LOOKS THAT KILL:
ACCESSORY RECEPTOR PHENOTYPES AND THE CYTOLYTIC ACTIVITIES OF CYTOTOXIC T-LYMPHOCYTES AND NATURAL KILLER CELLS

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Looks That Kill: Accessory Receptor Phenotypes and the Cytolytic Activities of Cytotoxic T-Lymphocytes and Natural Killer Cells

by

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A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

Immunology and Infectious Diseases/Faculty of Medicine

Memorial University of Newfoundland

May 2010

St. John's Newfoundland

Abstract

Natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) express activating and inhibitory accessory receptors specific for class I human leukocyte antigens (HLA-I) or stress induced antigens. Signals from the ligation of these receptors are integrated to modulate T-cell mediated cytolysis and to determine NK cell cytolytic activity. Signals generated from the ligation of inhibitory receptors also mediate several other functions, such as reducing apoptosis and activation induced cell death. In mouse models these inhibitory receptors 'license' NK cells. NK cells expressing an inhibitory receptor, from mice expressing the ligand, have the ability to mediate both general and antibody-dependent cellular cytotoxicity (ADCC). NK 'licensing' also appears to account for human NK cell activity. Data from two studies have demonstrated a role for licensing of general NK cell mediated cytolysis, but data regarding ADCC are conflicting. As activating and inhibitory receptors are of much importance to both NK and T-cells, we investigated the potential expression and/or involvement of these receptors in the HLA-I unrestricted cytolysis mediated against $CD4^+$ T-lymphocytes by a subset of $CD8^+$ CTL in HIV infection. The TCR-dependent and HLA-I-independent CTL demonstrated a phenotype that matches generalized changes on $CD8^+$ T-lymphocytes in progressive HIV infection. The CTL that killed activated uninfected $CD4^+$ T-lymphocytes lacked expression of the CD56 marker and the inhibitory NKG2A receptor. We also investigated the role of NK cell 'licensing' for ADCC in humans. The potency of NK cells expressing the inhibitory KIR3DL1 receptor was investigated in samples taken from individuals expressing and not expressing the HLA-BW4 ligand. Our results suggest NK cell licensing is involved in ADCC.

Acknowledgments

I thank my supervisor, Dr Michael Grant, for giving me the opportunity to work in his lab, and for helping me with the design and execution of these projects.

I thank the members of my supervisory committee, Dr Kensuke Hirasawa and Dr Thomas Michalak, for their time and effort.

I thank the members of Dr Grant's laboratory, Maureen Gallant, Katrin Zipperlen, Julia Pohling, Natasha Hollett and Stephen Penney, for all their help, and for making the lab a fun place to work.

I thank the remaining students and faculty of the Immunology and Infectious Disease group at Memorial University. Aside from teaching and discussing academic issues, you have thought me many valuable life lessons.

I thank Georgia Skardasi, for providing me with constant motivation and support.

Lastly, I thank and dedicate this thesis to my parents for their love and support.

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List of Abbreviations

ACD – Acid Citrate Dextrose

ADCC – Antibody Dependent Cellular Cytotoxicity

AICD – Activation Induced Cell Death

AIDS – Acquired Immune Deficiency Syndrome

APC – Allophycocyanin

BCR – B-Cell Receptor

BLCL - B Lymphoblastoid Cell Line

CFSE – Carboxyfluorescein Succinimidyl Ester

CMV – Cytomegalovirus

CTL – Cytotoxic T-Lymphocyte

DC – Dendritic Cell

EBV – Epstein Barr Virus

EDTA – Ethylene Diamine Tetra-Acetic Acid

FACS – Fluorescence Activated Cell Sorting

FCS – Fetal Calf Serum

FITC – Fluorescein Isothiocyanate

GALT – Gut Associate Lymphoid Tissue

gp160 – Glycoprotein 160

HAART – Highly Active Anti-Retroviral Therapies

HCV – Hepatitis C Virus

HHV – Human Herpes Virus

HIV – Human Immunodeficiency Virus

HLA-I – Human Leukocyte Antigen Class I

IFN-γ – Interferon-Gamma

IgG – Immunoglobulin Gamma

IL-10 – Interleukin-10

ITAM – Immunoreceptor Tyrosine-Based Activation Motif

ITIM – Immunoreceptor Tyrosine-Based Inhibitory Motif

KIR – Killer Immunoglobulin-Like Receptor

KIR-S - Killer Immunoglobulin-Like Receptor with Short Cytoplasmic Tails

KIR-L - Killer Immunoglobulin-Like Receptor with Long Cytoplasmic Tails

MCMV – Murine Cytomegalovirus

MHC-I – Major Histocompatibility Complex Class I

MICA/MICB – MHC-I Chain Related A or B Gene

NCR – Natural Cytotoxicity Receptors

NK Cells – Natural Killer Cells

NKR – Natural Killer Cell Receptors

PAMPs – Pathogen Associated Molecular Patterns

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PDC – Plasmacytoid Dendritic Cells

PE – Phycoerythrin

PerCP – Peridinin Chlorophyll Protein

PHA – Phytohemagluttinin

SCT – Single Chain Trimer

SHIV – Simian/Human Immunodeficiency Virus

SIV – Simian Immunodeficiency Virus

TCR – T-Cell Receptor

TGF-β – Transforming Growth Factor-Beta

TNF-α – Tumor Necrosis Factor-Alpha

ULBP – UL16 Binding Protein

Co-authorship Statement

Chapter 1

Chapter 1 consists of a literature review, which provides an introduction and overview for the experimental work described in the thesis. This review, "Natural killer cell receptors in human immunodeficiency virus infection: pathways to protection or doors to disappointment?", was published in *Current HIV Research* (2009 Sep; 7 (5): 487-96). Dr. Michael Grant and I jointly conceived the idea for this review. I was responsible for the literature review and manuscript preparation. Dr. Grant provided editorial assistance.

Chapter 2

Chapter 2 consists of a manuscript, "Distinct Phenotype of Unrestricted Cytotoxic T-lymphocytes from Human Immunodeficiency Virus-infected Individuals", which has been accepted for publication in the *Journal of Clinical Immunology.* Dr. Michael Grant and I jointly identified and designed this research project. I was responsible for conducting the research, data analysis and manuscript preparation. Dr. Grant provided editorial assistance.

Chapter 3

Chapter 3 consists of a manuscript, "Killer Cell Immunoglobulin-like Receptor 3DL1 Licenses CD16-mediated Effector Functions of Natural Killer Cells", which has been submitted for publication in the *Journal of Leukocyte Biology.* Dr. Michael Grant

and I jointly identified and designed this research project. I was responsible for conducting the research, data analysis and manuscript preparation. Dr. Grant provided editorial assistance.

Chapter 1

Natural Killer Cell Receptors in Human Immunodeficiency Virus Infection: Pathways to Protection or Doors to Disappointment

Abstract

In the absence of effective treatment, infection with the human immunodeficiency virus (HIV) ultimately leads to the acquired immune deficiency syndrome (AIDS). Many attempts have been made to prevent and attenuate HIV infection. While antiretroviral therapies for infected individuals have had great success, preventative and therapeutic vaccines focused on both humoral and cellular-mediated immunity have failed. Recently, several natural killer cell receptor (NKR) genotypes, in concert with certain class I human histompatibility-linked antigens (HLA) were found to be associated with protection from HIV infection and/or disease progression. These receptors are expressed on both natural killer (NK) cells and subsets of T lymphocytes. As HIV infection is often associated with attenuation of NK cells and much remains unknown about the basic functions of NKR, it remains undetermined whether the protective effect of these receptors relates to their expression on NK cells, T lymphocytes or both. This review summarizes current literature regarding NKR and HIV infection, and addresses several major questions remaining about the role of these receptors in protection against infection and disease progression.

1.1 Introduction

Human immunodeficiency virus (HIV) infection is a global health problem with approximately 30 million people worldwide infected and many times that number affected [1]. Since untreated HIV infection leads to acquired immune deficiency syndrome (AIDS), infected individuals with access to appropriate health care are treated with highly active antiretroviral therapy (HAART). While this treatment is highly effective, it is also expensive and unavailable in most underdeveloped countries [2]. Even in those individuals treated with HAART, multiple side effects and development of viral resistance are common problems [reviewed in 3 & 4]. As well, HAART cannot eradicate HIV, therefore, it can never cure HIV infection. Given the complex socioeconomic, political and scientific problems that limit the efficacy of HAART, development of a preventative or therapeutic vaccine remains an urgent goal for HIV/AIDS research.

Developing an effective HIV vaccine is a daunting task. The broadly neutralizing anti-HIV antibodies that protect rhesus macaques against chimeric simian/human immunodeficiency virus (SHIV) infection or disease progression have proven difficult to induce *in vivo* [5-9]. Other research using the simian immunodeficiency virus (SIV) model has demonstrated that $CD8^+$ cytotoxic T-lymphocytes (CTLs) are important regulators of viral replication and disease progression. Antibody-mediated depletion of these cells from macaques infected with attenuated SIV increases SIV RNA levels [10]. However, induction of HIV specific CTL in uninfected high-risk groups has not resulted in protection from infection. In fact, a recent trial actually observed a higher risk of HIV infection in a subset of vaccine recipients [11]. The difficulties encountered in developing a successful HIV vaccine imply that novel vaccination methodologies and/or a better

understanding of the immune system are necessary to design an effective vaccine. An important step in improving our understanding of immunological function is elucidating mechanisms of interaction between the innate and adaptive immune systems. The innate immune system is a large and complex system consisting of multiple soluble factors, cell types and effector mechanisms. Natural killer (NK) cells are one important component of the innate immune system that provide a critical link to the adaptive immune system.

Natural killer cells are lymphocytes that recognize and kill target cells, activate dendritic cells (DC) and secrete a broad range of cytokines and chemokines. Selective recognition by NK cells is mediated through expression of a variety of activating and inhibitory receptors, some of which are also expressed on CTL. Increased understanding of the functioning of NK cells and CTL expressing NK cell receptors (NKR) appears critical for the development of effective immunological therapies or vaccines against HIV, as demonstrated by relationships between certain NKR genotypes and protection from HIV infection and/or disease progression [12-14]. This review will focus on the role of NK cells and NKR in HIV transmission and disease progression. The ability of HIV to alter the phenotype and attenuate certain functional characteristics of NK cells will also be addressed. Lastly, the ability of NKR to influence CTL function and prominent contemporary research questions regarding NK cells and NKR expressed on CTL in HIV infection will be discussed.

1.2 HIV/AIDS

The 2008 Nobel Prize in Medicine was recently awarded to Luc Montagnier and Francoise Barre-Sinoussi for their discovery of HIV in 1983 [15]. This retrovirus, consisting of two copies of a single stranded RNA genome encoding nine genes, was

associated with development of AIDS in 1984 [16-19]. HIV is classified as a lentivirus, reflecting the lengthy incubation period between initial infection and development of AIDS. HIV infects $CD4^+$ T-lymphocytes, macrophages and DC through a multi-step interaction between individual components of viral envelope glycoprotein 160 (gp160) and several cell surface proteins [reviewed in 20]. Initially, the gp120 component of gp160 interacts with CD4. This results in a conformational change in gp120, allowing its interaction with either the CCR5 or CXCR4 co-receptor. This secondary interaction positions the virion closer to the plasma membrane and allows the fusogenic domain of gp41 to interact with the cell membrane. Subsequent fusion of the viral envelope and cell membrane allows the viral genetic material, included within the viral core, to be released inside the cell. Following capsule dissolution and reverse transcription, proviral DNA is transported to the nucleus and incorporated into cellular DNA, where it can remain silent or generate infectious virions.

Acute HIV infection is followed by the rapid depletion of $CD4^+$ T-lymphocytes in the gut-associate-lymphoid-tissue (GALT) [21]. This is followed by gradual depletion of the infected individual's peripheral $CD4^+$ T-lymphocytes, resulting in AIDS. Multiple mechanisms of $CD4^+$ T-lymphocyte destruction have been observed in HIV infection [reviewed in 22]. Although the relative contribution of these various mechanisms of $CD4^+$ T-lymphocyte depletion has not yet been determined, it is clear these mechanisms affect both infected and uninfected lymphocytes. Infected cells may be destroyed through loss of membrane integrity [23], increased susceptibility to apoptosis [reviewed in 24], cytolysis of infected cells by human leukocyte antigen class I (HLA-I) - peptide complex specific CTL [25] and cytolysis by NK cells [26]. Uninfected cells are destroyed through

the formation of syncytium [27], activation-induced cell death (AICD) [28], increased susceptibility to apoptosis [29] and recognition by CTL capable of lysing uninfected $CD4^+$ T-lymphocytes [30-33].

1.3 NK Cells

NK cells are large granular lymphocytes characterized by their ability to kill target cells without prior sensitization. These cells differ from other lymphocytes in their lack of clonotypic receptors encoded by rearranged germ-line T-cell receptor (TCR) or B-cell receptor (BCR) genes. Instead, NK cell clones stochastically express different combinations of numerous activating and inhibitory germ-line encoded receptors (Table 1.1) [reviewed in 34]. Therefore, individual NK cells can exhibit clonal behavior reflecting interactions between their idiosyncratic constellation of activating and inhibitory receptors and self-ligands expressed within variable contexts on normal and abnormal host cells. The ability of NK cells to kill target cells is determined by the overall balance between activating and inhibitory signals generated by ligation of these receptors. Therefore, activation of NK cells can be largely explained by a modified version of the 'missing-self hypothesis' (Fig. 1.1) - NK cells lyse target cells when activating NKR are engaged in the absence of inhibitory NKR ligation or when activating signals prevail over inhibitory signals [reviewed in 35]. It should be noted that cytolysis is not the sole purpose of NK cells, as human NK cells also produce cytokines and chemokines and promote maturation of DC, which is important for the initiation of an adaptive immune response [36 & reviewed in 37].

The major inhibitory NKR recognize classical and non-classical HLA-class I molecules. These receptors include the lectin-like NKG2A/CD94 heterodimer, which

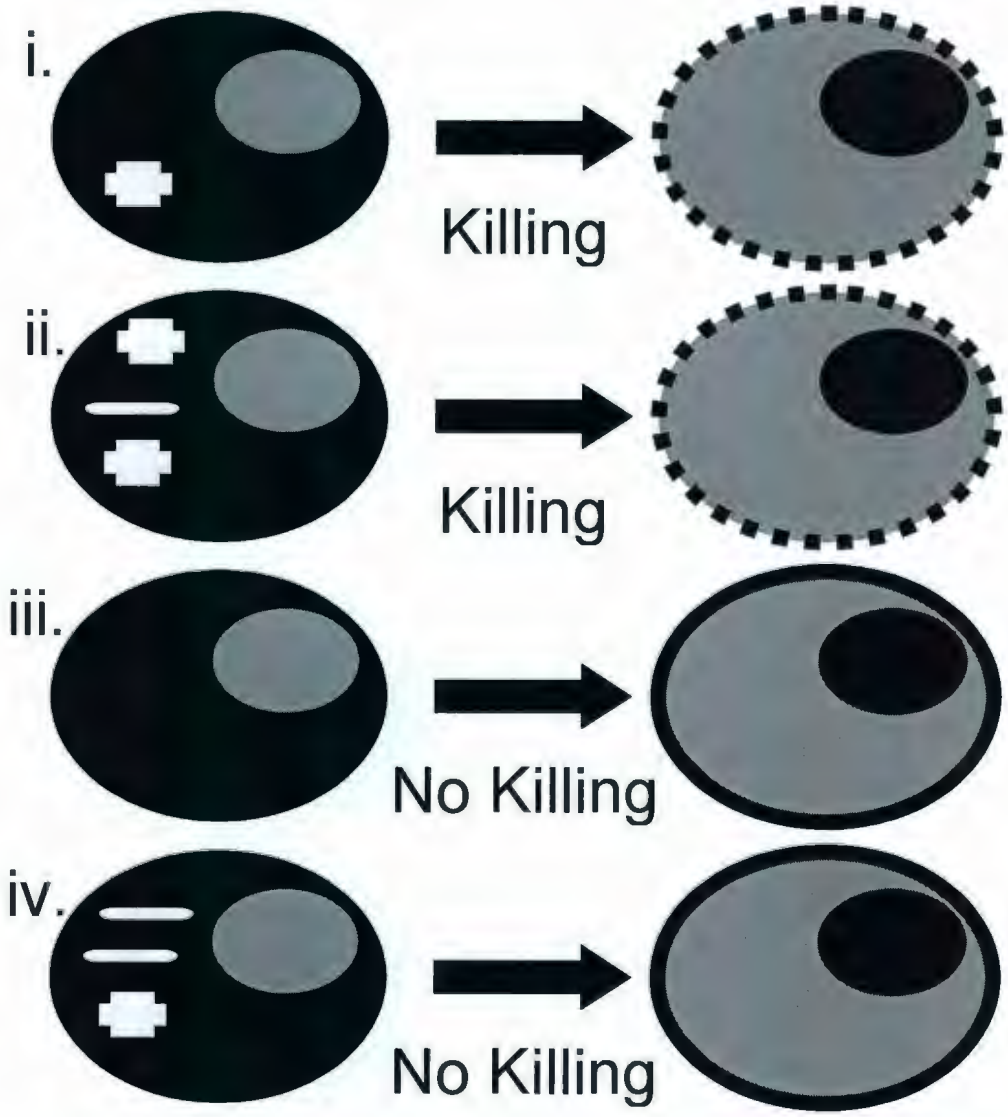

Fig. 1.1. The ability of an NK cell to kill a target cell is explained by a modified version of the 'missing self' hypothesis. NK cells kill target cells when (i) activating receptors are engaged in the absence of inhibitory receptors or when (ii) the signal propagated through activating receptors is stronger than that through the inhibitory receptors. NK cells will not kill target cells when (iii) there is an absence of activating receptor ligation or (iv) when the signal propagated through inhibitory receptors is stronger than that through the activating receptors.

