.C. Niemi, R.D. Goldfien, J.C. Hiserodt, and W.E. Seaman. 1991. NKR-P1, an activating molecule on rat natural killer cells, stimulates phosphoinositide turnover and a rise in intracellular calcium. *J Immunol.* 147:3244-50.

161. Ryan, J.C., E.C. Niemi, M.C. Nakamura, and W.E. Seaman. 1995. NKR-P1A is a target-specific receptor that activates natural killer cell cytotoxicity. *J Exp Med.* 181:1911-5.

162. Farrell, H.E., M.A. Degli-Esposti, and N.J. Davis-Poynter. 1999. Cytomegalovirus evasion of natural killer cell responses. *Immunol Rev.* 168:187-97.

163. Seo, N., Y. Tokura, S. Ishihara, Y. Takeoka, S. Tagawa, and M. Takigawa. 2000. Disordered expression of inhibitory receptors on the NK1-type natural killer (NK) leukaemic cells from patients with hypersensitivity to mosquito bites. *Clin Exp Immunol.* 120:413-9.

164. Mizuki, M., A. Eklund, and J. Grunewald. 2000. Altered expression of natural killer cell inhibitory receptors (KIRs) on T cells in bronchoalveolar lavage fluid and peripheral blood of sarcoidosis patients. *Sarcoidosis Vasc Diffuse Lung Dis.* 17:54-9.