A role for the anti-viral factor APOBEC3G in NK cell recognition of HIV-1 infected primary T lymphocytes

by

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List of Abbreviations

3DL1	KIR3DL1
3DS1	KIR3DS1
AID	Activation induced deaminase
AIDS	Acquired immunodeficiency syndrome
AMLV	Abelson murine leukemia virus
AP-1	Adaptor protein 1
APE1	AP endonuclease 1
APOBEC	Apolipoprotein B editing complex
APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
ATM	Ataxia–telangiectasia mutated
ATR	Ataxia–telangiectasia mutated and RAD3-related
ATRIP	ATR interacting protein
β_2M	Beta 2 microglobulin
BER	Base excision repair
C	Cytidine
CA	Capsid
CD16	IgG Fc receptor
CD56	Neural cell adhesion molecule
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CSR	Class switch recombination
CTL	Cytotoxic T lymphocyte
dsDNA	Double-strand DNA
Env	Envelope
FEN1	Flap-endonuclease 1
HAART	Highly active anti-retroviral therapy
HHR23A	Human homolog Rad23A
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HR	Homologous recombination
IN	Integrase
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin-like receptors
LILR	Leukocyte immunoglobulin-like receptors
MA	Matrix
MBD4	Methyl binding domain 4 protein
MHC-I	Major histocompatibility complex class I

MICA/B	MHC class I-related A/B
NC	Nucleocapsid
NCR	Natural cytotoxicity receptor
Nef	Negative factor
NHEJ	Non-homologous end joining
NK cells	Natural killer cells
NKG2D	Natural-killer group 2, member D
NLR	Nucleotide oligomerization domain-like receptors
PAMPs	Pathogen associated molecular patterns
PIC	Pre-integration complex
PLAP	Placental alkaline phosphatase
PP2A	Protein phosphatase 2A
PR	Protease
PRR	Pattern recognition receptor
RAET1	Retinoic acid early transcript 1
RLH	Retinoic acid-inducible gene I-like helicases
RPA	Replication protein A
RT	Reverse transcriptase
SHM	Somatic hypermutation
SLAM	Signaling lymphocyte activation molecule
SMUG1	Single strand selective mono-functional uracil-DNA glycosylase 1
ssDNA	Single-strand DNA
ssRNA	Single stranded RNA
TDG	Thymine/uracil mismatch DNA glycosylase
TLR	Toll-like receptors
TOPBP1	Topoisomerase-binding protein-1
U	Uracil
UDG	Uracil DNA glycosylase
ULBP	UL-16 binding protein
UNG1/2	Uracil DNA glycosylase 1/2
Vif	Virion infectivity factor
Vpr	Viral protein R

Abstract

Natural killer (NK) cells recognize virally infected cells through a balance of activating and inhibitory signals received from target cells. The NK cell recognition of human immunodeficiency virus type 1 (HIV-1) infected cells is inefficient due to multiple viral immune evasion strategies. The HIV negative factor (Nef) selectively downmodulates MHC-I surface expression, where expression of the NK cell inhibitory allotypes HLA-C and -E is not affected. Here I show NK cell recognition of HIV-infected cells is also limited by insufficient NK cell activating ligand expression. NK cell activating ligand expression is modulated by three HIV-1 accessory proteins: viral infectivity factor (Vif), viral protein R (Vpr) and Nef. Vpr upregulates NK cell activating ligand expression. However, the Vif and Nef accessory proteins limit these detrimental effects of Vpr expression.

The cellular cytidine deaminase APOBEC3G is an intrinsic restriction factor that suppresses HIV infection. It acts by causing G to A hypermutation and inhibiting viral genome synthesis. HIV counteracts APOBEC3G through the activity of Vif, which promotes the degradation of APOBEC3G. In other contexts, DNA damage like that caused by APOBEC3G is known to activate NK cell lysis by upregulating NK cell activating ligands. However, a possible effect of APOBEC3G-induced DNA damage on the cellular immune response has not yet been examined. This dissertation shows that APOBEC3G expression levels correlate with activation of DNA repair pathways and subsequent expression of NK cell activating ligands in primary T lymphocytes. Expression of Vif mitigates this effect and partially rescues infected cells from NK cell lysis. A Vif mutant unable to bind APOBEC3G was less effective at counteracting the effects of APOBEC3G on the DNA damage response and NK cell activating ligand expression. Interestingly, APOBEC3G and HIV infection activated an early stage in the DNA damage response; however downstream DNA repair signaling and upregulation of

NK cell activating ligands required HIV Vpr expression. These results define a new role for APOBEC3G acting not just as an intrinsic antiviral factor but also as an activator of the innate cellular immune response.

Chapter 1

Introduction

In recent years human immunodeficiency virus type 1 (HIV-1) has spread to an estimated 33.4 million people worldwide, a 20% increase since the year 2000[1]. These staggering numbers indicate that a greater understanding of HIV biology is needed to eradicate this growing epidemic. This dissertation addresses HIV's ability to evade recognition by the immune system, with the goal of revealing new ways to target HIV infected cells.

HIV-1 replication

HIV-1 is a member of the *Retroviridae* family of viruses that has a single stranded RNA (ssRNA) genome consisting of *gag*, *pol*, and *env* genes, which is characteristic of all retroviruses[2]. Additionally, HIV-1 encodes the *vif, vpr, vpu, nef, tat,* and *rev* accessory genes, the products of which enhance virus replication and fitness within the human host[3] (Figure 1.1). HIV-1 is further classified as a lentivirus due to a long period of clinical latency that is observed after the acute infection[4, 5] (discussed in greater detail below). HIV-1 infects cells expressing its primary receptor CD4 and one of its co-receptors, CXCR4 or CCR5 [6-12] (Figure 1.2). The primary cellular target of HIV-1 are $CD4^+$ T cells[13]. Following binding to the cell surface, fusion of the viral and cellular membranes occurs, releasing the viral genome into the cell cytoplasm[14, 15]. At this point the HIV-1 ssRNA genome is reverse transcribPed into a double stranded cDNA provirus by the viral reverse transcriptase (RT) enzyme and is trafficked to the nucleus as part of the pre-integration complex (PIC)[16, 17]. The viral integrase (IN) protein mediates proviral

integration into the cellular DNA[18]. The virus' ability to integrate into the cellular DNA allows it to persist for the life of the infected cell and to be replicated during normal cellular division. Integrated virus can be latent where it is virtually undetectable or transcriptionally active and produce new viral particles[19]. Viral mRNA transcripts and full-length genomes are generated by binding of the 5' LTR promoter by the cellular transcription machinery. Elongation of viral transcripts is enhanced by the HIV-encoded Tat protein[20]. Subsequently, the newly synthesized viral transcripts are spliced and translated to generate each of the nine HIV-1 gene products. Viral particles assemble at the plasma membrane with two copies of the ssRNA HIV-1 genome and several HIV-1 proteins, including the Gag precursor polyprotein, envelope (Env), protease (PR), IN, RT, virion infectivity factor (Vif), negative factor (Nef) and viral protein R (Vpr)[21]. Particles bud from the surface of the cell and mature, which involves PR cleavage of the Gag polyprotein into capsid (CA), matrix (MA) and nucleocapsid (NC) structural subunits[22]. The mature HIV-1 virions are infectious.