Table 1.1. Activating and inhibitory natural killer cell receptors and their ligands

Receptor	Activating/Inhibitory	Ligand	Reference
NKG2D	Activating	MICA/MICB[1] and ULBPs[2]	[35&40]
NKG2C	Activating	HLA-E	[38]
KIR-S	Activating	HLA-C and putatively HLA-A and B	[34]
NKp30	Activating	pp65[3]	[41]
NKp44	Activating	Viral hemagglutinins	[41]
NKp46	Activating	Viral hemagglutinins	[41]
CD16	Activating	IgG constant region	[42]
KIR-L	Inhibitory	Classical HLA-I	[34]
NKG2A	Inhibitory	HLA-E	[38]

[1]MICA/MICB, MHC-I chain-related A or B gene.
[2]ULBP, UL-16 binding proteins.
[3]pp65, CMV tegument protein.

recognizes the non- classical HLA-E molecule [38] and the killer cell immunoglobulin-like receptors with long cytoplasmic tails (KIR-L), which recognize classical HLA-A, B and C molecules [reviewed in 34]. These receptors share a common signaling mechanism involving the phosphorylation of intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM). Tyrosine phosphorylation of these regions attracts phosphatases such as SHIP-1, SHP-1 and SHP-2 that suppress NK cell activity by reversing phosphorylation events induced by activating NKR ligation [reviewed in 39]. Signals from inhibitory NKR counteract the ligation of activating NKR. These activating receptors recognize an assortment of ligands. The major human activating NKR include the lectin-like receptors NKG2D and NKG2C/CD94 [38 & 40], the killer cell immunoglobulin-like receptors with short cytoplasmic tails (KIR-S) [reviewed in 34], the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46 [reviewed in 41] and the low affinity IgG constant region receptor - CD16 [reviewed in 42]. Signals through activating NKR are induced through tyrosine phosphorylation of receptor-associated immunoreceptor tyrosine-based activation motifs (ITAM) or the phosphorylation of tyrosine residues on receptor-associated signaling subunits, such as DAP-10 [reviewed in 39].

There are distinct subsets of NK cells with separate functions. These subsets can be distinguished via CD56 and CD16 expression. Cytotoxic NK cells express both CD56 and CD16 and those that are $CD56^{bright}$ and $CD16^{+}$ are cytokine producers [reviewed in 43]. $CD16^{+}$ NK cells that do not express CD56 have no known function [44]. It is currently unknown if these NK cell subsets arise from distinct precursors or if they represent different developmental stages stemming from a common precursor. Until recently, little was known about what developmental stages determined if an NK cell

becomes cytotoxic. The 'licensing hypothesis' suggests an instructive role for interactions between HLA-class I molecules and inhibitory receptors in this process (Fig. 1.2) [reviewed in 45].

'Licensing' refers to an NK cell acquiring the ability to kill only appropriate target cells (i.e., it takes into account the necessity of NK cell self-tolerance). This process is said to occur during NK cell development and involves the interaction of an inhibitory receptor with its ligand. After this interaction, NK cells, expressing an inhibitory NKR recognizing a self-ligand, can lyse appropriate target cells. The target cells that are lysed may express reduced levels of the ligand recognized by the licensing inhibitory receptor, as occurs in virally infected and transformed cells. This hypothesis putatively explains how NK cells kill appropriate target cells while remaining self-tolerant. Experiments by Kim et al. [46] demonstrated evidence for the 'licensing hypothesis' in mice. They developed a single chain trimer (SCT)-K^b transgenic mouse that lacked expression of any other MHC-class I molecule. They next compared interferon-gamma (IFN-γ) production by the NK cells of the transgenic mouse to those in a major histocompatibility complex class I (MHC-I) and β-2-microglobulin knockout mouse. Stimulation by antibody cross-linking of the activating NK1.1 receptor lead to the production of IFN-γ in NK cells from the transgenic mice only. Furthermore, only NK cells expressing the Ly49C inhibitory receptor, specific for the SCT-K^b, produced IFN-γ. This was interpreted as selective licensing of NK cells expressing an inhibitory NKR specific for self-MHC. Kim et al. also demonstrated that licensing of NK cells requires signaling through the inhibitory NKR's ITIM. The introduction of a tyrosine-to-phenylalanine mutation, which abrogates

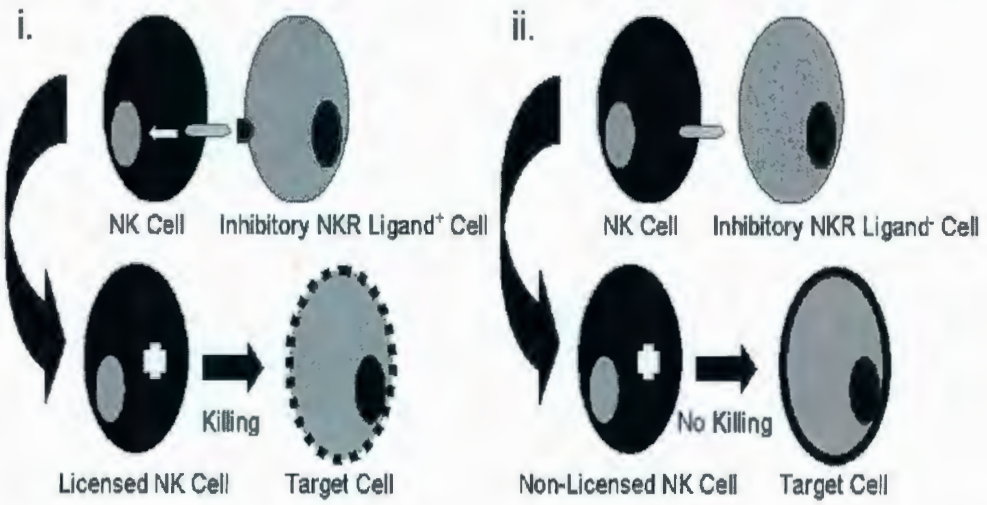

Fig.1.2. According to the 'licensing' hypothesis NK cells are licensed upon the engagement of an inhibitory NKR by its ligand. (i) In environments where such interactions occur, NK cells gain the ability to destroy appropriate target cells. (ii) In environments where no such interactions occur, NK cells are hyporesponsive to appropriate target cells.

signaling through the ITIM, removed a licensing effect of inhibitory NKR-cognate ligand interaction. The ITIM signal responsible for the licensing effect, however, appears to be distinct to the ITIM signals responsible for inhibiting NK cell cytotoxic triggering, as bone marrow chimera studies demonstrated preserved licensing of NK cells from SHP-1 deficient mice.

The importance of NK cells in human health is demonstrated by their ability to combat human herpes virus (HHV) and cytomegalovirus (CMV) infections and destroy tumor cells [47-49]. Although the importance of NK cells is relatively undefined in HIV infection, there is evidence these lymphocytes can play very important roles in protection against both initial infection and disease progression. Functional levels of NK cells remain higher in non-pathogenic immunodeficiency virus infections than in pathogenic immunodeficiency virus infections [50]. Maintenance of higher NK cell cytotoxicity levels is also associated with better prognosis in HIV-infected individuals [51], and high levels of NK cell function are found in exposed, but uninfected individuals [52]. In addition, specific NKR phenotypes, such as expression of the activating KIR3DS1 receptor and/or the inhibitory KIR3DL1 receptor, are associated with protection against HIV infection and disease progression when present in certain combinations with their known or presumed HLA ligands [12-14]. Higher degrees of antibody dependent cellular cytotoxicity (ADCC), a form of cytotoxicity mediated by $CD16^+$ NK cells against infected cells coated with antibodies, is also related to slower HIV disease progression [51]. Since NK cells are potentially important mediators of protection against HIV, it is important to appreciate potential negative effects of HIV infection on NK cell function.

1.4 Attenuation of NK Cell Function in HIV Infection

Infection with HIV has numerous detrimental effects on the function of NK cells. The capacity of NK cells to mediate direct cytotoxicity, mediate ADCC, interact efficiently with DC and secrete a normal complement of cytokines and chemokines are all seemingly reduced in HIV infection [reviewed in 53]. While some of these changes are induced through the direct interaction of NK cells with HIV, others reflect cytokine levels, virally induced changes in surface ligand expression on target cells and changes in the function of accessory cells that modulate NK cell functions. Multiple researchers have independently reported changes in the ability of NK cells from HIV-infected individuals to mediate general cytotoxicity, such as reduced cytolysis of K562 and P815 cell lines [54-55]. Decreases in general cytotoxicity have been linked to HIV infection related changes in HLA expression on target cells and changes in NKR expression patterns on NK cells. Viruses often reduce classical HLA class I molecule expression as a mechanism of escape from CTL. While these changes are an effective means of escaping CTL responses, they can increase the sensitivity of the infected cell to NK cell-mediated lysis by reducing inhibitory signaling. Changes in HLA expression have been observed within the context of HIV infection, both for classical and non-classical HLA-class I molecules [56-57]. To selectively avoid increased susceptibility to NK cell mediated lysis, HIV reduces expression of HLA class I molecules important for CTL mediated lysis of infected cells while increasing expression of non-classical HLA class I molecules that effectively inhibit NK cytotoxicity. Typically, HIV infection is associated with the downregulation of the classical HLA-A and HLA-B molecules, maintenance of classical HLA-C expression levels and an increase in the expression of the non-classical HLA-E

molecule [reviewed in 58]. Several HIV proteins mediate changes in HLA expression. Downregulation of HLA-A and B is mediated by HIV Nef, which binds to the cytoplasmic tails of these HLA molecules and interferes with HLA-I trafficking [59]. The increase in HLA-E is a result of a peptide from HIV p24 protein binding within the HLA-E peptide groove to stabilize its expression and increase the amount on the cell surface [56]. This altered HLA phenotype of HIV-infected cells may be an important contributor to the decreased NK cell mediated cytolysis of infected cells. Bonaparte et al. [60] demonstrated that NK cells kill autologous HIV-infected $CD4^+$ T-lymphocytes more effectively when the interaction between inhibitory receptors and HLA-C and E molecules is prevented. The same research group reported a similar regulatory role for the interaction between HLA-C and E and inhibitory receptors in anti-HIV ADCC [61]. Changes in the HLA expression of HIV-infected target cells, however, are not the only contributors to reduced NK cell cytotoxicity. Changes in the NKR expression pattern of NK cells also appear to make an important contribution to this phenomenon.

HIV-infected individuals exhibit increased numbers of NK cells with low expression of activating NKR and high expression of inhibitory NKR [55 & 62]. These changes in NKR expression have been recently reviewed [53]. They have been linked to the cytokine environment present in HIV infection, are related to HIV viral load and correlate with a decrease in the cytotoxic potential of NK cells. NK cells from HIV-infected persons exhibit a similar NKR phenotype to those cells cultured in interleukin-10 (IL-10), the level of which is commonly elevated in the serum of HIV-infected individuals [63]. Further evidence of an underlying relationship between cytokine levels and NK cell phenotypes comes from the effectiveness of HAART in reinstating normal

NK cell phenotypes, as HAART is also related to a decrease in the IL-10 present during HIV infection.

Reductions in the ability of NK cells from HIV-infected individuals to mediate cytotoxicity may also reflect changes in NK cell subset distribution. While levels of the $CD56^{bright}$ $CD16^{+}$ NK cells remain relatively constant during infection, decreases in the highly cytotoxic $CD56^{+}$ $CD16^{+}$ subset and increases in the non-functional $CD56^{-}$ $CD16^{+}$ subset have been reported [44 & 64]. If these subsets represent different developmental stages from a common precursor, these observations could reflect incomplete development of NK cells in HIV infection. Such a developmental problem could be induced from a change in the cytokine environment during chronic infection, or insufficient interactions with other cells necessary for efficient development. If developmental problems are responsible for the NK cell subset distribution in HIV infection, understanding of the driving force behind the relative increase in non-functional NK cells could enhance our knowledge of NK cell development.

It is likely that these changes in NK cell subset distribution are an important factor in the decrease in NK cell cytolytic function. While changes in HLA and NKR may play some role in the observed *in vivo* reduction of NK cell function during HIV infection, more recent research has demonstrated that NK cells are capable of lysing autologous HIV-infected targets in the presence of stimulated plasmacytoid dendritic cells (pDC), regardless of effector cell NKG2A expression [26]. These results suggest that the observed regulatory role of the HLA-E – NKG2A/CD94 interaction, in HIV associated general cytotoxicity and ADCC, may be more of a result of a particular *in vitro*

microenvironment, resulting from cell purification and a reduction in the ecological validity of the *in vitro* situation, rather than a true reflection of *in vivo* occurrences.

Other factors possibly responsible for the decrease in NK mediated cytotoxicity include: (i) interference in ADCC by increased soluble CD16 [65], (ii) direct HIV infection of NK cells [66], (iii) increased levels of other cytokines, such as transforming growth factor beta (TGF-β) [67], shown to have detrimental impacts on NK cell cytotoxic function [68] and (iv) direct interaction of soluble gp120 with NK cells [69]. Although many factors have been envisioned and hypothesized to interfere with NK cell function in HIV infection, the relative contribution of each variable remains largely unknown. Recently, NKp80 and NKG2A, in combination with CD16, were identified as markers of rhesus macaque NK cells [70]. As macaques are susceptible to infection with SIV, they may serve as the ideal model for investigating the *in vivo* mechanisms behind reduced NK cell cytotoxicity in immunodeficiency virus infection.

Other impacts of HIV infection on NK cell function include a reduction in the effectiveness of interactions between NK cells and DC. During the early immune response to infection, NK cells and DC interact with, and mutually activate one another. This interaction is important for effective functioning of both the innate and adaptive arms of the immune system. Upon recruitment to an inflamed area, NK cells become activated either through recognition of pathogen associated molecular patterns (PAMPS) or by ligands expressed on tumor or virus infected cells [reviewed in 71]. Following this, NK cells are further activated by IL-12 and IL-15, which are secreted by DC activated by recognition of pathogen products. This further NK cell activation facilitates the release of

numerous cytokines, including tumor necrosis factor–alpha (TNF-α) and IFN-γ from the NK cell, which induce antigen loaded DC to mature and migrate to sites where they will activate adaptive immunity. This NK/DC interaction may be a key factor in appropriate cooperation between adaptive and innate immune responses against HIV infection, as NK cells are activated by interacting with DC, while simultaneously, NK cells drive DC maturation and migration to sites where they can induce adaptive immune responses.

Another important aspect of the NK/DC interaction is the ability of NK cells to destroy immature DC cells that are not correctly undergoing maturation. This killing appears primarily mediated by the NKp30 receptor, as it is blocked by monoclonal antibodies against NKp30 [72]. NK cells lacking inhibitory KIR, but expressing the inhibitory NKG2A/CD94 receptor mediate this destruction of immature DC [reviewed in 71]. As such, the responsible NK cells are capable of killing immature DC that do not express high levels of HLA-I (classical or non-classical), but are inhibited from killing mature DC that have increased HLA-I (including HLA-E) expression. This ability of NK cells to kill immature DC is important in maintaining immunological efficacy. The killing of improperly maturing DC, as well as the induction of maturation in other DC, ensures that the DC population is maintained in a state that will be the most productive for inducing CTL effector responses. In the context of HIV infection, however, the effectiveness of this interaction is reduced, which may have detrimental effects on the adaptive immune response.

The irregular interaction between NK cells and DC in HIV infection is demonstrated by the inability of NK cells from HIV infected individuals to kill immature

DC and reduced IL-12 secretion by HIV-infected DC after CD40 ligand stimulation [73-74]. A recent review of the effects of HIV infection on the NK-DC interaction suggested that the irregular interaction may be a result of changes in NK cell subset distributions, NKR expression patterns and/or an overall degradation in NK cell functional activities [reviewed in 75]. Regardless of the mechanism behind this irregular interaction, the inability of NK cells and DC to efficiently cross-talk could play a large role in the inability of the immune response to control HIV infection. The inability of DC to properly activate NK cells could reduce cytolysis of HIV-infected cells through both ADCC and general cytotoxicity. This inefficient communication could also be an impediment to generation of an effective CTL response against HIV. Fewer activated DC would result in the activation of fewer CTL, and this may account for the inability of anti-HIV CTL responses to keep pace with HIV mutations as progressive defects in NK cell function accumulate. The interaction between NK cells and DC provides a mechanism through which changes in the innate immune response, during HIV infection, could negatively affect the adaptive CTL immune response.

The ability of NK cells to secrete other soluble factors, such as chemokines, is also affected during HIV infection. Production of the CCL5 chemokine by NK cells is reduced in HIV-infected individuals with viremia [76]. This reduction in chemokine production may be detrimental in HIV infection. As this chemokine is capable of interaction with the CCR5 HIV co-receptor, production at higher levels may prevent the infection of new cellular targets [reviewed in 53].

The study of NK cells and their interaction with HIV infection reveals numerous potential benefits of NK cell activity against HIV. Recent studies have suggested

particular NKR phenotypes, especially those including the activating KIR3DS1 or inhibitory KIR3DL1 receptors, are protective against HIV infection and progression to AIDS when present within particular HLA environments [12-14]. Individuals expressing the activating KIR3DS1 receptor have also been shown to have higher NK cell function and NK cells with a greater ability to inhibit HIV replication in HLA-BW4$^+$ cell lines [77-78]. Others have linked the degree of ADCC mediated by NK cells to protection from HIV disease progression [51]. In contrast, a recent study demonstrated no effect of CD16$^+$ NK cell depletion during primary SIV infection in rhesus macaques [79]. This study raises the question of whether expression of NKR on NK cells is how these receptors may conduct their most important functions in HIV infection. Most NKR are also expressed on CTL, and many of them play important roles in CTL mediated activities [reviewed in 80]. It remains possible that protection from HIV infection and disease progression reflects effects of specific NKR expression on CTL in general, and specifically on anti-HIV CTL.

