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

BY

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THESIS

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

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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 (LU₁₀) and decreased to 28.18 LU₁₀ and 18.42 LU₁₀ 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

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>ART</td>
<td>Antiretroviral Therapy</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
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<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<tr>
<td>DiO</td>
<td>3,3′-Dioctadecyloxycarbocyanine perchlorate</td>
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<tr>
<td>DMSO</td>
<td>DimethylSulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>FCS</td>
<td>Fetal Bovine Serum</td>
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<td>FDC</td>
<td>Follicular Dendritic Cells</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>HLA</td>
<td>Human Leucocyte Antigen</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IQR</td>
<td>Interquartile Range</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor Tyrosine-based Inhibitory Motifs</td>
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<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based Activation Motifs</td>
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<tr>
<td>KIR</td>
<td>Killer Immunoglobulin Type Receptor</td>
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<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>LU&lt;sub&gt;10&lt;/sub&gt;</td>
<td>Lytic Units at 10% Lysis</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
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<tr>
<td>NASBA</td>
<td>Nucleic Acid Based Amplification System</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
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<tr>
<td>NKC</td>
<td>Natural Killer Complex</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev Response Element</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>μl</td>
<td>Microlitres</td>
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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 immunodeficiency 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 cytolysis 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).
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 stoichiometry (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.
**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 identical 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 transmembrane 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 Immunodeficiency 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 \textit{vif}, \textit{vpr}, \textit{vpu} and \textit{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 expedite 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

Promotes infectivity

Pathogenicity, CD4 down-regulation, kinase association

Viral envelope

nef

5' LTR

3' LTR

TAR

Binding site for host transcriptional factors

Protease, integrase, ribonuclease, Reverse transcriptase

Promotes infectivity

Structural gene expression regulatory element

Transcriptional activator
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 autoimmune 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 particularly 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 seropositive 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 seropositive 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 propagate 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 levels 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 Nelfinavir, 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 seropositive 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 $2.70 \log_{10}$ 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 $3\times10^6$ 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 cm² tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) with RPMI/10 medium at 37°C in a humidified 5% CO₂ incubator. Cells were monitored daily and maintained at a density of no more than 3x10⁵ 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 1x10⁶ cells/ml in RPMI/10. The yield of viable PBMC after cryopreservation 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% CO₂, after which time they were used as effectors in cytotoxicity 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'-Dioctadecyloxycarbocyanine 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% CO₂ 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% CO₂ 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 (LU₁₀) were calculated as described by Pross et al. (150).
E. Immunophenotyping

Whole blood samples were drawn into sodium heparin vacutainer tubes. One hundred microlitres 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.

<table>
<thead>
<tr>
<th>HIV-1 RNA conc. (log₁₀ copies/ml)ᵃ)</th>
<th>Baseline</th>
<th>Week 8</th>
<th>Week 24</th>
<th>Week 48</th>
<th>HIV seronegative donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.84 ±0.73</td>
<td>2.76 ±0.55</td>
<td>2.70 ±0.00</td>
<td>2.70 ±0.00</td>
<td></td>
</tr>
</tbody>
</table>

Number of individuals with < 2.70 log₁₀0/10 copies/ml of HIV-1 RNA

<table>
<thead>
<tr>
<th>Absolute CD4+ T cell count (cells/μL)ᵃ)</th>
<th>Baseline</th>
<th>Week 8</th>
<th>Week 24</th>
<th>Week 48</th>
<th>HIV seronegative donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>552 ±330</td>
<td>557 ±293</td>
<td>594 ±254</td>
<td>661 ±215</td>
<td></td>
</tr>
</tbody>
</table>

NK cell percentageᵇ) 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 log₁₀ copies/ml of HIV-1 RNA were assigned a value of 2.70 for the purpose of calculations.

ᵇ) Median, IQR
\textbf{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 \textit{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.
Figure 4. Representative contour plot for determination of NK cytolytic 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.
Figure 5. NK cytolytic activity expressed in lytic units at 10% lysis (LU₁₀) for ten ART naïve patients at baseline, weeks 8, 24, and 48 of therapy. The median LU₁₀ at each time point is represented by the solid bar (—) and the median LU₁₀ 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/μL, 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/μL. 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

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group size</td>
<td>6</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>CD4+ T cell count</td>
<td></td>
<td>525 ±171</td>
<td>207 ±161</td>
</tr>
</tbody>
</table>
  (cells/μL) <sup>a)</sup> |         |                      |         |
| Baseline CD4+ T cell   |         | 250 ±164             |         |
  count (cells/μL) <sup>a,b)</sup> |         |                      |         |
| HIV-1 RNA conc.        |         | 1.70 ±0.00           | 4.90 ±0.36|
  (log<sub>10</sub> copies/ml) <sup>a)</sup> |         |                      |         |
| Baseline HIV-1 RNA     |         | 4.75 ±0.55           |         |
  conc. (log<sub>10</sub> copies/ml) <sup>a,b)</sup> |         |                      |         |

Group A - HIV seronegative  
Group B - HIV seropositive, viral load <50 log<sub>10</sub> copies/ml for greater than one year  
Group C - HIV seropositive, untreated

<sup>a)</sup> Mean ±SD  
<sup>b)</sup> Baseline values represent values prior to the initiation of ART
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 costimulatory 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 cytotoxicity observed in this cohort. This up-regulation of CD161 during uncontrolled viral replication may be an immediate response by which cytolytic 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


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