HIV disease progression

HIV disease occurs in a series of stages and ultimately leads to immune system destruction and death of the infected individual due to an inability to suppress opportunistic infections (reviewed in [23]) (Figure 1.3). The acute phase of infection is characterized by an initial spike in viremia that is limited by the cellular immune response (reviewed in [24]). However, unlike many viral infections, the immune response is unable to completely clear the infected cells due to virally encoded immune evasion strategies. The remaining virus load level is known as the viral set point[24] (Figure 1.3, black arrow). Following the acute infection, patients infected with HIV have a stage of clinical latency that can last for several years and is characterized by normal CD4$^+$ T cell counts and little detectable viremia[4]. Over time HIV gradually weakens the immune system of the infected individual by killing CD4$^+$ T cells[24]. As CD4$^+$ T cell numbers decline, virus replication increases, leading to an inability to control infections. This leads to an increased susceptibility to opportunistic infections and acquired immunodeficiency syndrome (AIDS). The inability of AIDS patients to clear these

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infections ultimately results in the death of the individual.

To limit viral replication and spread, highly active anti-retroviral therapies (HAART) have been developed to target stages in the viral life cycle[25]. Although HAART is effective at preventing the spread of infection, long-lived HIV-infected cellular reservoirs remain unaffected by drug treatment[25]. Failure of drug treatment is also common due to the acquisition of drug resistant mutations[25]. Therefore, elimination of HIV from the body will likely require more than HAART alone.

Cellular immune response to viral infections

Cytotoxic T lymphocytes (CTLs) detect peptide antigens presented by major histocompatibility complex class I (MHC-I) proteins on the surface of cells[26, 27]. MHC-I proteins are made of a human leukocyte antigen (HLA) α chain that is non-covalently linked to an invariant β_2-microglobulin (β_2M) light chain[27]. The α chain folds into three domains: α_1, α_2 and α_3[27]. The folding of the α_1 and α_2 domains forms a peptide-binding cleft that presents peptide antigens to responding CTLs while the α_3 domain interacts with β_2M and spans the plasma membrane[27]. Humans encode six different MHC-I HLA genes, including *HLA-A, -B, -C, -E, -F* and *-G*[26]. HLA-A, -B and -C proteins make up the classical MHC-I molecules that are highly polymorphic within the human population. According to current EMBL reports there are 739 different HLA-A, 1,268 different HLA-B and 513 different HLA-C alleles within the human population[28]. Each person expresses two HLA-A, -B and -C alleles (one per chromosome) that are likely different given the level of polymorphism. HLA-E, -F and -G are termed MHC-IB molecules and are almost completely conserved[26]. Short peptides derived from proteins expressed by the cell, including those of any intracellular pathogens, are presented to circulating CTLs by MHC-I molecules[27]. CTLs express antigen-specific T cell receptors that distinguish which peptide-MHC-I complexes are foreign and lyse infected cells[27].

HIV-specific CTLs recognize infected cells, the earliest CTL responses detecting Nef and Env epitopes[24]. However, the HIV Nef gene product selectively downmodulates

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expression of the MHC-I allotypes HLA-A and –B, which limits this response[29, 30]. Nef functions as an adapter protein by binding the cytoplasmic tail domains of HLA-A and -B molecules and linking them to the cellular adaptor protein 1 (AP-1), which redirects MHC-I molecules to a lysosomal degradation pathway[31-33]. Therefore HIV-infected cells are not efficiently recognized by CTLs.

Inhibiting the presentation of viral peptides to responding CTLs by downmodulating MHC-I is a common strategy employed by viruses that establish chronic infections[34]. However, natural killer (NK) cells lyse cells with reduced MHC-I[35]. Whether NK cells lyse a target cell depends on a balance of inhibitory and activating signals[35] (Figure 1.4). NK cell signaling is mediated by inhibitory and activating receptors that recognize specific ligands on the surface of target cells and transmit a signal via immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based activation motif (ITAM) containing molecules[35]. Inhibitory receptors detect specific MHC-I allotypes and some non-MHC-I ligands; these receptors include the killer cell immunoglobulin-like receptors (KIR) with long cytoplasmic tail domains, the leukocyte immunoglobulin-like receptors (LILR) and the CD94-NKG2 C-type lectin receptors[35]. Like MHC-I, there are several different allotypes of KIR that are expressed among the human population[36]. However, their expression on NK cells within each person is variegated, as NK cells can express some or all of the KIRs encoded[36]. HLA-C and -E molecules, which are resistant to Nef-mediated downmodulation, reduce NK cell recognition of HIV-infected cells by providing inhibitory signals to the responding NK cell[37]. However, an effect of HIV on NK cell activating signals has not been studied.

NK cell activation is mediated by receptors that recognize activating ligands expressed on the surface of target cells[35]. There are two major families of NK cell activating receptors, the natural cytotoxicity receptors (NCRs) and the natural-killer group 2, member D (NKG2D) receptor; however some CD94-NKG2 and the KIRs with short cytoplasmic tail domains also transmit activating signals due to the presence of ITAM signals[35]. In addition to these primary activating receptors, NK cells express a number of activating co-receptors that can stimulate lysis in conjugation with one of the

primary activating signals[38]. Members of the co-receptors include the signaling lymphocyte activation molecule (SLAM) family of receptors and NKp80. NK cell activating ligand expression can be induced during viral infection and cellular transformation. Little is known about the ligands recognized by the NCRs, however NKG2D recognizes a number of distinct but structurally similar ligands[39]. Members of the NKG2D ligands include MHC class I-related (MIC) A and B and members of the UL-16 binding protein (ULBP) and retinoic acid early transcript 1 (RAET1) protein families[39]. NKG2D activating ligands are upregulated under conditions of cellular stress including DNA damage[40], heat shock[41-43], oxidative stress[44, 45], histone deacetylase inhibition[46, 47] and during T cell stimulation[48, 49]. Infection by a number of viruses including CMV, HTLV, Adenovirus Ad5, Abelson murine leukemia virus (AMLV) and HIV have also been shown to stimulate NKG2D ligand expression[50-54]. However many of these and other viruses encode specific mechanisms to limit the expression of NKG2D ligands[55-61], suggesting the importance of limiting NK cell activation to evade NK cell recognition.