1.5 The Expression of NKR on CTL

Although mainly studied in the context of their expression on NK cells, activating and inhibitory NKR are also expressed on subsets of T-lymphocytes. These receptors are generally expressed on CD8$^+$ T-lymphocytes with an effector/memory phenotype, but are also expressed on some CD4$^+$, γδ TCR$^+$ and cord blood T-lymphocytes [81-83]. These receptors tend to be expressed on antigen experienced T-cells, and maintenance of this expression may require constant exposure to antigen [84]. Consistent with the phenotype of NKR expressing CD8$^+$ T-lymphocytes and the mechanism of NKR expression induction and maintenance, NKR expressing T-cells are present at higher frequencies in

older individuals and persons with chronic viral infections [85 & reviewed in 86-87]. Although inhibitory receptors are most frequently induced on activated T-lymphocytes, activating receptors are also observed. Such receptors, when expressed, can either mediate cytotoxicity or co-stimulate TCR mediated cytotoxicity [88-89]. The expression of the appropriate adaptor protein corresponding to the activating NKR appears to determine if the receptor mediates direct cytotoxicity or co-stimulates TCR mediated cytotoxicity. In the presence of the appropriate adaptor protein, the receptor may mediate direct cytotoxicity, whereas in the absence of this adaptor protein, the activating NKR may still co-stimulate TCR activation [88].

Inhibitory receptors expressed on T-lymphocytes appear to have several roles. Signaling through these inhibitory receptors can prevent AICD, decrease the T-lymphocyte's activation level and prevent TCR-triggered cytotoxicity [90 & Reviewed in 91]. This last function of inhibitory NKR on T-lymphocytes is contradicted, however, by evidence suggesting NKR only interfere with late events following TCR engagement, allowing granule exocytosis to occur [92]. A higher frequency of NKR expressing T-lymphocytes are found in chronic viral infections such as HIV infection and HIV-infected individuals express inhibitory KIR on more $CD8^+$ T-lymphocytes than non-infected persons [93]. Although KIR expression levels on $CD8^+$ T-lymphocytes fall in aviremic infected individuals and/or individuals receiving HAART, they do not generally return to levels observed in uninfected individuals. The study reporting this also demonstrated that $CD8^+$ T-lymphocytes expressing inhibitory KIR have decreased TCR induced proliferation, cytokine production and granule exocytosis relative to KIR^- T lymphocytes. These decreases, although possibly induced by inhibitory KIR expression, occurred even

without KIR ligation. The authors of the study suggest the reason for this decrease in TCR stimulation induced activities is recruitment of the inhibitory KIR to the TCR induced immunological synapse. Positioning of the inhibitory KIR within the immunological synapse and recruitment of phosphatases may explain the decrease in TCR induced activity. The plausibility of this explanation and other potential explanations will be discussed in the prominent questions section of this review. While this study provided information regarding $CD8^+$ T-lymphocytes expressing inhibitory KIR, little information is available regarding expression of activating KIR on T-lymphocytes and much remains unresolved about how or if inhibitory KIR on HIV specific CTL directly reduce their functions in the presence and/or absence of relevant HLA-I molecules.

1.6 Prominent Questions

While much has been elucidated about the functions of NK cells in healthy and HIV-infected individuals, many questions remain unanswered. In this section of the review we will discuss some of the most prominent contemporary questions regarding NK cell and NKR function in HIV infection.

Can Inhibitory KIR Decrease CTL Function Independent of Ligation?

As previously described, Alter et al. [93] recently demonstrated that CTL expressing KIR were inefficiently triggered through their TCR independent of ligation of inhibitory receptors. The authors suggested the KIR might inhibit T cell stimulation due to positioning of the inhibitory KIR within the immunological synapse and recruitment of cellular phosphatases by the ITIM within. Previous research investigating CTL expressing inhibitory NKR found inhibitory NKR localized to the periphery of the

immunological synapse during early TCR driven events, including cytotoxicity. These receptors only tend to move to the center of the synapse, where they mediate inhibitory functions, late in the interaction [92].

Another possibility is that expression of KIR on CTL simply reflects the status or natural history of the T-cell. Since KIR expression may be maintained through constant exposure to antigen, KIR^+ cells may be refractory to TCR stimulation *ex vivo*. A history of extensive proliferation and previous activation, both factors that induce lower responsiveness to stimulation, may also describe T cells expressing inhibitory KIR. This would reflect a similar situation to that observed with CD28 expression in HIV infection, as infected individuals have high levels of $CD8^+CD28^-$ CTL. These lymphocytes increase early after infection and reflect an increased number of anti-HIV effector CTL. HLA-I tetramers have previously been used to demonstrate this expansion of $CD28^-$ anti-HIV CTL [94].

To fully understand the role of inhibitory KIR on anti-HIV CTL, a more detailed phenotypic and functional analysis of KIR^+ T cells in different environments is necessary. It may also be informative to analyze the positioning of the inhibitory KIR within the immunological synapse. The role of the inhibitory KIR could also be investigated by creating a KIR-L expressing CTL cell line and compare its TCR mediated activities to the same cell line with the KIR-L expression inhibited. This study would elucidate if KIR-L expression is responsible for suppressed TCR activity, or if other cellular factors are responsible.

What is The KIR3DS1 Ligand?

The KIR3DS1 molecule is an activating receptor expressed on NK cells and some T-cells. In the context of HLA-BW4 80I, this receptor has been linked to reduced HIV transmission and protection from progression to AIDS [13-14]. The gene encoding this receptor shares greater than 95% homology with the inhibitory KIR3DL1 receptor, which binds the HLA-BW4 epitope. Due to the similarity between the KIR3DL1 and KIR3DS1 receptors it has been assumed that the KIR3DS1 receptor also binds the HLA-BW4 epitope. No physical evidence, however, has been offered supporting an interaction between KIR3DS1 and HLA-BW4. Cells expressing the activating NKR are not capable of binding HLA-BW4 tetramers loaded with HIV peptides [95]. This begs the question of what KIR3DS1 can interact with? One possibility is the receptor can only bind the HLA-BW4 ligand when it is loaded with specific peptides and that the right HLA-I-peptide combination has not yet been investigated. The plausibility of this explanation is highlighted by similar observations with the KIR3DL1/HLA-BW4 interaction. KIR3DL1 demonstrates an array of reactivities with different HLA-BW4 alleles, and these response levels are influenced by the peptides used to load the HLA-BW4 molecules [96]. Another possibility, similar to that offered by Alter et al. [93] to explain how inhibitory NKR may reduce CTL TCR triggered functions, is that KIR3DS1 may not require ligation. Currently, it is unknown where the KIR3DS1 receptor locates within the immunological synapse. If appropriately localized, this receptor may be able to increase levels of activation through its ITAM. This possibility could be investigated using an NK cell or T-cell clone expressing KIR3DS1. A comparison of versions of the clone expressing the receptor, having the receptor silenced with siRNA or expressing a version of the receptor

with the extracellular region truncated may reveal the relevance of ligation in this receptor's functionality. The plausibility of this explanation is reflected in ligation independent regulation of T-cell activation thresholds by CD5 [97].

What is The Role of 'Licensing' on NK Cell Function in HIV Infection?

The inhibitory KIR3DL1 NKR is also correlated with protection from HIV infection [12]. Several potential mechanisms exist to account for this as of yet unexplained protection. It is possible that NK cells expressing this NKR are capable of recognizing and lysing HIV-infected cells because of the HLA-B downregualtion induced by HIV [57]. Alternatively, it is possible that these cells are better mediators of cytotoxicity because they are licensed in individuals expressing an HLA-BW4 epitope. Supporting this second explanation is the observation that individuals expressing both HLA-B57 (BW4 80I) and high expressing alleles of KIR3DL1 are the most likely to be protected against infection [12]. While it remains unknown whether licensing explains the KIR3DL1/HIV disease protection relationship, the role of NK cell licensing, in general, in HIV infection has been largely ignored. Human studies suggest licensing may account for some of the differences between people in general NK cell mediated cytotoxicity [98-99]. However, there is conflicting evidence regarding the role of licensing in the ADCC capability of NK cells. It should be noted that these studies investigated ADCC using plate bound anti-CD16 antibody and rabbit anti-mouse antibody labeled P815 cells. It is possible human NK cell licensing of ADCC is more detectable using an ecologically valid model featuring NK cells recognizing the Fc of IgG bound to a target cell. As such, much remains to be answered about NK cell licensing in general and in the context of HIV infection. Future studies should investigate if HIV-infected individuals with higher

numbers of licensed NK cells maintain higher levels of NK function than individuals with lower numbers of licensed cells. Questions of the role of licensing in NK cell mediated ADCC could also be investigated using anti-HIV ADCC models. Levels of ADCC mediated against $CD4^+$ T-lymphocytes coated with gp120 and anti-gp120 antibody could be compared in NK cells expressing an inhibitory receptor from individuals that express the cognate ligand of this receptor and those that do not.

What is the Role of NKR on HLA-I Independent CTL that Lyse Uninfected $CD4^+$ T-Lymphocytes?

Untreated infection with HIV or SIV is associated with CTL capable of killing uninfected $CD4^+$ T-lymphocytes in an HLA unrestricted fashion [30-33]. These CTL are $CD3^+$, $CD8^+$, TCR $\alpha\beta^+$, $CD4^-$, $CD16^-$ and $CD28^-$. Their cytolytic activity is blocked by antibody against CD3 and $\alpha\beta$ TCR molecules. The presence of these CTL is associated with progression to AIDS, as they are observed only in pathogenic immunodeficiency virus infections and have been associated with various markers of disease activity or progression in HIV-infected individuals (i.e., viral load, β-2 microglobulin serum levels and $CD8^+$ and $CD4^+$ T-lymphocyte counts).

Previous descriptions of distinct HLA-unrestricted CTL have noted CD56 and NKG2D as the most prominent NKR identified on such cells [100-101]. The NKG2D receptor has been implicated in co-stimulating anti-viral HLA-I restricted CTL and in mediating HLA independent TCR independent cytolysis [89 & 101]. As NKR appear to mediate important roles in HLA-unrestricted cytolysis, future investigations of this

potentially important CTL subset in HIV infection should investigate the role of a variety of activating and inhibitory NKR.

1.7 Conclusion

The interaction of NKR, expressed on NK cells and CTL, with HIV is a blossoming area of investigation. The expression of certain combinations of NKR with their ligand counterparts has been associated with protection from HIV infection and disease progression. The mechanisms of this protection, including the functioning effector cell (i.e., NK cell or CTL), have yet to be elucidated. On a much larger scale, the role of NKR in CTL functions remains poorly understood. While this review has highlighted much work that has been completed regarding NKR on NK cells and CTL in the context of HIV infection, it has also highlighted how little we currently understand and how much work remains to be completed.

Throughout this review we have highlighted much of the background research that has been conducted regarding NKR on NK cells and CTL. We have also addressed some of the prominent research questions regarding the role of these receptors in controlling HIV. The answers to these, and similar, research questions may have an impact on the future of HIV vaccine and therapeutic research. A greater understanding of NK cell function may make it possible to address virus transmission with prophylactic therapies enhancing NK cells in the mucosal immune system, or make it possible to offer therapies increasing NK cell function in already infected individuals. Similarly, a greater understanding of NKR expressed on CTL may be beneficial for designing a CTL-based vaccine or modulating the high levels of immune activation observed during HIV infection.

1.8 Study Objectives

This body of work investigated the role of NKR in adaptive immune responses and the ontogeny of ADCC effector functions of NK cells. First, the NKR phenotype of CTL that mediate immunopathology during HIV infection was investigated. We examined if NKR phenotypic changes that typically occur during HIV infection could be used as phenotypic markers of these CTL and if such changes on CTL in general could be implicated in the development of these autoreactive lymphocytes. Secondly, the role of NK cell licensing in CD16 mediated effector functions was examined. While the role of licensing is appreciated for the establishment of general NK functions, its role in ADCC is questionable. We investigated if expression of KIR3DL1 and its HLA-Bw4 ligand was associated with higher CD16-mediated effector functions of KIR3DL1^{+} NK cells.

The results from these studies have many potential applications and benefits. The first component of this body of work helped with the phenotyping of an autoreactive subset of CTL, which are correlated with HIV disease progression. The information gathered regarding these CTL is not only helpful with further phenotyping these cells, but could also be beneficial in understanding the origin and function of these cells. The second component of this thesis identified a model of ADCC and employed this model to investigate the role of NK cell licensing in CD16-mediated effector functions. A greater understanding of NK cell licensing may be beneficial for the development and enhancement of novel immunotherapies.

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Chapter 2

Distinct Phenotype of Unrestricted Cytotoxic T-lymphocytes from Human Immunodeficiency Virus-Infected Individuals

Abstract

Human immunodeficiency virus (HIV)-infected individuals have $CD8^+$ cytotoxic T-lymphocytes (CTL) that kill activated uninfected T-lymphocytes. These CTL are independent of classical class Ia human histocompatibility-linked leukocyte antigens (HLA-Ia). To further characterize these CTL, we investigated their restriction to non-classical class Ib HLA-E molecules and their expression of natural killer cell receptors (NKR) that are often affected in HIV infection. We found no role for HLA-E in CTL-mediated killing of activated uninfected T- lymphocytes. The non-HLA-restricted CTL did not express NKG2A, an inhibitory NKR that binds HLA-E, nor CD56, a prominent marker on previously described non-HLA-restricted CTL. This $NKG2A^-CD56^-$ phenotype of HLA-unrestricted CTL that kill uninfected activated T-lymphocytes matches generalized changes on $CD8^+$ T-lymphocytes that occur in progressive HIV infection, suggesting these phenotypic changes may reflect pathological evolution of the $CD8^+$ T cell repertoire. These CTL represent a unique phenotypic and functional subset with potential relevance to HIV pathogenesis.

2.1 Introduction

Cytotoxic T-lymphocytes (CTL) kill cells infected with intracellular pathogens via T-cell receptor (TCR)-mediated recognition of class I human histocompatibility-linked leukocyte antigens (HLA-I) loaded with pathogen-derived peptides. In addition to the clonotypic TCR, CTL express numerous activating and inhibitory natural killer cell receptors (NKR) that can modulate their behaviour [1-2]. Human and murine studies show that CTL provide protection against infection with intracellular pathogens, contribute to the clearance of acute infections and control persistent infections [3-5]. However, protection, clearance and control represent only one end of a spectrum of possible interactions between CTL and pathogens. At the opposite end, pathogens such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) establish chronic infections and replicate liberally, despite anti-viral CTL [6 & reviewed in 7]. In these situations, numerous phenotypic and functional changes accumulate in CTL, such as increased expression of the inhibitory NKG2A receptor in HCV infection [8]. Whether such phenotypic changes help drive the establishment of, or are secondary to chronic infection remains controversial.

Untreated HIV infection ultimately progresses to the acquired immune deficiency syndrome (AIDS), despite a strong anti-viral CTL response [6]. This failure reflects, at least in part, evasion of CTL via generation and selection of escape mutations [9]. There is often development of CTL-mediated immunopathology together with a loss of protective immunity and in HIV infection [10-14]. Numerous changes in the function and expression patterns of NKR on CTL also occur in HIV infection. While some changes in NKR expression are purely phenotypic with no direct effect on CTL function others

affecting activating or inhibitory receptors could directly alter CTL function. Such NKR alterations in viremic HIV-infected individuals as decreased expression of the CD56 marker and the NKG2A/CD94 inhibitory receptor respectively, illustrate these two possibilities [15-16]. Since these alterations are characteristic of chronic HIV infection, they may be useful for either signifying or explaining abnormal CTL function. This makes HIV infection an important system for studying the impact of phenotypic and functional changes to CTL.

The CTL against activated uninfected T-lymphocytes that occur in human HIV infection and pathogenic simian immunodeficiency virus (SIV) infections are a striking example of abnormal CTL function. Their specificity and distribution suggests they may contribute to $CD4^+$ T-lymphocyte depletion [10-14]. While their $CD8^+CD3^+\alpha\beta TCR^+CD28^-$ phenotype is conventional, their antigen recognition features are not. They kill both autologous and allogeneic target cells and are not inhibited by pan anti-HLA-I antibodies. One possibility is that they recognize a non-classical HLA class Ib molecule such as HLA-E, which although similar to classical HLA-I molecules in structure and function [reviewed in 17], has only three known alleles (two identical at the protein level). Therefore, HLA-E-restricted CTL are generally cross-reactive and appear non-HLA-restricted. This, and the increased expression of HLA-E during HIV infection led us to investigate its role in killing of activated uninfected T-lymphocytes by the HLA-I unrestricted CTL. Since numerous phenotypic and functional changes in NKR accompany HIV infection, we also investigated if these might contribute to the behaviour of these CTL or aid in their identification. Our results suggest that certain changes to CTL function and phenotype that occur during HIV infection may reflect and effect diversion

of the $CD8^+$ T cell response away from virus-specific and protective towards autoreactive and pathological.

2.2 Materials and Methods

Study Subjects

Study subjects were HIV-infected individuals attending the St. John's General Hospital HIV Clinic, St. John's, Newfoundland, Canada and HIV-uninfected laboratory personnel. Informed consent was obtained for their participation, and the Memorial University Faculty of Medicine Human Investigation Committee provided ethical approval for the study.