NK cell response to HIV infection

The role of NK cells in limiting HIV infection is best shown by studies of the allelic frequencies of *HLA* and *KIR* molecules among populations. All HLA-B molecules express either the Bw4 or Bw6 serological epitope, which is determined based on the amino acids at positions 77-83 [62]. HLA-B Bw4 molecules are recognized by the inhibitory KIR3DL1 (3DL1) receptor and thus inhibit NK cell lysis[36]. Despite having roughly 97% homology to the extracellular portion of 3DL1, the activating KIR3DS1 (3DS1) receptor does not recognize HLA-B Bw4 molecules[63]. However, the expression of HLA-B Bw4 molecules with isoleucine at position 80 (HLA-B Bw4-80Ile) and 3DS1 correlates with disease progression, where individuals expressing both Bw4-80Ile and 3DS1 progressed to AIDS at a slower rate than those with either one or neither of these molecules[64]. The resistance to HIV-infection observed in these individuals is likely explained by increased 3DS1 expression among Bw4$^+$ individuals compared with Bw4$^-$ [63]. Likewise, higher expression of NK cell activation markers, including 3DS1, was

observed in a cohort of HIV-exposed uninfected individuals compared with HIV-infected controls[65], suggesting that NK cell activation potential may confer protection to HIV.

HIV-1 viremia affects NK cell receptor expression and the frequency of NK cell subpopulations. NK cells from HIV-infected individuals express decreased levels of the activating NCRs NKp30, NKp44, and NKp46 and are less cytotoxic than those from healthy donors[66, 67].

NK cells are divided into two main subtypes based on their neural cell adhesion molecule (CD56) and IgG Fc receptor (CD16) surface expression[68]. $CD56^{dim} CD16^+$ NK cells are highly cytotoxic, whereas $CD56^{bright} CD16^{low}$ NK cells are less cytotoxic and serve a regulatory function[68]. In a healthy individual the vast majority of circulating NK cells are the cytotoxic $CD56^{dim}$ subset[68]. However, HIV viremic individuals have an outgrowth of a rare and non-cytotoxic $CD56^- CD16^+ KIR^+$ population of NK cells[69, 70]. This outgrowth of $CD56^- CD16^+ KIR^+$ cells was not observed in HIV-infected individuals undergoing HAART[67], indicating active virus replication is required. The total number of NK cells expand during acute HIV infection and then retract to normal levels during chronic infection[71]. The expansion during acute HIV infection is observed due to increased $CD56^{dim} CD16^+$ NK cells that decrease to normal levels during chronic infection[71]. However, the percentage of $CD56^{bright} CD16^{low}$ NK cells decreases and dysfunctional $CD56^- CD16^+$ cells expand during chronic infection[71]. Because these changes in the NK cell population occurs as infection progresses, this dissertation focuses on understanding the susceptibility of HIV-infected cells to healthy NK cells to shed light upon the earliest infection events and analyzes HIV's effects on NKG2D ligand expression.

APOBEC/AID cytidine deaminases

NKG2D ligands are upregulated under conditions of cellular stress. One form of stress that cells regularly encounter is DNA damage. The RNA base uracil (U) is found in cellular DNA via the misincorporation of dUTP or deamination of cytidine (C)

residues and plays an important role in adaptive and innate immune responses (reviewed in [72]). The level of misincorporation depends on the concentration of cytosolic dUTP; it is estimated that 10^4 dUTP residues are incorporated into cellular DNA during replication[73]. Because uracil incorporation can be mutagenic, cells express dUTPase and uracil DNA glycosylase (UDG) enzymes to limit the pool of dUTP and remove incorporated uracil residues, respectively. There are several UDG enzymes that have overlapping function. They include mitochondrial UNG1, nuclear UNG2, nuclear single strand selective mono-functional uracil-DNA glycosylase (SMUG1), thymine/uracil mismatch DNA glycosylase (TDG), and methyl binding domain 4 protein (MBD4)[74]. UNG and SMUG1 typically remove uracil from single stranded DNA (ssDNA) but also have enzymatic activity against uraciliated double stranded DNA (dsDNA)[74]. TDG and MBD4 serve to remove uracil from dsDNA resulting from dUTP misincorporation[74]. The mechanism of nucleotide removal and DNA repair is discussed in greater detail in the next section.

Cytidine deamination occurs either spontaneously or is mediated by cellular cytidine deaminases[72]. The apolipoprotein B editing complex (APOBEC) family of cytidine deaminases is important in innate and adaptive immune responses and includes activation induced deaminase (AID) and APOBEC3 isoforms[75]. AID has been shown in B cells to initiate antibody somatic hypermutation (SHM) and class switch recombination (CSR) in lymph node germinal centers[76, 77]. However, AID is ectopically upregulated in retrovirally infected B cells through an ill-defined mechanism involving the transcription factor NFκB[52, 78]. The DNA damage induced as a result of AID expression induces NKG2D ligand expression[52]. It is unclear however, whether the DNA damage signal induced by AID expression that leads to NKG2D ligand expression is of cellular or viral origin.

Several of the APOBEC3 isoforms have been shown to be important intrinsic factors in limiting retroviral infection and endogenous retroelement translocation[79-84]. The APOBEC3G (A3G) and APOBEC3F (A3F) isoforms (A3G being the most anti-viral) are important factors for limiting HIV-1 replication[85, 86]. A3G has both deaminase-

dependent and independent mechanisms of inhibiting HIV infection. Firstly, A3G is incorporated into budding virions and deaminates the viral genome during the first strand cDNA synthesis step of reverse transcription[87-89]. This results in the accumulation of G-to-A hypermutations of the viral genome and production of defective virions. A3G also inhibits the completion of reverse transcription, which results in the production of abnormal HIV cDNA fragments that are unable to integrate[90]. Deaminase domain mutants also limit HIV-1 replication[91, 92], which revealed a second deaminase-independent mechanism of A3G inhibition. This mechanism has been attributed to A3G's ability to inhibit translocation of RT along template RNA[93]. More recently, a role for A3G in enhancing CTL recognition of HIV-infected cells has been revealed, which suggested that the anti-viral effects of A3G may extend beyond inhibiting replication[94].

A3G expression varies from one cell type to another and exists in RNA-bound high molecular mass (HMM) and unbound low molecular mass (LMM) forms[95]. Analysis of the enzymatic activity of these forms revealed that the HMM form was less catalytically active than the LMM form[96]. Catalytic activity could however be restored with RNase digestion of the A3G-ribonucleoprotein (RNP) complex[96]. Furthermore, the abundance of each A3G form differed among resting and activated T cells and correlated with HIV infectivity, where activated T cells predominately expressed HMM A3G and resting T cells predominately expressed LMM A3G[96]. However, enzymatically active A3G must also be expressed in activated T cells as A3G-dependent hypermutation of the HIV-1 genome is observed in virus derived from these cells.

Cells detect pathogen associated molecular patterns (PAMPs) via toll-like receptors (TLR), nucleotide oligomerization domain-like receptors (NLR), and retinoic acid-inducible gene I-like helicases (RLH)[97-99]. In the case of viral infections, the most common PAMP is viral nucleic acid found either in endosomal compartments or in the cytoplasm (reviewed in [100]). The recognition of these pathogen markers leads to proinflammatory cytokine and type I interferon (IFN) secretion, which modulates anti-viral immune responses. Several of these cytokines, including IFN-α, IFN-γ, IL-2 and IL-15, have been shown to upregulate A3G expression[101-103]. Additionally, it was

recently reported that Influenza virus RNA induces A3G expression[104] and type I IFNs produced by TLR3 stimulated dendritic cells upregulate expression of a LMM form of A3G[105]. Thus, cells have the capacity to upregulate the intrinsic antiviral factor A3G in response to viral infections.

To limit the detrimental effects of A3G packaging into viral particles, the HIV-1 *vif* gene product targets A3G for proteasomal degradation in virus producing cells[87, 106, 107] (Figure 1.5). Vif functions as an adaptor protein to link A3G to a Cullin5/ElonginB/ElonginC E3 ubiquitin ligase complex[108]. This leads to poly-ubiquitination of A3G, targeting it to the proteasome for degradation[109-111]. However, Vif's effects on A3G are not absolute, as hypermutated genomes have been isolated from individuals infected with full-length HIV-1[112, 113]. In fact, minimal mutations induced by A3G are thought to benefit HIV-1 by promoting the acquisition of drug resistance[114, 115].

Cellular DNA damage response and NKG2D ligand expression

DNA mutations generated by nucleotide misincorporation during replication or breaks induced by environmental stress are commonly encountered by cells (reviewed by[116]). Limiting the detrimental effects of DNA damage is vital for cells to maintain genomic integrity. Cells achieve this stability through intricate mechanisms of arresting the cell cycle to halt DNA replication and repair damaged DNA (discussed below). In the event of irreparable damage, these same mechanisms trigger apoptosis in the damaged cell to prevent the passage of mutations to daughter cells.

As stated previously, uracil residues are incorporated in DNA as a result of dUTP misincorporation and cytidine deamination. Cells detect incorporated uracils and remove them via the base excision repair (BER) mechanism during S phase of the cell cycle (Figure 1.6) (reviewed by [117]). Uracils are first removed by the UDG enzymes UNG2, SMUG1, TDG and MBD4, resulting in an abasic site (AP-site). Because replication through the AP-site could potentially be mutagenic, AP-endonuclease 1 (APE1) cleaves the residual deoxyribosephosphate (dRP) and the DNA is repaired by short patch or long

patch mechanisms. In short patch repair, the dRP is removed and the missing nucleotide is filled in by DNA polymerase β (Polβ) and the ends are joined by DNA ligase. In instances where the dRP is not removed by Polβ, a long patch repair mechanism is used. In long patch repair, DNA polymerase δ/ε adds 2-8 nucleotides, which results in the displacement of a DNA "flap". This flap is then cleaved by flap-endonuclease 1 (FEN1) and the DNA is ligated. In the event of hyperuracilation of the genome, DNA fragmentation may result due to the removal of uracils located close to one another on opposite DNA strands[118]. These DNA fragments may activate DNA damage responses due to exposed stretches of ssDNA or free dsDNA ends (or double strand breaks, DSBs).

Exposed stretches of ssDNA are quickly stabilized by replication protein A (RPA) (Figure 1.7a) (reviewed in [119]). The RPA coated ssDNA then recruits the RAD9– RAD1– HUS1 (also known as 9-1-1) protein complex, the ataxia–telangiectasia mutated and RAD3-related (ATR) kinase, and ATR interacting protein (ATRIP) to the junction of the ssDNA and the exposed 5' primer. Loading of the 9-1-1 complex to the site of DNA damage leads to the recruitment of topoisomerase-binding protein-1 (TOPBP1), which then binds to and phosphorylates ATR. Activated ATR then phosphorylates a number of targets including checkpoint kinase-1 (Chk1) and the histone H2AX. These activated effectors ultimately lead to cell cycle arrest, replication fork stability and removal of the mutated DNA.

DSBs are encountered less frequently by cells yet have highly deleterious potential. In response to a DNA DSB, mammalian cells activate a signaling cascade aimed at recognizing the site of damage, propagating the signal and initiating repair of the damage. Central in the early events of DSB repair is the MRE11-RAD50-NBS1 (MRN) complex, which surveys the genome and associates at the ends of dsDNA breaks (Figure 1.7b) (reviewed in [120]). Once at the site of DNA damage, the MRN complex activates the ataxia–telangiectasia mutated (ATM) kinase. ATM is normally found in the nucleus as a dimer in association with protein phosphatase 2A (PP2A). Upon activation, PP2A dissociates and ATM monomers trans-autophosphorylate and associate at the site of damage[121]. Phosphorylated ATM then phosphorylates the histone H2AX (the

phosphorylated form referred to as γH2AX), which serves as a scaffold to recruit downstream repair proteins to the site of damage. Concurrently, ATM phosphorylates checkpoint kinase-2 (Chk2), which inhibits cell cycle progression to allow for repair of the damaged DNA. Cells undergoing replication most commonly repair DSBs through a mechanism of homologous recombination (HR) with the sister chromatid serving as template[122]. Quiescent cells typically repair DSBs by resecting the DNA ends and ligating them to one another via a mechanism of non-homologous end joining (NHEJ)[122]. The resected ends generate a stretch of ssDNA that can be recognized by RPA and activate ATR.[123, 124]

Although its role in HIV-1 infection remains unclear, a consistent phenotype of HIV-1 Vpr expression is arrest of the cell cycle at the G_2/M transition[125, 126]. Vpr expression activates the canonical ATR response, indicated by the accumulation of DNA repair foci containing γH2AX and RPA and the subsequent activation of Chk1[127]. Vpr has also been shown to activate ATM, indicated by γH2AX expression and Chk2 phosphorylation[128]. Vpr functions as an adaptor protein, linking cellular targets to a viral protein R binding protein (VprBP or DCAF1), damaged DNA binding protein 1 (DDB1) and cullin 4A (Cul4A) E3 ubiquitin ligase complex[129-131]. Although the ability of Vpr to colocalize with ubiquitinated proteins and target them for proteasomal degradation is necessary for γH2AX focus formation and cell cycle arrest[132], the repertoire of proteins targeted by Vpr is incompletely defined. Identification of the protein targets will allow for further characterization of the Vpr-mediated cell cycle arrest and lead to a greater understanding of the role of Vpr in HIV-1 infection. It has recently been shown that Vpr-mediated activation of an ATR response induces the expression of NKG2D activating ligands[133, 134], which may be a consequence of degrading one or many cellular targets.