Generation of effector cells

Blood was drawn by forearm venipuncture into vacutainers containing acid-citrate dextrose (ACD) anti-coagulant (Becton Dickinson). Peripheral blood mononuclear cells (PBMC) were isolated via density gradient using Ficoll-Paque Plus lymphocyte isolation solution (GE Healthcare) and resuspended at 1.0 X 10^6 cells/ml in lymphocyte medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2 X 10^{-5} M 2-mercaptoethanol (all from Invitrogen). Effector cells were prepared from the freshly isolated PBMC of HIV-infected individuals as previously described [11]. In brief, approximately 10% of the PBMC were stimulated for three days in lymphocyte medium supplemented with 10 μg/ml phytohemagluttinin (PHA) (MP Biomedicals) while the rest were cultured in plain lymphocyte medium. After three days, PHA-activated cells were washed twice in phosphate buffered saline (PBS) containing 1% FCS and combined with the remaining 90% of PBMC left in unsupplemented lymphocyte medium. Following

three days co-culture, 10 U/ml interleukin-2 (IL-2) (Hoffmann La Roche) was added. The cells were used as effectors in ^{51}Cr release assays after seven days expansion with IL-2.

Cytotoxicity Assays

Target cells were prepared from HIV-uninfected individuals by culturing PBMC for three days at 1.0 X 10^6 cells/ml in lymphocyte medium supplemented with 10 μg/ml PHA. Target cells were labeled by incubation in a small volume at 37°C for 90 minutes with 100 μCi $Na_2{}^{51}CrO_4$ (MP Biomedicals). Labeled cells were washed once in 10 ml PBS with 1% FCS and three additional times with 5 ml of the same buffer. Target cells were then counted and resuspended in lymphocyte medium at 1 X 10^5 cells/ml. Chromium-release cytotoxicity assays were conducted in 96-well round bottom plates (Becton Dickinson). Cytotoxicity against the activated lymphocytes was measured with intact effector cells and effector cells depleted of $CD56^+$ cells. Effector cells were combined with targets at 50:1, 25:1 and 12.5:1 ratios in duplicate. Maximum release was obtained by adding 1N hydrochloric acid to targets and minimum release obtained by incubating targets with lymphocyte medium alone. Antibody blocking studies were conducted by adding anti-CD3 (Clone: OKT3, ATCC), pan anti-HLA-I (Clone: PA2.6, ATCC), anti-HLA-E (Clones: MEM-E/07 and MEM-E/08, Santa Cruz Biotechnology) or anti-TCR αβ (Clone: WT31, Santa Cruz Biotechnology) to 5 μg/ml in lymphocyte medium with effector to target (E:T) ratios of 50:1. Each well contained 5000 targets and was made up to a final volume of 300 μl with lymphocyte medium. After 5 hours of incubation, 125 μl cell free supernatant from each well was transferred to kimble tubes (Fisher Scientific) and the ^{51}Cr release counted in a Wallac 1280 gamma counter. Percent

specific lysis was calculated using the following formula: [(test release – minimum release) / (maximum release – minimum release)] X 100.

Cell Depletions

Effectors were depleted of $CD56^+$ cells using the CD56 Easy Sep separation kit (Stem Cell Technologies) following the manufacturer's protocol. The efficacy of these depletions (>95%) was confirmed by flow cytometry. Whole and depleted cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD56 (eBioscience) antibody for 20 minutes at 4°C. Cells were then washed with fluorescence-activated cell sorting (FACS) buffer containing 5 mM ethylene diamine tetra-acetic acid (EDTA) (Sigma), 0.5% FCS and 0.2% sodium azide (Sigma) in PBS. Labeled cells were resuspended in 1% paraformaldehyde (Sigma) in PBS and stored at 4°C until analyzed on a Becton Dickinson FACScalibur flow cytometer. It should be noted that the antibodies used for depletion and staining were different clones.

Flow Cytometry

Target cells (stimulators) were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) prior to co-culture with effector cells so that they were easily distinguishable from effectors by flow cytometry. Approximately 2 X 10^6 cells were resuspended at 1.0 X 10^6 /ml in PBS containing 5 mM EDTA. CFSE (1 μM) was added to the cells at 0.2 μl/ml and the cells were incubated at 37°C for 15 minutes. Labeled cells were then washed five times with ice-cold lymphocye medium. Effector cells were then combined with CFSE labeled target cells at a 20:1 ratio. After 1 hour at 37°C in 5% CO_2, Brefeldin A (Sigma) was added at 10 μg/ml and the cells left for an additional four hours.

Cells were then washed with FACS buffer and labeled with three colour combinations of FITC-conjugated anti-CD3 (Biolegend), anti-CD56 (eBioscience), or anti-CD8 (Dako), phycoerythrin (PE)-conjugated anti-CD3 (Becton Dickinson), anti-CD8 (eBioscience) or anti-NKG2A (R&D Systems) and peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 or anti-CD8 (Biolegend) antibodies. Antibody labeled cells were incubated at 4°C for 30 minutes and then washed with FACS buffer. Cells were then fixed with fixation buffer, permeabilized with permeabilization buffer (both from IntraStain kit, Dako) and incubated with allophycocyanin (APC)-conjugated anti-IFN-γ antibody (Biolegend) for 20 minutes in the dark at room temperature. Cells were then washed and resuspended in 1% paraformaldehyde in PBS until analysis on a FACscalibur flow cytometer. Extracellular flow cytometry was also conducted on target cells to confirm HLA-E expression. Cells were incubated with 5 μg of each anti-HLA-E antibody for 20 minutes at 4°C. Antibody labeled cells were than washed with FACS buffer and incubated with APC-conjugated goat-anti-mouse IgG (Invitrogen) for 20 minutes at 4°C. Cells were once again washed with FACS buffer and incubated with PE-conjugated anti-CD3 (Becton Dickinson) for 20 minutes at 4°C. The cells were washed once more and resuspended in 1% paraformaldehyde in PBS until analysis on a FACScalibur flow cytometer.

2.3 Results

Generation of CTL that kill activated uninfected T-lymphocytes

Effector CTL against activated uninfected T-lymphocytes were generated from freshly isolated PBMC of HIV-infected individuals as described and tested against activated T lymphocytes from uninfected individuals. The CTL killed target cells in a TCR-dependent and HLA class Ia-independent manner as demonstrated by inhibition with anti-CD3 and anti-TCR αβ antibodies and lack of inhibition by pan anti-HLA class I antibody. Representative data from 2 of a total of 24 HIV-infected individuals tested is shown in Fig. 2.1.

Role of HLA-E in CTL killing of activated uninfected T-lymphocytes

Although these effector cells are not restricted to classical HLA class Ia molecules, they could be restricted to the non-classical, highly conserved HLA class Ib molecule, HLA-E, which increases in expression during HIV infection [Reviewed in 17]. To test this possibility, we first confirmed surface expression of HLA-E on the activated PBMC used as targets in our cytotoxicity assays by extracellular flow cytometry. Over 99% of the target cells expressed HLA-E (Fig. 2.2a). Antibody blocking of HLA-E on target cells had no effect on cytotoxicity in 5/5 individuals tested in this manner (Fig. 2.2b). One of the blocking antibodies used (Clone: MEM-E/08) was previously shown to inhibit HLA-E restricted killing [18], therefore, this lack of inhibition indicates these CTL are not restricted to HLA-E.

Phenotypic analysis of CTL against activated uninfected T-lymphocytes

We next used extracellular and intracellular flow cytometry to directly analyze the phenotype of effector cells recognizing activated uninfected T lymphocytes. Effector cells were co-cultured for 5 hours with CFSE-labeled, PHA-activated T-lymphocytes at a 20:1 ratio and stained for surface markers (CD3 and CD8) and intracellular IFN-γ. Stimulator cells were excluded from analysis on the basis of CFSE incorporation and high fluorescence intensity (Fig. 2.3a) and effector cells responding to the activated uninfected T-lymphocyte targets were identified by production of IFN-γ (Fig. 2.3b). As the responding CTL are capable of killing HLA-E expressing T-lymphocytes and NKG2A/CD94 inhibits cytotoxicity via ligation of HLA-E [19], we investigated if these CTL expressed the NKG2A/CD94 inhibitory receptor. Previously described HLA class I unrestricted CTL were shown to prominently express CD56 [20], therefore, we also investigated CD56 expression. The vast majority of CTL responding to activated uninfected T-lymphocytes distinctly lacked expression of both CD56 and NKG2A in 5/5 tested samples (Fig. 2.4a and 4b).

Depletion of $CD56^+$ effector cells

Since our flow cytometry results suggested an unexpected CD56-negative phenotype for the HLA-unrestricted CTL, we depleted our effector cell populations of $CD56^+$ cells before cytotoxicity assays. Depletions using antibody-conjugated magnetic beads removed greater than 95% of $CD56^+$ cells (Fig 2.5a). Intact and $CD56^+$ cell depleted effector cell populations tested against activated uninfected T-lymphocyte target cells in cytotoxicity assays mediated similar levels of TCR-dependent cytolysis in 4/4

individuals tested in this manner (Fig. 2.5b). This corroborated the results of phenotypic analysis by flow cytometry indicating that the HLA-unrestricted CTL against uninfected activated T-lymphocytes do not express CD56.

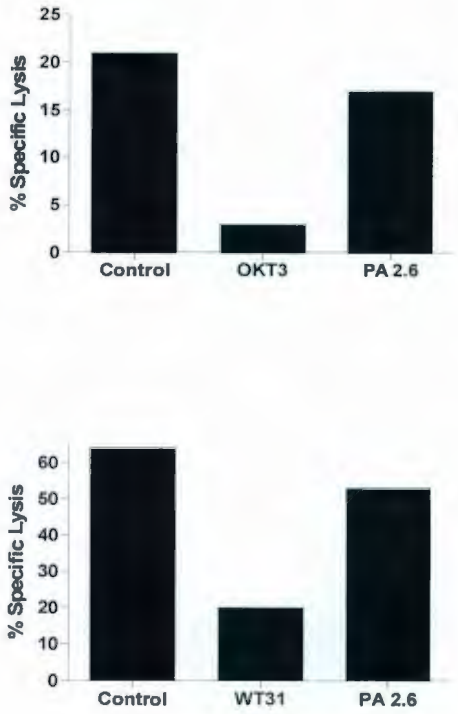


Figure 2.1. Effects of TCR and HLA Class I blocking on killing of activated uninfected T-lymphocytes by CTL from HIV-infected individuals. Representative results from 2 out of a total of 24 individuals tested are shown. Graphs show percent specific lysis by CTL at a 50:1 E:T ratio and the effect of antibodies against CD3 (OKT3), αβ TCR (WT31) and HLA-I (PA2.6).

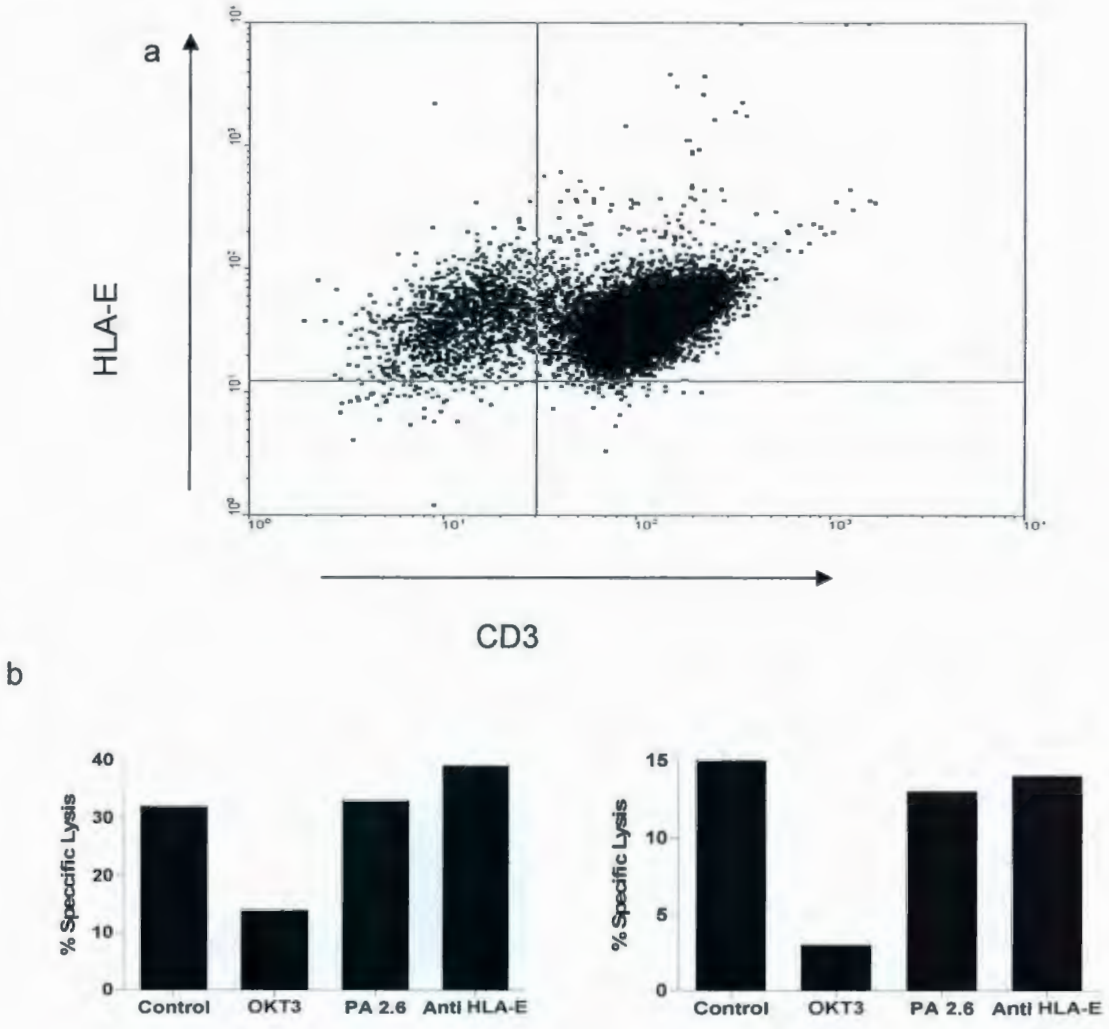
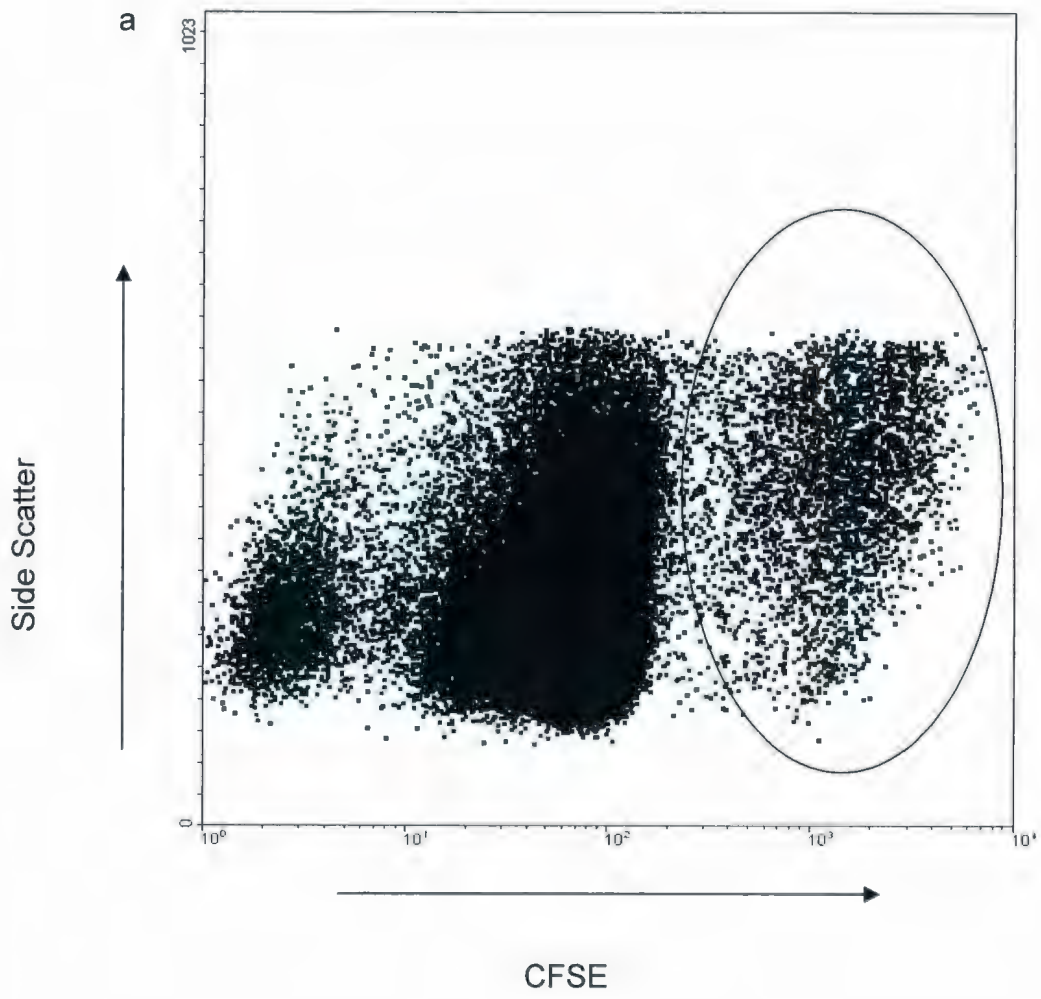