Although their degradation does not appear to explain the G_2/M cell cycle arrest phenotype, several cellular targets of Vpr have been identified. Many of the proteins identified are part of cellular DNA repair responses, including UNG2, SMUG1 and HHR23A[135-139]. As stated previously, UNG2 and SMUG1 are UDG enzymes that remove uracil from DNA as a result of misincorporation or cytidine deamination.

HHR23A is the human homolog of the yeast Rad23 protein and is part of the nucleotide excision repair (NER) pathway, detecting DNA damage as a result of UV irradiation[140]. In the absence of Vpr, UNG2 is incorporated into newly formed HIV-1 virions through an interaction with integrase and is thought to limit HIV infection by degrading the genome[141]. Consistent with this hypothesis, it has been shown that Vpr partially rescues HIV-1 infectivity in the absence of Vif by reducing the detrimental effects of A3G[139] and that virion-associated UNG2 degrades A3G-edited HIV DNA[142]. Mutating the tryptophan at position 54 of Vpr disrupts UNG2 binding and partially reverses Vpr's ability to enhance infectivity[135, 143]. However, it was shown that HIV replication is unaffected by endogenous levels of UNG2 either in the presence or absence of A3G[144], which suggests A3G's capacity to inhibit HIV infectivity is not solely due to degradation of the genome.

Summary of dissertation

This dissertation improves our understanding of HIV's ability to evade immune recognition by NK cells. Although it is clear that HLA-C and –E molecules are unaffected by Nef and inhibit NK cell lysis[37], the role of NK cell activation in HIV infection is less defined. Ligands recognized by the NK cell activating receptor NKG2D are upregulated under conditions of cellular stress, including viral infection and DNA damage. Indeed the DNA damage response induced by the HIV-1 Vpr protein upregulates NKG2D ligand expression[133, 134]. However, I show that NKG2D ligand expression is limiting in HIV infection due functions of the Vif and Nef proteins.

One form of genotoxic stress that cells regularly encounter is the presence of uracil in their DNA, which occurs by misincorporation or cytidine deamination. Since the cytidine deaminase AID activates the DNA damage response and induces NKG2D ligand expression[52], it seemed likely that A3G expression would have a similar outcome. In fact, I find in this dissertation that A3G expression activates a DNA damage response and upregulates NKG2D ligands. However, maximal NKG2D ligand expression in HIV-infected T cells requires Vpr expression, which I attribute to Vpr's effects on the

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expression of the DNA repair proteins UNG2 and HHR23A. Therefore, the data presented in this dissertation suggest that A3G induces DNA damage that is stabilized by Vpr-mediated degradation of UNG2 and HHR23A.

This dissertation is organized into two additional chapters and two appendices. Chapter 2, which has been submitted for publication, analyzes the modulation of NKG2D ligands and NK cell recognition of HIV-infected primary T cells. It also further investigates the upregulation of NKG2D ligands and activation of the DNA damage response by A3G. In Chapter 3 I discuss these findings in detail and offer several avenues of future investigation. Preliminary evidence is presented in Appendix 1 supporting the hypothesis that the degradation of DNA repair enzymes by Vpr enhances NKG2D ligand expression. While much of this dissertation is focused on the inhibition of NKG2D ligand expression by Vif, Appendix 2 presents preliminary data on the mechanism of Nef-mediated NKG2D ligand inhibition.

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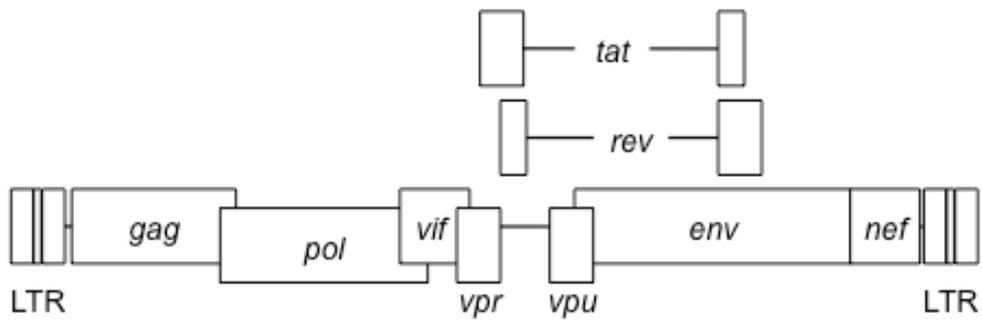
Figure 1.1: HIV-1 genome

Shown is a map of the HIV-1 ssRNA genome that is flanked by long terminal repeat (LTR) regions.
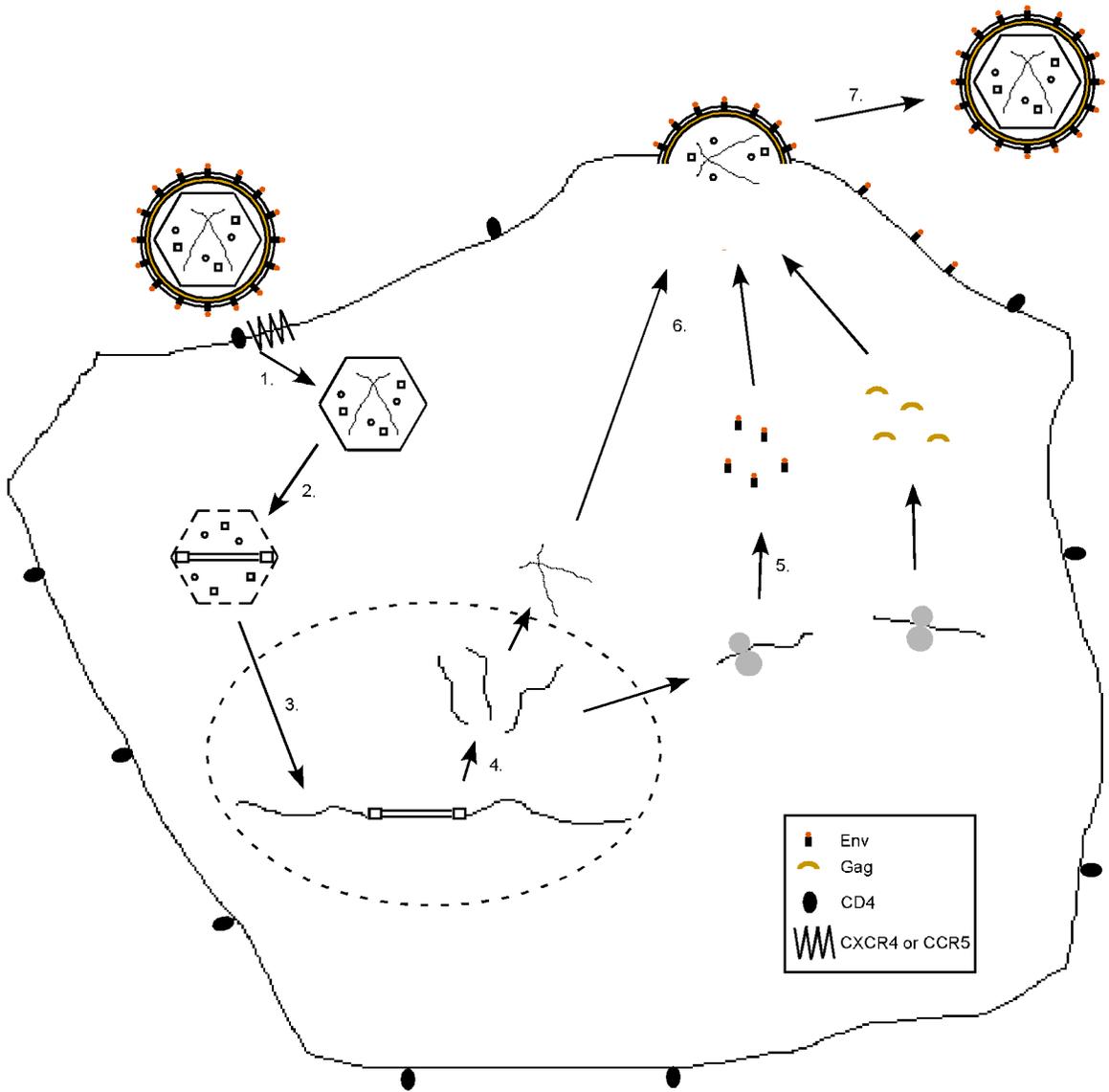
Figure 1.2: HIV-1 Replication

1. Receptor binding and membrane fusion. 2. Reverse transcription. 3. Integration. 4. Transcription and genome synthesis. 5. Translation. 6. Particle assembly and budding. 7. Virion release and maturation.
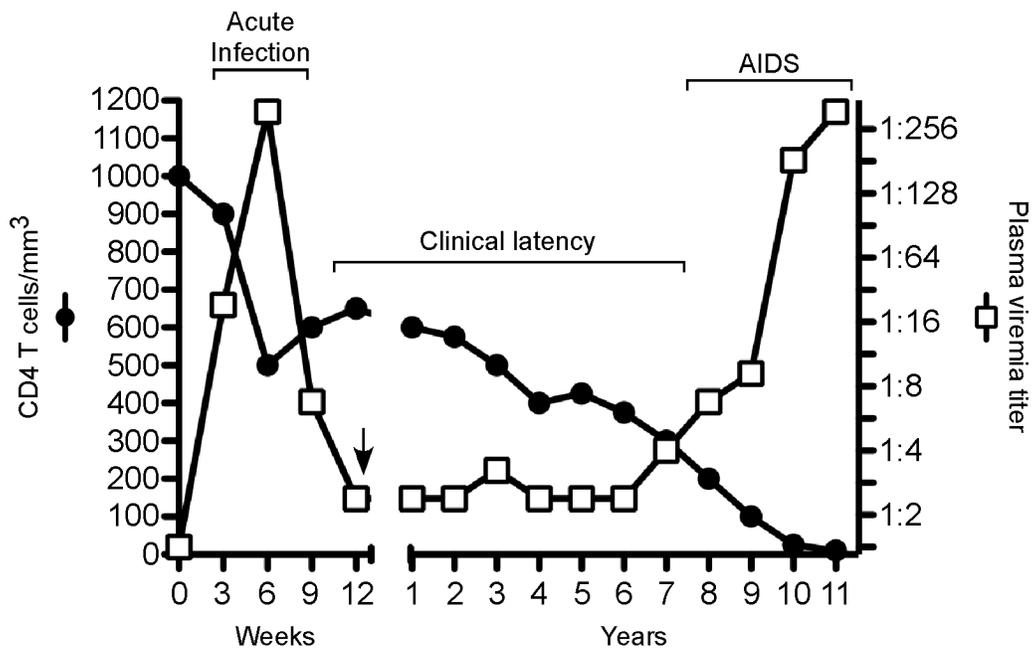
Figure 1.3: Natural course of HIV-1 infection

Plot of CD4$^+$ T cells/mm^3 (black circles) and plasma viremia titer (white squares) versus time. Viral set point is indicated by the black arrow. Adapted from[23].
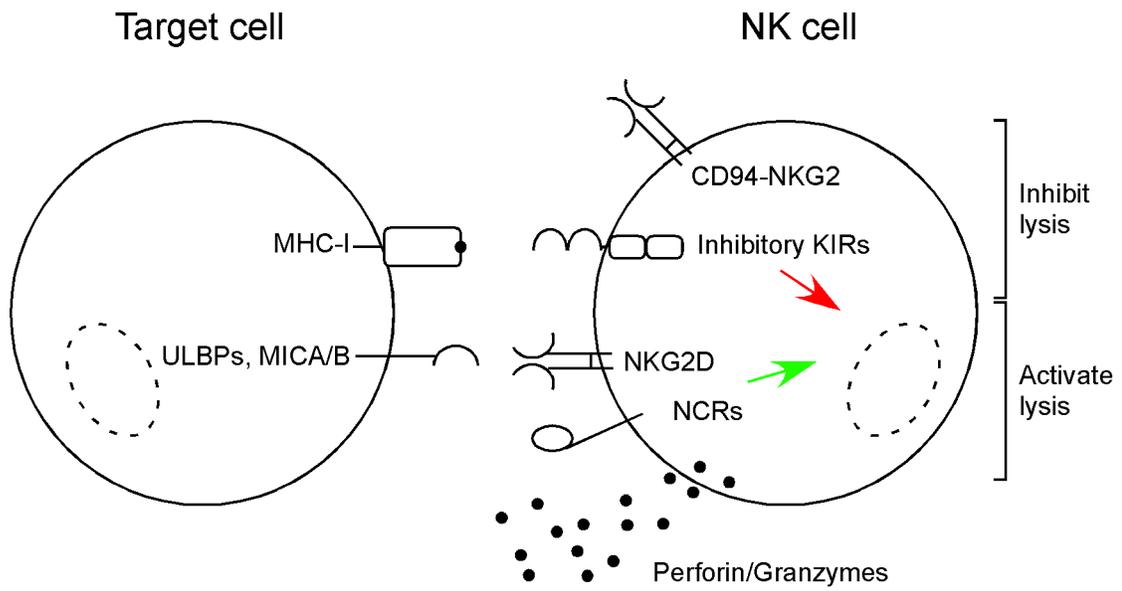
Figure 1.4: Natural killer cell recognition