Figure 2.2. Effect of anti-HLA-E antibodies on CTL-mediated killing of activated uninfected T-lymphocytes. (a) Flow cytometry demonstrates expression of HLA-E on >99% of target cells (3-day PHA-stimulated PBMC). (b) The bar graphs compare specific lysis of untreated target cells to specific lysis of those pretreated with anti-HLA-E antibody at 50:1 E:T ratios. Representative results from 2 of 5 individuals tested are shown.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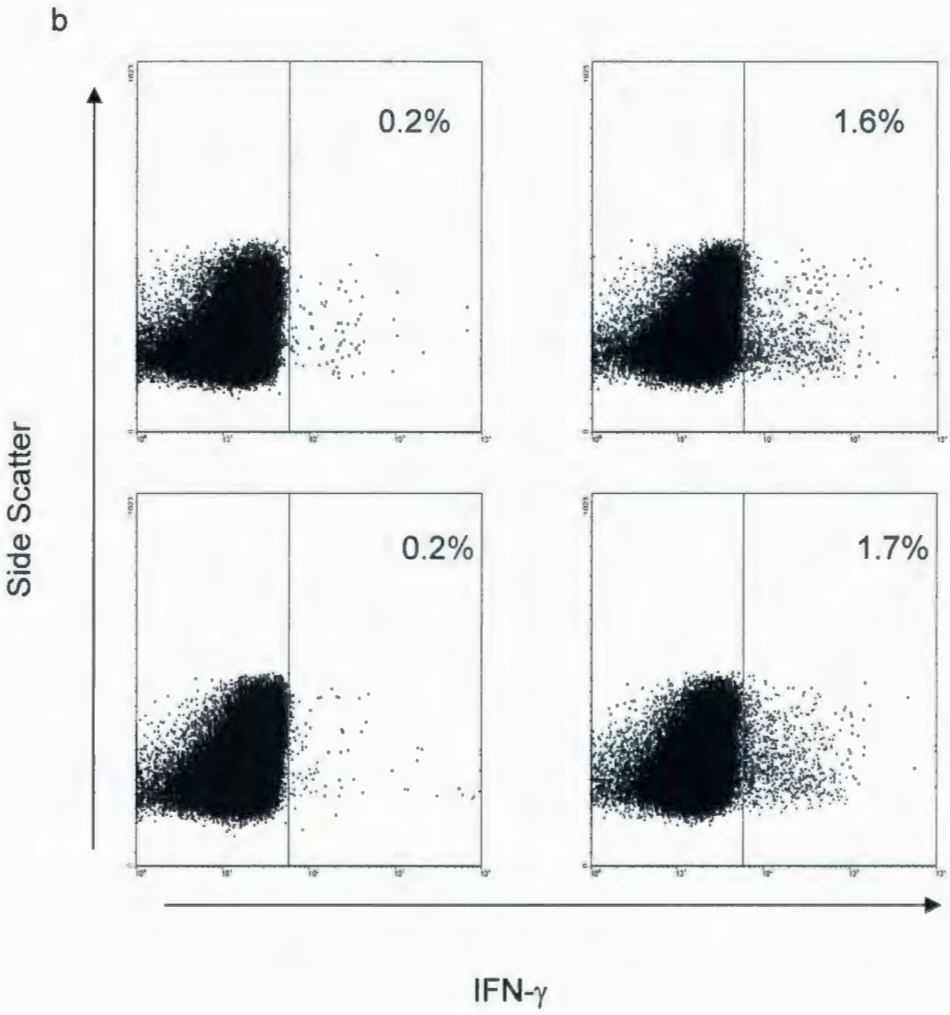

Figure 2.3. Detection of intracellular IFN-γ in effector cells stimulated with activated uninfected T-lymphocytes at a 20:1 ratio. (a) Prior to co-culture, activated uninfected stimulator cells were stained with CFSE for exclusion from analysis. (b) Intracellular IFN-γ production by unstimulated (left hand plots) and stimulated (right hand plots) effector cells was measured after gating on $CD3^+CD8^+$ lymphocytes. Representative results from 2 of 7 individuals tested are shown.

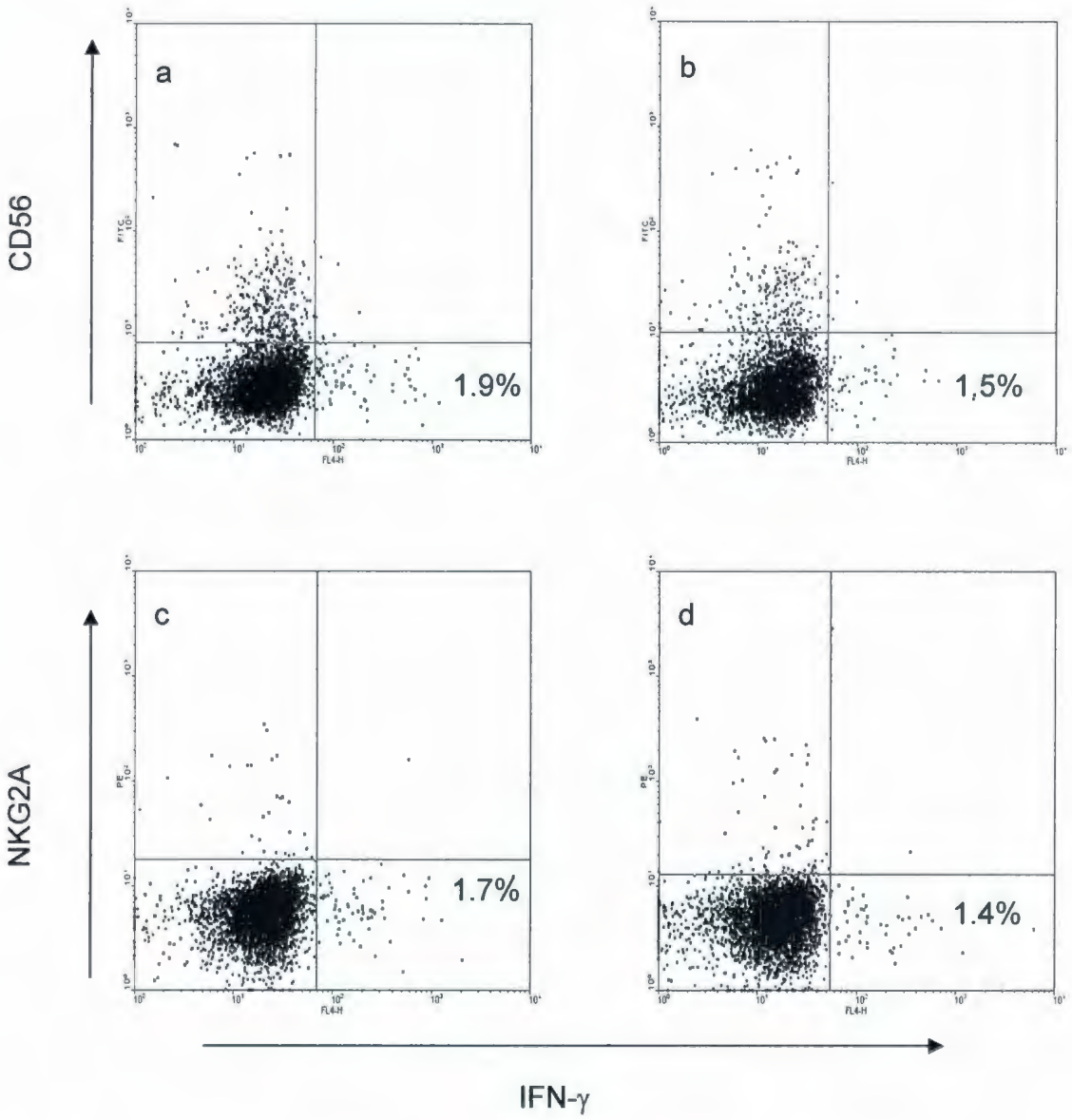

Figure 2.4. Assessment of CD56 (a, b) and NKG2A (c, d) expression on effector cells producing IFN-γ in response to activated uninfected T lymphocytes. Cells expressing CD3 and CD8 were gated for analysis. Representative results from 2 of 5 individuals tested are shown.

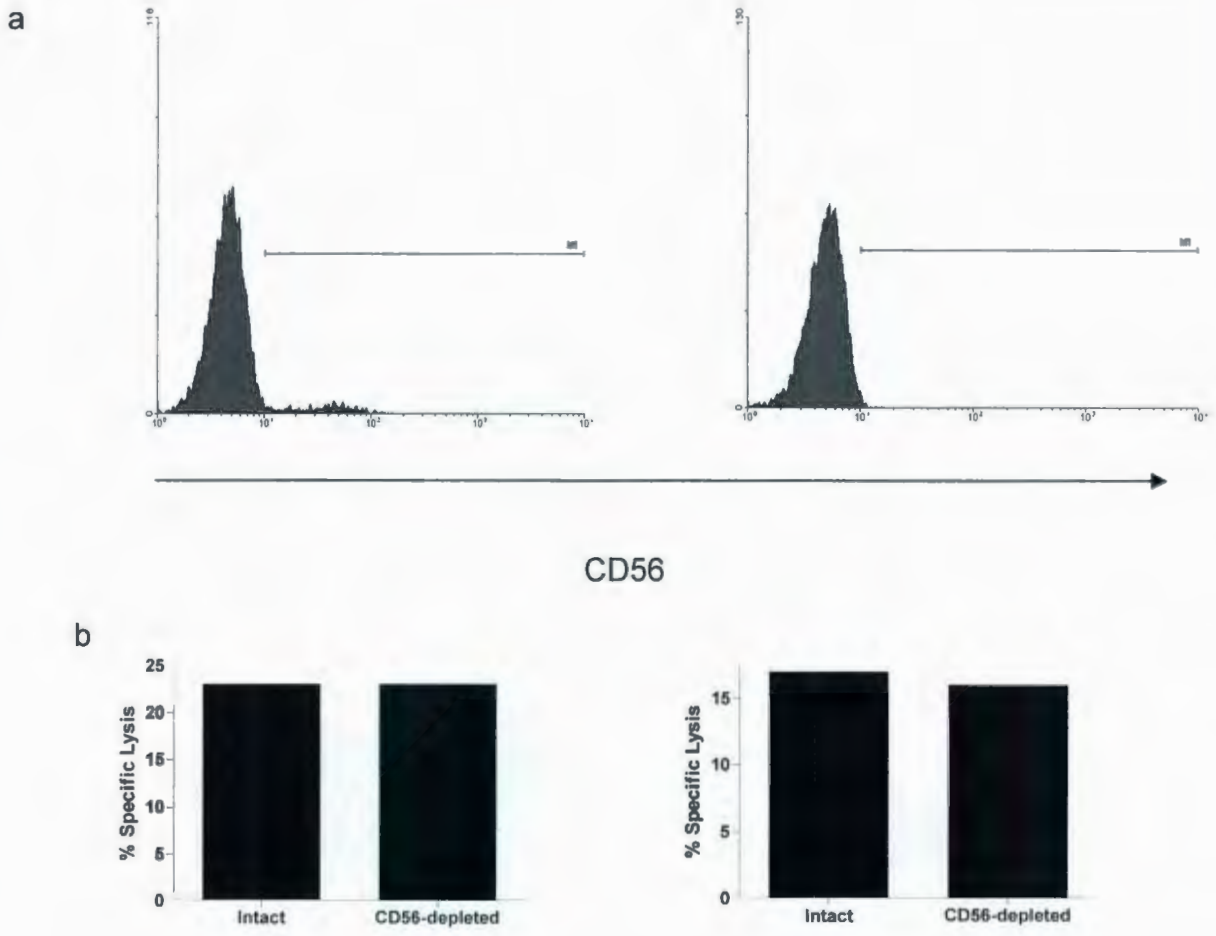

Figure 2.5. Effect of depleting $CD56^{+}$ cells on specific lysis of activated uninfected T-lymphocytes. (a) Effector cells that killed activated uninfected T-lymphocytes were depleted of $CD56^{+}$ cells with antibody conjugated magnetic beads. (b) Specific lysis of activated uninfected T-lymphocytes by intact and CD56-depleted effector cells was then compared at an E:T ratio of 50:1. Representative results from 2 of 6 individuals tested are shown.

2.4 Discussion

In this study, we further characterized unusual, previously described CTL that specifically kill activated uninfected T-lymphocytes. These CTL are associated with HIV viremia [14], therefore, several characteristic phenotypic changes that occur on CTL during progressive HIV infection were investigated as distinguishing markers and possible functional modifiers. As previously reported, CTL against activated uninfected T-lymphocytes were readily generated by in vitro stimulation of PBMC from HIV-infected individuals [11]. Antibody blocking studies confirmed the CTL were not restricted to classical HLA class Ia molecules and showed for the first time that they are also not restricted to the highly conserved, non-classical class Ib molecule, HLA-E. The CTL responding against activated uninfected T-lymphocytes were directly identifiable by flow cytometry through their IFN-γ production. We used this technique in concert with specific subset depletion to demonstrate that, unlike previously described HLA non-restricted CTL, these CTL lack expression of CD56 [20]. They also did not express NKG2A/CD94, the inhibitory NKR that binds HLA-E molecules.

The observation that CTL against activated uninfected T-lymphocytes lack expression of CD56 and NKG2A/CD94 corresponds to general alterations in NKR expression seen on CTL in HIV infection [15-16]. Reflection of this phenotype by HLA non-restricted autoreactive, potentially pathological $CD8^+$ T cells suggests that this alteration in accessory receptor expression pattern in HIV infection may represent evolution of adaptive cellular immunity away from being primarily protective towards becoming autoreactive and pathological. Lack of CD56 has no known direct functional

consequences, but serves as a phenotypic distinction from other CTL. However, lack of NKG2A/CD94 may serve both as a phenotypic marker and contribute to the behaviour of this $CD8^+$ T cell subset.

The inhibitory NKG2A/CD94 receptor is a negative regulator of CTL and NK cell activity through its recognition of the non-classical HLA-E molecule [19]. Increased HLA-E expression in HIV-infected individuals [Reviewed in 17] might functionally impair CTL expressing NKG2A/CD94 and thus, select against them. A changing pattern of inhibitory receptor expression on CTL might also favour expansion of non-HLA-restricted autoreactive T cells normally inhibited by HLA-E expression on activated T lymphocytes. Therefore, down-regulation of NKG2A/CD94 and other inhibitory accessory receptors on CTL in HIV-infected individuals could directly contribute to the selective expansion of CTL against activated uninfected T-lymphocytes and thus, to establishment of pathogenic autoimmunity in HIV infection.

The CTL we studied were previously shown to be unrestricted to classical HLA class I molecules and in this study, we excluded a role for the non-classical HLA-E molecule in either target recognition or negative regulation of the CTL. Unlike other previously described HLA-unrestricted CTL, these cells did not express CD56 [20]. Thus, not only are they atypical in their lack of HLA restriction, but are also atypical of other HLA-unrestricted CTL in their lack of CD56 expression. Their distinct specificity, unusual phenotype, and association with $CD4^+$ T-lymphocyte depletion, viremia and immune activation in HIV infection [14] make the origin, regulation and impact of this CTL population important to determine. While the present study investigated the immunological characteristics of these CTL in HIV-infected humans, animal models of

immunodeficiency virus infection may be better suited to elucidate the origin and regulation of these cells. Such studies could be done in SIV-infected macaques, where these CTL also occur in pathogenic infections [13]. Another possibility would be to investigate development of these cells in a recently described HIV infection model in mice reconstituted with human lymphoid cells [21]. Studies of the impact of transferring these CTL from infected to uninfected animals could address important questions regarding their role in disease progression, as could *in vivo* depletion of these CTL.

The CTL described in this study represent both a unique observation, with relevance to basic immunology, and a potentially important aspect of HIV infection. Further studies are required to understand the origin of these lymphocytes and their potential role in development of AIDS. Knowledge acquired through such studies could benefit numerous areas of HIV research, including pathogenesis and vaccine design.

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Chapter 3

Licensing of Antibody Dependent Cellular Cytotoxicity Functions in Natural Killer Cells Through KIR3DL1-HLA-Bw4 Interactions

Abstract

Natural killer (NK) cells are a subset of lymphocytes that mediate cytolysis of transformed and virally infected cells through reduced human leukocyte class I antigen (HLA-I) expression, recognition of stress-induced ligands and recognition of the constant regions (Fc) of immunoglobulin gamma (IgG) antibodies bound to tumor or pathogen-associated antigens. According to the licensing hypothesis, NK cells become cytotoxic only after an interaction between an inhibitory receptor and its ligand. Although this hypothesis is supported by murine and human studies, evidence that licensing is required for antibody dependent cellular cytotoxicity (ADCC) in humans is ambiguous. While inhibitory receptor interactions with HLA-C are associated with licensing of ADCC functions, interactions between the KIR3DL1 inhibitory receptor and its HLA-Bw4 ligand may be insufficient. We investigated the impact of KIR3DL1 and HLA-Bw4 co-expression on ADCC using an ecologically valid and robust system of antibody labeled Epstein-Barr Virus (EBV) transformed B-lymphocytes. General levels of ADCC were significantly higher in a group of KIR3DL1^{+} individuals expressing HLA-Bw4 than in a comparable group not expressing HLA-Bw4. Flow cytometry demonstrated that following stimulation with ADCC targets, a significantly higher frequency of KIR3DL1^{+} NK cells produced interferon-γ (IFN-γ) in HLA-Bw4^{+} individuals than in HLA-Bw4^{-} individuals. These results indicate a role for KIR3DL1/HLA-Bw4 interactions in licensing NK cells for ADCC effector functions.

3.1 Introduction

Efficient control of viral infection and malignancy requires both the innate and adaptive immune systems. During early immune responses, natural killer (NK) cells recognize and lyse virally infected and transformed cells through general cytotoxicity, triggered by altered expression of self-ligands (1-2). Following an adaptive immune response, NK cells also kill cells by antibody dependent cellular cytotoxicity (ADCC), through cross-linking of the CD16 $FC\gamma_{iii}$ receptor by immunoglobulin gamma (IgG) bound to target cells (reviewed in 3). Upon exposure to appropriate target cells, NK cells also secrete cytokines and chemokines and aid in the maturation of dendritic cells (DC) (4 & reviewed in 5). Cytotoxic NK cell activity provides direct early protection against infections and cytokine and chemokine production helps initiate adaptive immune responses. Adaptive immunity can work efficiently when NK cells mature DC, which drive the efficient development of cytotoxic T-lymphocyte (CTL) responses (reviewed in 6).

Both the early responses of NK cells and the longer-term responses of antigen specific CTL are regulated by their expression of a variety of activating and inhibitory receptors known as natural killer cell receptors (NKR). These non-rearranged germ-line encoded receptors are classified on the basis of their structure (reviewed in 7). As each NK cell clone can express a different combination of NKR, the activity of individual clones is regulated by its personalized cell surface constellation of activating and inhibitory NKR (reviewed in 8). The cumulative activating/inhibitory signal generated by

ligation of these receptors regulates NK cell killing of target cells and production of cytokines and chemokines (reviewed in 9). Although the role of NKR in regulating the activity of mature effector NK cells and T-cells has been heavily documented, it has only recently been proposed that inhibitory NKR are also important in NK cell ontogeny.

Inhibitory NKR regulate cytolytic competency in NK cells through a process referred to as licensing (reviewed in 10). According to the licensing hypothesis, NK cells acquire responsiveness to appropriate targets only after they engage the ligand of an inhibitory receptor. Therefore, NK cells from individuals that express both an inhibitory NKR and its ligand should be more functional than those from individuals expressing the NKR in the absence of its ligand. Both murine and human studies support this hypothesis (11-13), however, the evidence for licensing in humans is still somewhat contradictory when general cytotoxicity and ADCC are compared. When donors expressed the inhibitory killer immunoglobulin-like (KIR) NKRs that interact with human leukocyte histocompatibility-linked antigen (HLA)-C and the corresponding HLA-C ligand, their NK cells exhibited greater general and CD16-mediated effector functions than NK cells from those individuals lacking the appropriate HLA-C ligand (12). Similarly, expression of KIR3DL1, an inhibitory KIR that interacts with the HLA-Bw4 public epitope, was associated with higher NK cell cytotoxicity and IFN-γ production upon exposure to HLA-I deficient target cells when the NK cells were isolated from HLA-Bw4^{+} donors (13). However, NK cells expressing KIR3DL1 exhibited no such donor dependent difference in IFN-γ production following CD16 stimulation. As the evidence supporting licensing in general cytotoxicity models was similar for these two inhibitory NKR ligand pairings,

divergent results regarding ADCC could reflect either different roles for the different receptors or methodological discrepancies. Both studies stimulated NK cells with plate bound anti-CD16 antibody, but only the HLA-C/KIR study investigated licensing using IgG coated target cells. Detection of a licensing effect for ADCC may be more reliable with an ecologically valid experimental system using IgG coated target cells than with plate-bound anti-CD16-mediated cross-linking. Therefore, in this study we employed IgG-coated target cells to assess the licensing capacity of KIR3DL1/HLA-Bw4 interactions towards NK-mediated ADCC.

Freshly isolated PBMC from KIR3DL1$^+$ donors were tested for their ability to kill IgG pan anti-HLA class I coated target cells. Intrinsic ADCC levels and the direct activity of KIR3DL1$^+$ cells were compared between HLA-Bw4 expressing and non-expressing individuals. This study addressed directly, in an ecologically valid system, the impact of inhibitory receptor and ligand co-expression on NK cell licensing for ADCC.

3.2 Materials and Methods

PBMC Isolation

Venous blood was drawn from the forearm veins of healthy volunteers into vacutainers containing acid-citrate-dextrose (ACD) anti-coagulant. Peripheral blood mononuclear cells (PBMC) were isolated via density gradient using Ficoll-Paque PLUS lymphocyte isolation solution (GE Healthcare) and suspended at 1.0 X 10^6 cells/ml in RPMI medium supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 1% penicillin/streptomycin, and 2 × 10^{-5} M 2-mercaptoethanol (all from Invitrogen).

KIR genotyping

DNA was isolated from either BLCL or fresh PBMC using the illustra genomicPrep Mini Spin kit (GE Healthcare). To determine KIR3DL1 genotype status, 200 ng genomic DNA was then used for gene-specific PCR as previously described (14). The KIR3DL1 primers (forward 5' CCA TCG GTC CCA TGA TGC T 3' and reverse 5' AGA GAG AAG GTT TCT CAT ATG 3') were used at 0.5 μM each in a 50 μl reaction volume using 0.2 mM dNTPs, 1.5 mM magnesium chloride, 1X Taq buffer and 2.5 U Taq polymerase (all from Invitrogen). The PCR was performed on a PTC-100 thermalcycler (MJ Research) under the following conditions: initial denaturation for 5 min at 95° C, then 20 s at 97° C, 45 s at 62° C and 90 s at 72° C for the first 5 cycles followed by 25 cycles of 20 s at 95° C, 45 s at 60° C and 90 s at 72° C. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

ADCC ^{51}Cr Release Assay

Approximately 1.0 X 10^6 $Bw6^{+/+}$ BLCL were labeled with 5 μg of W6/32 (ATCC), or left unlabeled, and incubated on ice for 30 minutes. After two washes with PBS plus 1% FCS, the BLCL were labeled with 100 μCi of $Na_2{}^{51}CrO_4$(MP Biomedicals) and incubated at 37°C for 90 minutes. Labeled cells were washed once in 10 ml of PBS with 1% FCS and three additional times with 5 ml of the same buffer. Target cells were then counted and resuspended at 1 X 10^5 cells/ml.

Chromium-release assays were conducted in 96-well round bottom plates (Becton Dickinson) with freshly isolated PBMC as effectors. Effectors were combined with targets at 50:1, 25:1 and 12.5:1 ratios in duplicate. Maximum release was obtained by combining targets with hydrochloric acid and minimum release was obtained by combining targets with medium alone. Each well contained 5000 targets and was made up to 300μl with additional medium. Percent specific cytotoxicity was calculated using the following formula: [(test release – minimum release) / (maximum release – minimum release)] X 100.

Antibody purification and pepsin digest

W6/32 antibody was purified from hybridoma supernatant using an anti-mouse IgG-agarose column (Sigma). Briefly, W6/32 supernatant was loaded onto the IgG-agarose column and washed with 0.01 M sodium phosphate buffer containing 0.5 M NaCl (pH 7.2). The antibody was then eluted with 0.1 M glycine with 0.15 M NaCl (pH 2.4). Peak fractions were pooled and neutralized using 1 M Tris. The purified W6/32 antibody

was then dialyzed (dialysis tubing with 12,000 – 14,000 Da MWCO source) into distilled water over night at 4 °C. Following dialysis, the diluted antibody was concentrated using a SpeedVac centrifuge. The dried W6/32 antibody was resuspended in 200 mM sodium acetate (pH 4.0) and 10 μg pepsin (Sigma) per 200 μg antibody was added. The mixture was incubated for 6 hours at 37 °C and the reaction was stopped by adding 1/10 the reaction volume of 2 M Tris. The antibody fragments were then dialyzed (12,000 – 14,000 Da MWCO) into distilled water over night at 4 °C and analyzed using SDS – polyacrylamide gel electrophoresis to assess complete and proper fragmentation during the digest.

Biotinylation of W6/32 Fab_2

W6/32 Fab_2 fragments were dialyzed (12,000 – 14,000 Da MWCO) into 100 mM sodium carbonate (pH 9.5) over night at 4°C and 200 μg biotin (Sigma) were added per 1mg antibody fragments. The mixture was then incubated for 4 hours in the dark at room temperature. The biotinylated fragments were then dialyzed (12,000 – 14,000 Da MWCO) into PBS over night at 4°C and the antigen binding capacity of the W6/32 Fab_2 fragments assessed by flow cytometry using Streptavidin-PE (Caltag) for detection.

Flow Cytometry

Cell surface expression of KIR3DL1 on the NK cells of those individuals geneotyped as KIR3DL1$^+$ was confirmed by flow cytopmetry. Whole PBMC were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-KIR3DL1 (Miltenyi Biotec) and peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 (Biolegend) antibodies for 20 minutes at 4°C. Cells were then washed with fluorescence-activated cell sorting (FACS) buffer containing 5 mM ethylene diamine tetra-acetic acid (EDTA)

(Sigma), 0.5% FCS and 0.2% sodium azide (Sigma) in PBS. Labeled cells were resuspended in 1% paraformaldehyde (Sigma) in PBS and stored at 4°C until analyzed on a Becton Dickinson FACScalibur flow cytometer.

To enumerate KIR3DL1$^+$ NK cells producing IFN-g, BLCL were first labeled as above with W6/32. Effector cells were then combined with W6/32 labeled target cells at a 20:1 ratio. After 1 hour at 37°C in 5% CO_2, Brefeldin A (Sigma) was added at 10 μg/mL and the cells left for an additional four hours. Cells were then washed with fluorescence-activated cell sorting (FACS) buffer, and were labeled with FITC-conjugated anti-KIR3DL1 and PerCP-conjugated anti-CD3 antibodies. Antibody labeled cells were incubated at 4°C for 30 minutes and then washed with FACS buffer. Cells were then fixed with fixation buffer, permeabilized with permeabilization buffer (both from IntraStain kit, Dako) and incubated with allophycocyanin (APC)-conjugated anti-IFN-γ antibody (Biolegend) for 20 minutes in the dark at room temperature. Cells were then washed and resuspended in 1% paraformaldehyde in PBS until analysis on a FACscalibur flow cytometer.

Statistical Analysis

All statistical analyses were performed using Prism Graphpad Version 4.03. The data sets for the two groups were compared using non-paired one-tail Student's t-tests, with differences considered significant at $p < 0.05$. All data was demonstrated to be normally distributed using the Kolmogorov-Smirnov and Shapiro-Wilk tests.

3.3 Results

Pan anti-HLA class I antibody induces ADCC

Freshly isolated PBMC were used as effector cells against HLA-Bw6$^{+/+}$ BLCL labeled and unlabeled with pan anti-HLA class I antibody, W6/32. Effector cells lysed W6/32 coated, but not unlabeled, BLCL in chromium release assays (Fig. 3.1). As this cytolysis could be due to interference with the interaction of inhibitory receptors with HLA class I molecules or the recognition of the IgG constant region by CD16, we created W6/32 Fab_2 fragments via pepsin digestion to distinguish between these two possibilities. The Fab_2 fragments were biotinylated, used to label BLCL and detected with PE labeled strepavidin to demonstrate binding (Fig 3.2a). However, when BLCL labeled with W6/32 Fab_2 fragments were used as target cells in chromium release assays as above, no cytolysis was detected (Fig 3.2b). This demonstrates the Fc region of W6/32 is important in the cytolysis of antibody labeled cells, and that the mechanism of cytolysis is ADCC.

W6/32 induced ADCC is higher in KIR3DL1$^+$ individuals that also express HLA-BW4

All PBMC donors were positively genotyped for KIR3DL1 and shown to express the receptor on similar percentages of total CD3$^-$ lymphocytes (Fig. 3.3). These individuals were then HLA-typed and grouped as expressers of HLA-Bw4 or HLA-Bw6 homozygotes (Table 3.1). Freshly isolated PBMC from all individuals were used as effectors against HLA-Bw6$^{+/+}$ BLCL labeled or unlabeled with W6/32. Cytolysis at an E:T ratio of 50:1 varied over a broad range from 9% to 88%. Effectors from HLA-Bw4$^+$ individuals (X = 52%, SD = 23%) mediated significantly higher levels of cytotoxicity than those from HLA-Bw6$^{+/+}$ participants (X = 22%, SD = 10%) ($p < 0.01$) (Fig. 3.4).

This suggests the HLA-Bw4^{+} participants had a higher number of NK cells licensed to mediate ADCC than the HLA-Bw6$^{+/+}$ participants

Phenotypic Analysis of responding cells by flow cytometry

The ability of NK cells to produce IFN-γ upon exposure to target cells is an effector function correlated with cytolytic potential and regulated by licensing. As such, if NK cells were licensed for ADCC effector functions through the KIR3DL1 receptor, more IFN-γ production would be expected in KIR3DL1^{+} NK cells from HLA-Bw4^{+} individuals than HLA-Bw6$^{+/+}$ individuals after stimulation with ADCC target cells. Upon exposure to W6/32 labeled BLCL CD3^{-} lymphocytes produce IFN-γ (Fig. 3.5). As such, we investigated the ability of KIR3DL1^{+} CD3^{-} cells, from both groups of participants, to produce IFN-γ upon exposure to W6/32 labeled BLCL (Fig. 3.6a). Significantly higher percentages of KIR3DL1^{+} CD3^{-} cells produced IFN-γ in PBMC isolated from HLA-Bw4 expressing individuals (X = 23%, SD = 13%) than in HLA-Bw6 homozygous individuals (X = 4%, SD 4%) ($p < 0.01$) (Fig. 3.6b). These results corroborate the cytotoxicity assays in demonstrating there are more licensed NK cells in HLA-Bw4^{+} individuals. More specifically, the flow cytometry results demonstrate a much higher frequency of KIR3DL1^{+} NK cells licensed to mediate ADCC in individuals expressing HLA-Bw4 than in HLA-Bw6 homozygous individuals.

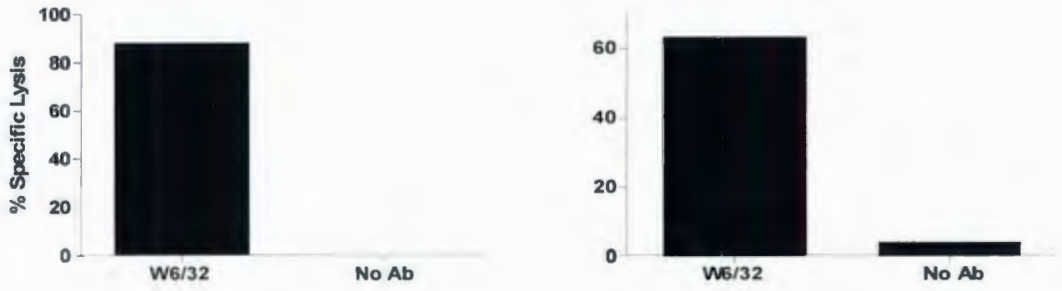


Figure 3.1. Effect of coating HLA-Bw6$^{+/+}$ EBV-transformed BLCL with pan-anti-HLA class I antibody W6/32 on cytolysis by freshly isolated PBMC. Representative results from 2 of 17 tested individuals are shown. The graphs show percent specific lysis of BLCL at a 50:1 E:T ratio in the presence and absence of W6/32.

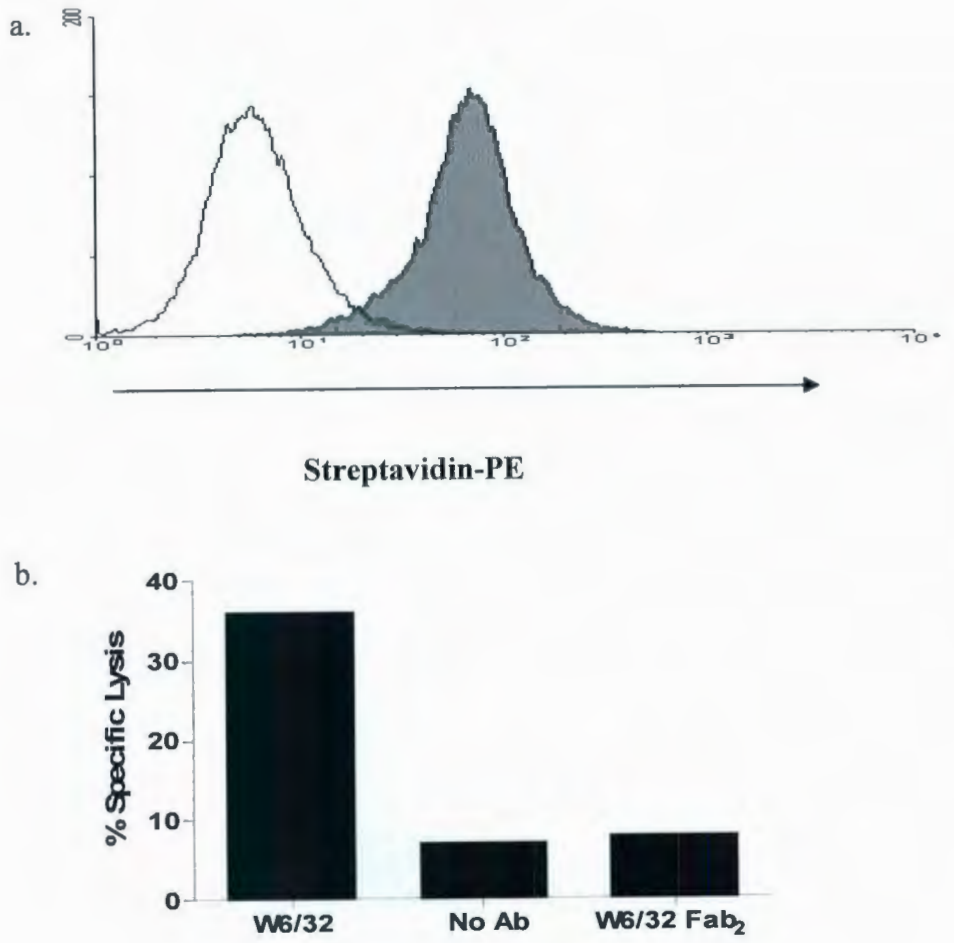


Figure 3.2. Effect of coating HLA-Bw6$^{+/+}$ EBV-transformed BLCL with Fab_2 fragments of pan-anti-HLA class I antibody W6/32 on cytolysis by freshly isolated PBMC. (a) Flow cytometry demonstrates the binding of biotinylated W6/32 Fab_2 fragments to BLCL with streptavidin-PE. The unshaded peak shows binding of streptavidin-PE alone. (b) The graph compares percent specific lysis mediated by PBMC at a 50:1 E:T ratio against HLA-Bw6$^{+/+}$ BLCL treated with intact W6/32, W6/32 Fab_2 fragments or no antibody.