NK cell inhibitory receptors transmit signals that inhibit NK cell lysis (red arrow) and activating receptors transmit signals that induce NK cell lysis (green arrow).
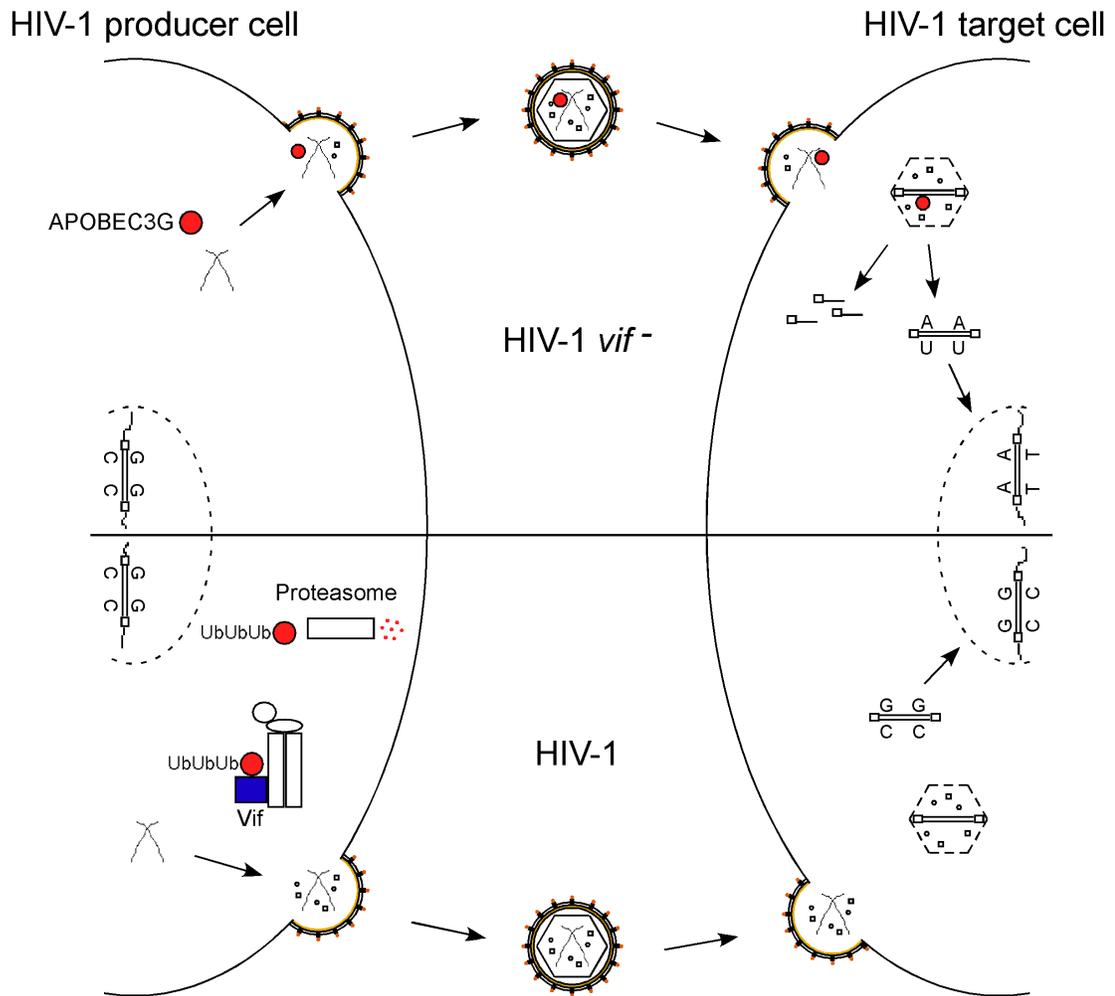
**Figure 1.5: HIV-1 Vif degrades APOBEC3G to limit virion incorporation**

APOBEC3G is incorporated into budding virions in the absence of HIV-1 Vif and inhibits infection of the target cell by inducing G to A hypermutation and inhibiting RT elongation. HIV-1 Vif targets A3G for proteasomal degradation by linking it to the Cul5/EloB/C E3 ubiquitin ligase complex, rendering the virus produced infectious. Adapted from[145].
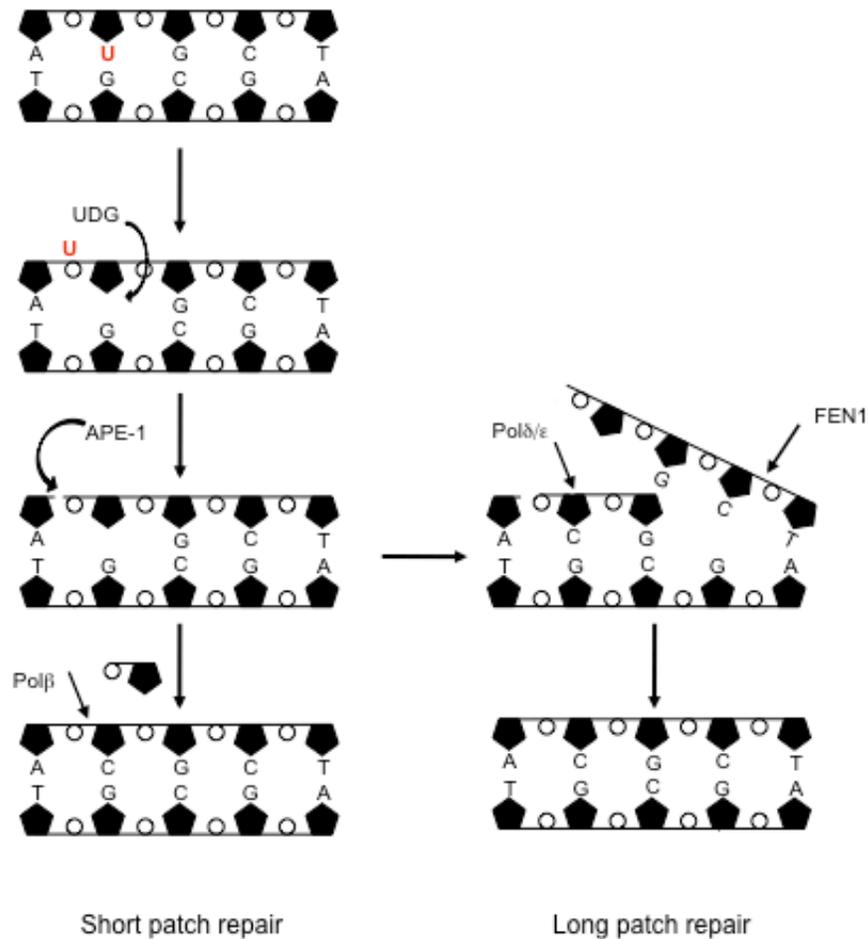
Figure 1.6: Base excision repair pathway