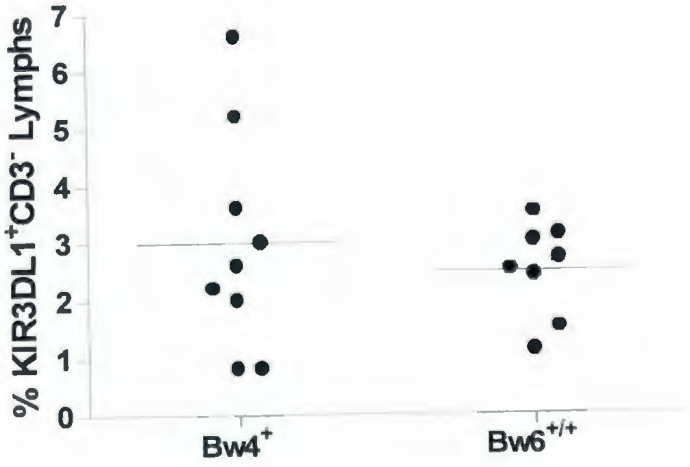


Figure 3.3. Relative frequencies of KIR3DL1^{+}CD3^{-} lymphocytes in Bw4 and Bw6 individuals. The scatter plot shows the percentage of total lymphocytes that are CD3^{-} KIR3DL1^{+} for each individual with means for each group represented by horizontal lines.

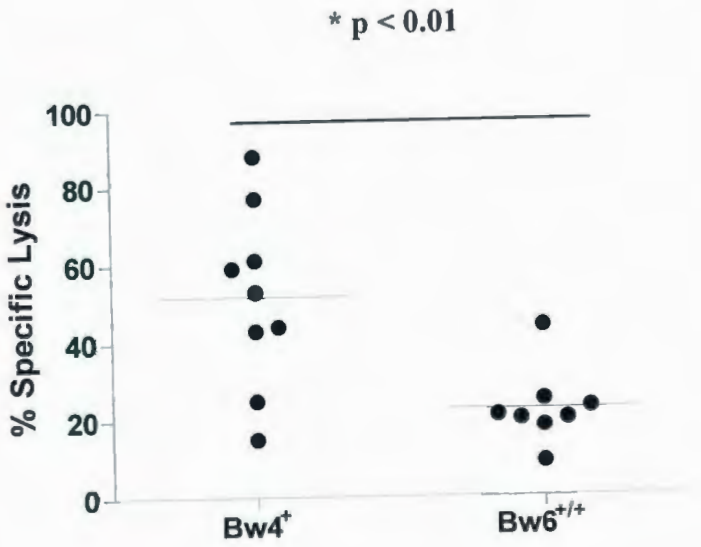


Figure 3.4. Comparison of W6/32 mediated ADCC by PBMC from HLA-Bw4^{+} and HLA-Bw6$^{+/+}$ individuals. The scatter plot shows percent specific lysis of HLA-Bw6$^{+/+}$ BLCL treated with W6/32 by lymphocytes from HLA-Bw4^{+} and HLA-Bw6$^{+/+}$ individuals at a 50:1 E:T ratio. Mean specific lysis values for the groups are shown by horizontal lines within each group and significant difference between the means shown above a line spanning the 2 groups.

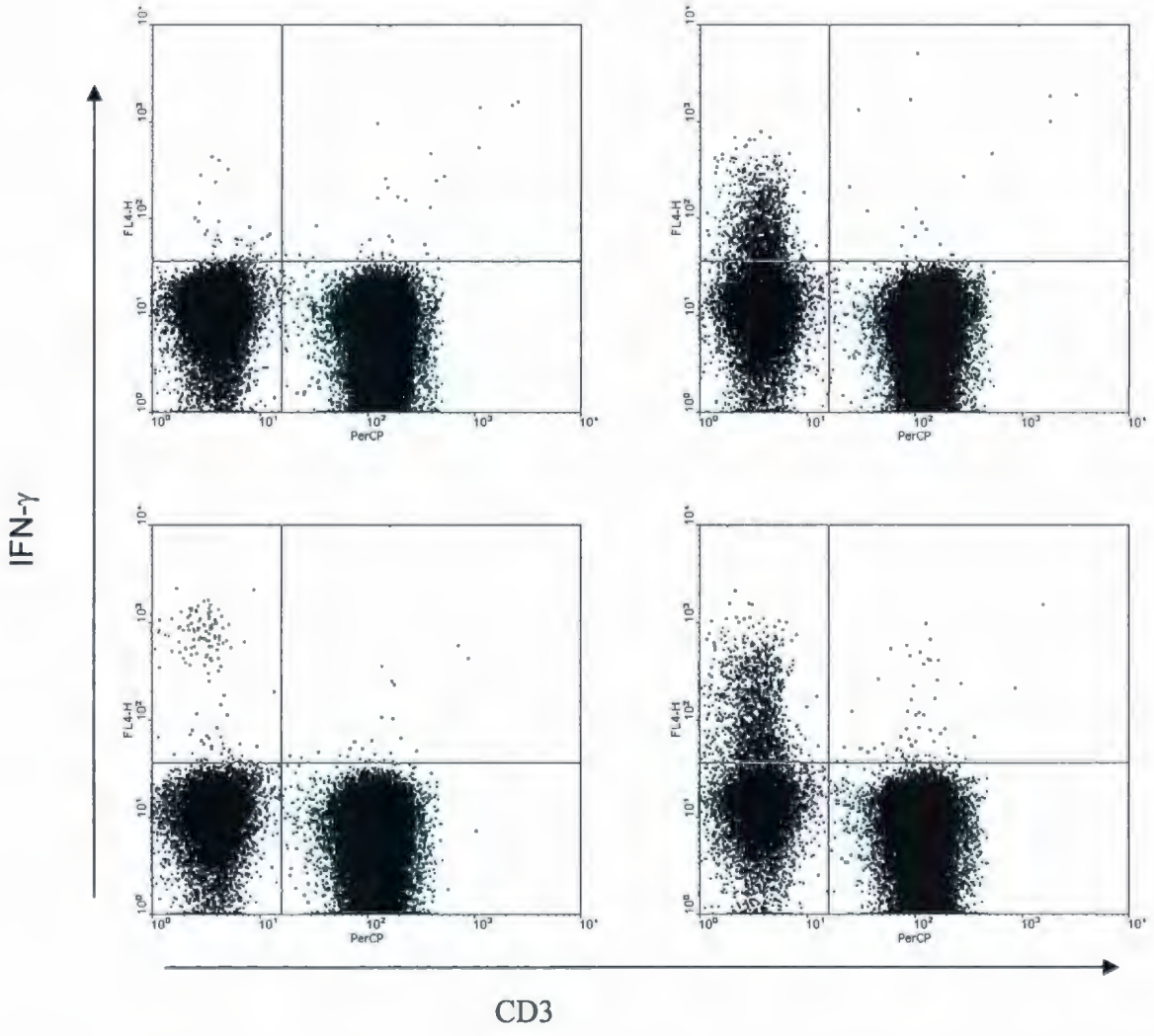
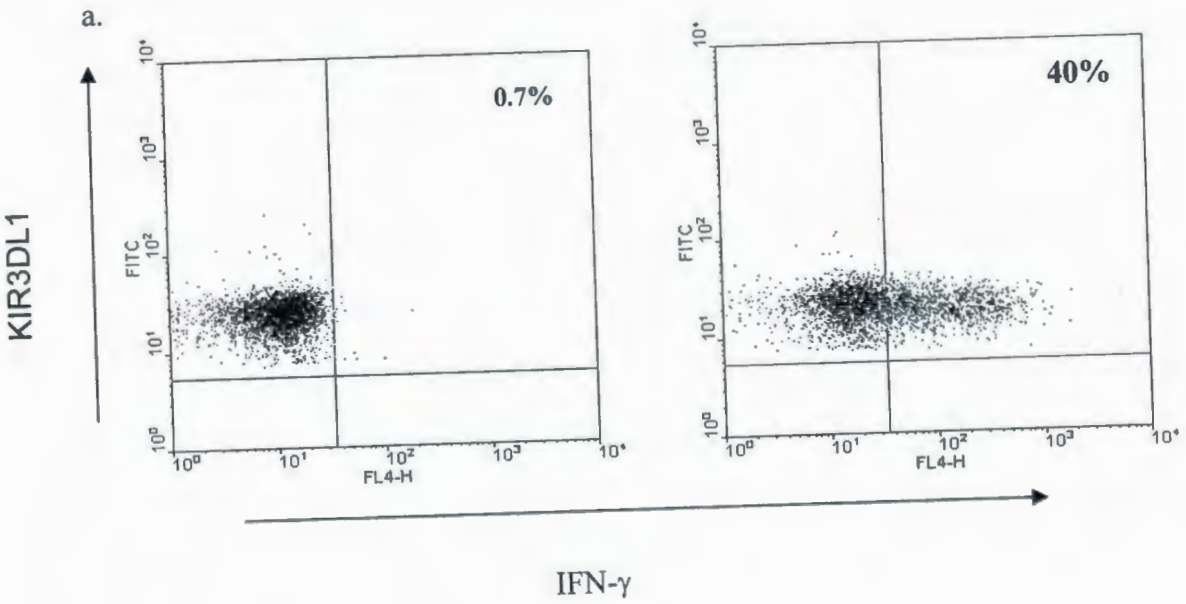


Figure 3.5. Detection of IFN-γ produced by $CD3^-$ PBMC cells following incubation with W6/32 labeled HLA-$Bw6^{+/+}$ BLCL at a 20:1 E:T ratio. Intracellular IFN-γ production by unstimulated (left hand plots) and stimulated (right hand plots) show representative results from 2/17 individuals tested.


a.
KIR3DL1
0.7%
40%
FITC
FL4-H
IFN-γ

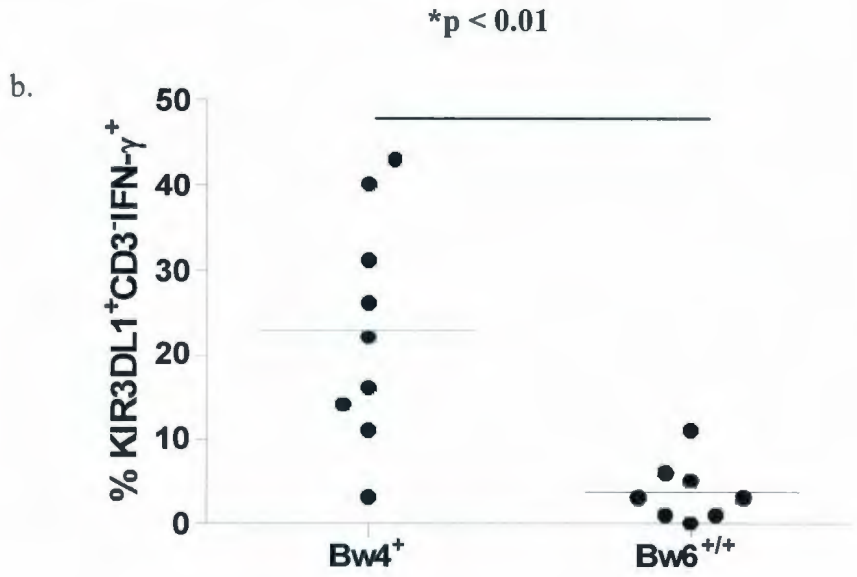


Figure 3.6. Detection of intracellular IFN-γ produced by KIR3DL1^{+}CD3^{-} PBMC following incubation with W6/32 labeled HLA-Bw6$^{+/+}$ BLCL at a 20:1 ratio. (a) Intracellular IFN-γ was detected in unstimulated (left hand plots) and stimulated (right hand plots) PBMC after gating on CD3^{-}KIR3DL1^{+} lymphocytes. (b) The scatter plot shows the percentage of KIR3DL1^{+}CD3^{-} lymphocytes producing IFN-γ following stimulation as above in HLA-Bw4^{+} and HLA-Bw6$^{+/+}$ individuals. Means are shown by horizontal lines within the groups and significant difference between the means shown above a horizontal line spanning the 2 groups.

Table 3.1 – HLA-A & B and KIR3D Genotypes of Study Participants

Sample ID	HLA-A & B[1]	KIR3D Locus[2]
001	A1, A2; B18, **B49**	$L1^{++}$
002	A2; **B44**, **B51**	L1/S1
003	A3, A28; **B27**, B62	$L1^{++}$
004	**A25**, **A32**; B39, **B63**	L1/S1
005	A3; **B 51**, B61	$L1^{++}$
006	A2, **A32**; **B44**, **B57**	L1/S1
007	A2, A11; B14, **B47**	L1/S1
008	A2; **B13**, B18	L1/S1
009	A2, A3; **B27**, B45	$L1^{++}$
010	A2, A3; B62, B60	$L1^{++}$
011	A1; B8, B62	$L1^{++}$
012	A2, A3; B7, B40	$L1^{++}$
013	A1; B8	$L1^{++}$
014	A3, A11; B18	$L1^{++}$
015	A1, A2; B8, B62	$L1^{++}$
016	A2, A11; B7, B15	L1/S1
017	A2, A3; B7, B18	$L1^{++}$

[1] HLA-A & B alleles containing the Bw4 epitope are in bold.
[2] $L1^{++}$ – Homozygous for KIR3DL1. L1/S1 – Heterozygous, contains one copy of KIR3DL1 and one copy of KIR3DS1.

3.4 Discussion

In this study we investigated the licensing of NK cell CD16 mediated effector functions through the inhibitory KIR3DL1 receptor via interaction with its HLA-Bw4 ligand. We developed a robust ADCC system, appropriate for testing the impact of this interaction, using $Bw6^{+/+}$ BLCL and the murine IgG_{2a} pan anti-HLA class I antibody, W6/32. In this system, the mean level of ADCC was significantly higher in the group of individuals expressing both KIR3DL1 and HLA-Bw4 than in those expressing KIR3DL1 that were HLA-Bw6 homozygous. Direct analysis of $KIR3DL1^+$ NK cells by flow cytometry demonstrated substantially higher responsiveness of $KIR3DL1^+$ NK cells on a per cell basis in $HLA\text{-}Bw4^+$ individuals than in HLA-Bw6 homozygous individuals. A significantly higher percentage of $KIR3DL1^+$ NK cells derived from $HLA\text{-}Bw4^+$ individuals than from HLA $BW6^{++}$ individuals produced IFN-γ when exposed to anti-HLA class I coated ADCC targets.

This study provides the first evidence that the KIR3DL1 inhibitory receptor is involved in licensing NK cell ADCC effector functions. While a previous report demonstrated a licensing effect of KIR3DL1 towards general NK cytotoxicity, no differences in ADCC functions were observed in $KIR3DL1^+$ NK cells from $HLA\text{-}Bw4^+$ and HLA-Bw6 homozygous participants (13). The discrepancy between these and our results could be accounted for by the methodological differences between the two investigations. While the previous report stimulated NK cells by cross-linking CD16 with plate bound anti-CD16 antibody, our investigation used antibody labeled BLCL to stimulate NK cells. As co-stimulation is often involved in ADCC, it may be significant

that our methodology provided stimulation with not only the CD16 ligand, but potentially with other ligands of co-stimulatory receptors that may be necessary for efficiently triggering ADCC. Our methodology provides a robust, but ecologically valid situation that may yield more physiologically relevant results than with direct cross-linking of CD16.

The differential presence of licensed NK cells in humans may explain the associations between specific NK cell phenotypes and protection against initial infections and disease progression. For example, specific combinations of KIR3DL1 and HLA-Bw4 alleles occur at higher frequencies in groups of individuals who have been exposed to the human immunodeficiency virus (HIV), but remain uninfected (15). In HIV-infected individuals, co-expression of KIR3DL1 and its HLA-Bw4 ligand is also associated with slower disease progression (16). These observations could be accounted for by increased numbers of licensed NK cells within these individuals that could eliminate and/or control HIV infections. This possibility, and the role of licensing in general, requires further studying in the context of HIV infection, as a greater understanding of the role licensing plays in the protective effects of NK cells against HIV infection and in controlling established HIV infections could help guide development of protective vaccines and/or microbicides.

The ability to investigate a role for NK cell licensing in a vaccination or microbicide protocol is limited by the lack of information currently available regarding either the mechanism or durability of the licensing process. For example, it was recently suggested that NK licensing is a dynamic process that may be reversible (17). One potential method for elucidating such information on licensing of NK cells could be the

development of *in vitro* protocols for licensing hyporesponsive/non-licensed NK cells. Such experiments would aid with establishing the mechanism and durability of the licensing process, and also with the design of potential 'immunogens' that could be used to enhance NK cell function via vaccination or microbicide exposure.

Cytolysis mediated through CD16, ADCC, can be distinguished from cytotoxicity mediated by other NKR in how it bridges the innate and adaptive immune responses. While other NKR directly recognize self-molecules, such as HLA class I, or stress-induced molecules, CD16 indirectly recognizes the non-self components of pathogens through binding the constant region of anti-pathogen IgG antibodies. This quality of ADCC introduces the possibility of exploiting ADCC to prevent and/or control infection via therapeutic or protective vaccination. The design of such vaccines would need to consider two factors, the ability of the antibodies to induce ADCC and the functional capabilities of the NK cells. This framework involves consideration of the non-traditional idea of inducing non-neutralizing antibodies as a component of an effective vaccine, as such vaccines would only require the induction antibodies that can recognize pathogen components expressed on the surface of infected cells. While a vaccine inducing such non-neutralizing antibodies may not provide protection equally in all individuals, protection could be enhanced if immunogens capable of licensing hyporeactive NK cells were included.

While our study provides evidence of the licensing of CD16 mediated effector functions, many questions remain unresolved. This study evaluated the licensing of NK cells on the level of inhibitory NKR and HLA-Bw4 genotypes. Much evidence suggests that the function of the inhibitory KIR3DL1 receptor is influenced by the allelic version

of the receptor an individual possesses, as well as the ability of that version to interact with the allelic version of the HLA-Bw4 molecules expressed (18). As such, further study investigating the differential abilities of different KIR3DL1 and HLA-Bw4 allelic interactions to license NK cells is required. The results of such investigations could elucidate the optimal conditions for licensing NK cells.

The licensing process not only endows the ability to mediate effector functions upon NK cells, but it also maintains tolerance to self within this lymphocyte subset. As only NK cells expressing an inhibitory receptor to self are licensed, tolerance to self-cells expressing the normal constellation of HLA class I molecules is ensured. This makes intuitive sense when considering general cytotoxicity, which utilizes receptors recognizing altered expression of self-molecules. However, it is less clear at first as to why licensing would be necessary or advantageous for CD16 mediated effector functions. CD16 exploits the adaptive immune system, and recognizes antibodies directed against pathogens, which have already been vetted via the B-cell tolerance pathway. Antibodies against self, however, have been documented in healthy individuals and individuals with several infections (19-21). Therefore, the requirement for NK cells to be licensed to mediate ADCC can serve as a barrier for these autoreactive antibodies to be utilized for effector functions. Due to licensing, antibodies that bind self-molecules on healthy cells will not trigger an ADCC response, as the NK cell will be inhibited by the ligation of an inhibitory receptor by the normal expression of HLA class I. However, because pathogens often downregulate the expression of HLA class I to avoid the adaptive immune response (22), antibodies that recognize infected cells will be recognized by CD16 and lysed by the NK cell.

While NK cell licensing can be viewed as a secondary protection mechanism ensuring autoreactive antibodies are not utilized in autoimmunity, it is also likely that the licensing process, due to the evolutionary history of these cells, regulates all NK cell functions. NK cells are considered as the evolutionary precursors to the B and T lymophocytes of the adaptive immune system. In the organisms where these cells were present in the absence of adaptive immunity, tolerance would still need to be maintained via licensing and similar mechanisms. As such, it is possible that the licensing of NK cell CD16 mediated effector functions is a carry over from an adaptation to a previous *in vivo* environment.

Demonstrating licensed NK cells is a key step in understanding the process of licensing and exploring its potential pragmatic applications. The present study provided evidence for the licensing of ADCC capabilities through the inhibitory KIR3DL1 receptor. Further elucidation of the basic properties of this process could lead to the development and enhancement of immunological therapies. A greater understanding of licensing and NK ontogeny could also help explain the mechanisms via which specific NK cell phenotypes provide protection against initial infections and disease progression. The licensing hypothesis provides a model through which many of the properties of NK cells can be better understood and applied.

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Chapter 4

Conclusion

This project investigated non-clonotypic activating and inhibitory receptors expressed on T-lymphocytes and natural killer (NK) cells. First, changes in the expression patterns of the CD56 marker and the inhibitory NKG2A natural killer cell receptor (NKR) on cytotoxic T-lymphocytes (CTL), during human immunodeficiency virus (HIV) infection, were investigated as phenotypic markers and contributory factors to the emergence of an autoreactive CTL subset that kills activated uninfected T-lymphocytes. Secondly, we investigated if host co-expression of an inhibitory NKR and its ligand was associated with the licensing of antibody dependent cellular cytotoxicity (ADCC) capabilities in NK cells. Our results suggest that inhibitory receptors are involved in both regulating adaptive immune responses and in the ontogeny of the CD16-mediated effector functions of NK cells.

Chronic HIV infection is associated with progressive loss of protective immunity and development of immune pathology, including a subset of CTL that kill activated uninfected T-lymphocytes (1-5). HIV infection is also associated with several alterations in accessory receptor expression patterns, including decreased numbers of CTL expressing the CD56 marker (6) and the inhibitory NKG2A/CD94 receptor (7). As such, we investigated if these alterations could provide phenotypic markers or contribute to the behaviour of CTL capable of killing activated uninfected T-cells. CTL that killed activated uninfected T-lymphocytes lacked expression of CD56 and NKG2A/CD94. This finding corresponds to the previous observation of increased levels of CTL lacking expression of CD56 and NKG2A/CD94 in HIV-infected individuals. The results from this study suggest some of the alterations in receptor expression that develop during chronic HIV infection may relate to evolution of the adaptive immune response from primarily

protective to autoreactive and pathological. The observation of lack of expression of NKG2A/CD94 on autoreactive CTL from HIV infected individuals is potentially valuable not only for explaining how alterations in inhibitory receptors can modify CTL function, but also for the development of a pathology model for the progression from HIV infection to the acquired immune deficiency syndrome (AIDS).

CTL that kill activated uninfected T-lymphocytes are found in human HIV infections and simian immunodeficiency virus (SIV) infections that progress to AIDS (1-5). The association of these CTL with disease progression does not end with their appearance in only pathogenic infections, as they are also associated with several markers of disease progression, including $CD8^+$ T-lymphocyte counts, serum beta-2-microglobulin levels and viral loads. Since the majority of $CD4^+$ T-lymphocytes destroyed in immunodeficiency virus infection are uninfected (8) and these CTL are associated with disease progression, this cellular subset could be important in the pathogenesis of HIV infection. Therefore, understanding how changes in accessory receptors induced on CTL during chronic viral infection could lead to the development of such an autoreactive subset of CTL may also elucidate a mechanism of pathogenesis in HIV infection.

The inhibitory NKG2A/CD94 receptor is a regulator of CTL and NK cell activity. This receptor recognizes the non-classical class I human leukocyte-histocompatibility linked antigen (HLA) molecule, HLA-E (9). As HLA-E expression is increased in HIV infected individuals (reviewed in 10), HIV infection may drive the selection of CTL that have reduced expression of NKG2A/CD94. This will result in CTL that recognize their cognate antigen and respond without being inhibited by the increased levels of HLA-E.

Therefore, CTL that lack expression of NKG2A would be selected, as they would be more likely to respond appropriately after recognizing cognate antigen. However, such a change in inhibitory receptor expression on CTL may allow TCR with cross-reactivity with self to recognize and respond to self. These changes in the expression patterns of inhibitory accessory receptors on CTL in HIV infected individuals may contribute to the development of CTL capable of killing activated uninfected T-lymphocytes and the establishment of pathogenic autoimmunity.

In the second part of this project we investigated if co-expression of the killer immunoglobulin like receptor (KIR) 3DL1 with its ligand, class I HLA-BW4, licensed NK cells to mediate ADCC. As previous research investigating licensing of ADCC through KIRs yielded contradictory results (11-12), we applied an ecologically valid experimental system to address potential methodological problems. Our results demonstrated that KIR3DL1^{+} NK cells had higher ADCC and interferon gamma (IFN-γ) production when isolated from individuals expressing the HLA-BW4 ligand. This observation suggests inhibitory NKR ligation plays a vital role in the ontogeny of NK cells. Establishing that the licensing of NK cell activities occurs through inhibitory receptors is important for furthering our understanding of basic NK cell biology and for creating and enhancing NK cell based therapies.

Currently hematopoietic transplants are used as therapies against several forms of leukemia (reviewed in 13). NK cells that develop from these transplants, from donors expressing both an inhibitory receptor and its ligand, become alloreactive when the recipient lacks expression of the receptor's ligand. This observation is explained by a

combination of the licensing hypothesis and the missing-self hypothesis. The NK cells are licensed for cytotoxicity in the donor. However, upon transfer to the recipient they are unable to be inhibited due to the missing ligand, or missing self. Thus, when an activating receptor is stimulated the cell will become activated due to the lack of inhibitory signals. A greater understanding of the licensing process may allow the development of *in vitro* licensing protocols and a simplification of this therapy. Instead of using transplantation, hyporeactive or non-licensed NK cells could be harvested from the individual with the leukemia. These cells could be licensed *in vitro* and reintroduced within the individual. Upon reintroduction these cells should mediate an anti-leukemia effect similar to that observed after transplantation.

The development of *in vitro* licensing protocols could also provide therapeutic avenues for several infections, including HIV infection. Co-expression of KIR3DL1 and its ligand HLA-BW4 is associated with protection from HIV infection in uninfected individuals that have been exposed to HIV (14). This suggests that NK cells expressing the KIR3DL1 receptor are somehow providing protection against initial infection. As this protective effect coincides with expression of HLA-BW4, it is possible that licensing can explain this observation. As such, the ability to induce licensing *in vitro* and reintroduce KIR3DL1^{+} NK cells, from individuals lacking HLA-BW4, may provide protection against initial infection with HIV.

Enhancement or creation of new therapies based on the licensing of NK cells is highly speculative. Much remains unknown about the licensing process, including if it is reversible. Some suggest that to remain licensed NK cells may need constant exposure to the ligands of their inhibitory receptors (15). If this were the case, NK cells licensed *in*

vitro would lose their ability to mediate effector functions after reintroduction into the original host. If NK cell licensing is to be harvested for the purposes of creating and/or enhancing therapies, much more information is required about the mechanism and stability of licensing. Although understanding licensing is of much importance for harvesting the full potential of NK cells, a detailed understanding of other NK cell properties is also essential. The recent observation of memory in NK cells is a novel property that could be of much importance.

Evidence for memory in NK cells is seen in both infection models and the adoptive transfer of *in vitro* cytokine-activated cells (16-17). Murine NK cells provide protection against murine cytomegalovirus (MCMV) infection through the activating Ly49H receptor. This receptor recognizes the viral m157 protein, which is expressed on the surface of infected cells. This infection model was recently employed to investigate memory in NK cells. The investigators found a preferential expansion of Ly49H expressing NK cells. They also observed heightened levels of Ly49H expressing NK cells in both the lymphoid and non-lymphoid organs for up to 37 days post infection. These cells were able to produce cytokines and degranulate upon reactivation, and provided protection against MCMV infection in adoptively transferred hosts. The properties of these cells suggest they are memory NK cells. They are expanded by recognition of a specific ligand by an activating receptor, and are capable of protecting adoptively transferred hosts from initial infection with the original pathogen. Evidence for memory NK cells has also been provided by *in vitro* activation of NK cells with cytokines and reintroduction into animals. These cells are detectable by production of larger quantities of IFN-γ than naïve NK cells upon stimulation. In this experiment, however, memory NK

cells did not demonstrate higher levels of cytotoxicity than naïve NK cells. These two independent studies demonstrate that NK cells have the ability to exhibit memory. The results suggest that two independent types of memory may be present in NK cells, one induced through activating receptor ligation and another through cytokine stimulation. These two types of memory appear to have different characteristics (Fig. 1), which may have diverse implications. With greater understanding these types of memory may have many different applications.

The NK cell memory induced by triggering activating receptors confers upon the lymphocytes an ability to recognize and kill appropriate target cells faster than naïve cells (16). In an MCMV infection model, this increased killing potential protected adoptively transferred animals from initial infection. This type of NK cell memory could potentially be harvested to protect against many infections. However, much remains unknown about this novel NK cell characteristic. It is unknown if activating NKR must recognize non-self components for this type of memory to be induced or if it is just a certain threshold of activation that is required. These questions could be determined via *in vitro* experiments with cross-linking of activating NKR or by transplantation of NK cells into donors that express the self-ligands for activating receptors, but not the inhibitory receptors. Regardless of the basic characteristics of this form of memory, many applications are possible. There is even potential to use this observation to generate designer NK cell phenotypes that upon introduction to an *in vitro* environment could confer protection against infection or tumor development.

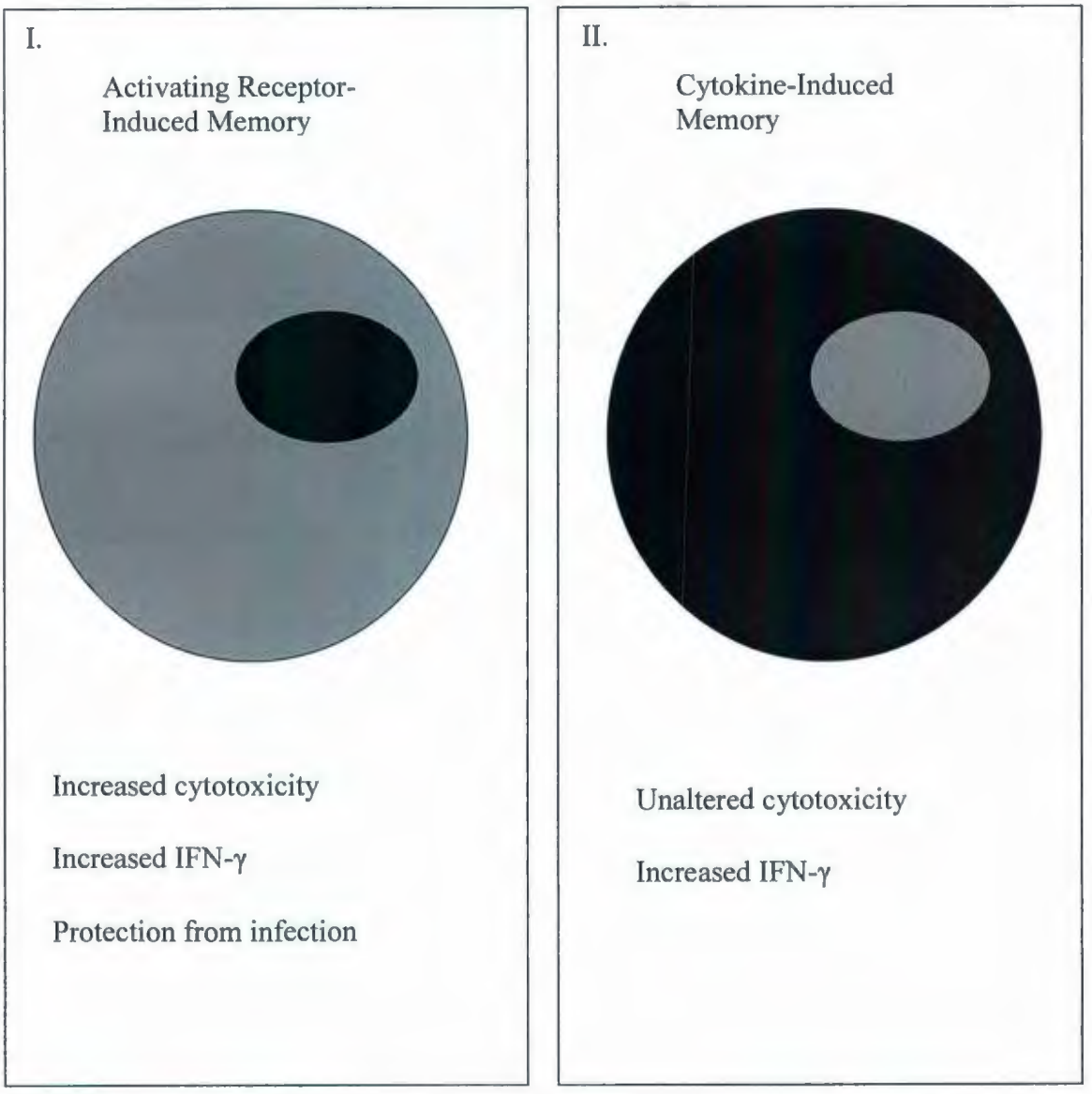

Figure 4.1. NK cell memory can be induced by (I) triggering through activating receptors or (II) through stimulation with cytokines. These two mechanisms of memory induction produce NK cells that exhibit different characteristics.

The NK cell memory induced by cytokine stimulation appears to predispose NK cells to produce higher levels of IFN-γ and not influence cytotoxicity potential (17). This form of memory could also be used for many therapeutic purposes. The activation of such memory NK cells could act as an addition to vaccination protocols, as IFN-γ activates NK cells to produce TNF-α and the two cytokines act together to induce the maturation and migration of antigen loaded dendritic cells (DC). As such, this form of NK cell memory could be beneficial for enhancing the induction of adaptive immunity.

Although the two types of NK cell memory have many potential applications, the ecological validity of their *in vivo* existence remains questionable. As activating NKR tend to recognize self-ligands, the presence of an activating receptor that recognizes a peptide from a pathogen is most likely a chance occurrence. Thus, the development of NK cell memory in MCMV infection could be due to an unrealistic triggering of the activating receptor in the absence of inhibitory signals, which would be decreased due to the down-regulation of MHC-I ligands by MCMV (18). Also, it is unknown if the induction of memory by *in vitro* cytokine stimulation could occur *in vivo*. The culture conditions may simply reflect an environment that cannot be recreated within an organism. As such, it remains unknown if NK cell memory is an ecologically valid concept, or if it represents an experimental artifact.

The present study provided evidence that NKR are valuable in the development of ADCC or CD16-mediated effector functions in NK cells and that they could serve as phenotypic markers of and functional contributors to the development of T-cell mediated immune pathology in HIV infection. Increasing the understanding of accessory receptor

function is a potentially valuable tool for developing and enhancing medical therapies for many conditions. Although it remains unknown if many of the observations regarding NK cell function are ecologically valid, many valuable contributions can arise from these observations.

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