Simplified model of short patch and long patch base excision repair of uracil incorporation. Incorporated uracils are removed by uracil DNA glycosylase (UDG) enzymes and result in the formation of an abasic (AP) site. The AP site is repaired by cleavage of the DNA backbone by AP-endonuclease 1 (APE-1), which activates short patch or long patch repair mechanisms. DNA Polymerase β (Polβ) removes the abasic nucleotide and replaces it with the correct base in short patch repair. DNA Polymerase δ/ε (Polδ/ε) adds a stretch of 2-8 nucleotides in long patch repair, which displaces a flap of ssDNA that is removed by Flap-endonuclease 1 (FEN1). Adapted from[117].

Figure 1.7: ATR and ATM DNA damage responses

(a) Stretches of ssDNA are stabilized by replication protein A (RPA), which allows for the recruitment of several effector proteins to initiate repair. The ATR kinase is integral to this response and is phosphorylated by TOPBP1 at the site of DNA damage. Activated ATR can then phosphorylate the histone H2AX and Chk1. Phosphorylated Chk1 inhibits cell cycle progression and allows for repair of the damaged DNA. (b) The free ends of a dsDNA break are bound by the MRN complex of proteins, which then recruits phosphorylated ATM monomers to the site of DNA damage. ATM then phosphorylates H2AX, which serves as a scaffold to recruit additional effector proteins to the site of damage and to amplify the response. Activated ATM phosphorylates Chk2, which inhibits cell cycle progression and allows for repair of the DNA. Adapted from[119].
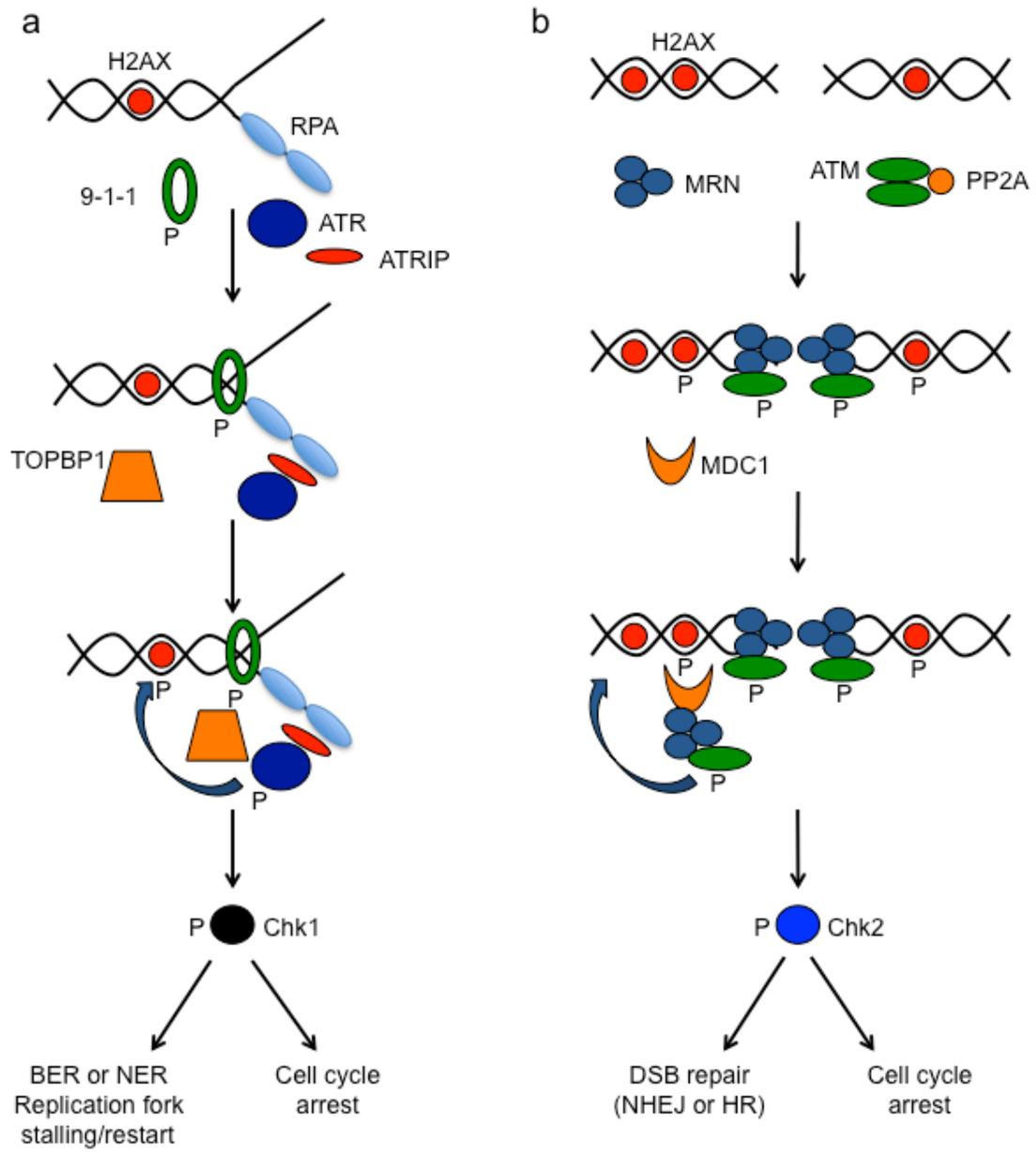
Figure 1.7: ATR and ATM DNA damage responses

Chapter 2

The antiviral factor APOBEC3G enhances natural killer cell recognition of HIV-infected primary T cells

Abstract

APOBEC3G (A3G) is an intrinsic inhibitor of human immunodeficiency virus (HIV) replication, which causes G-to-A hypermutations and inhibition of viral genome synthesis. HIV counteracts A3G through the activity of viral infectivity factor (Vif), which degrades A3G. Here, we show that A3G expression activates DNA damage response pathways and upregulates NK cell activating ligand expression. Vif mitigates these effects of A3G and reduces NK cell lysis. In HIV-infected cells, A3G-dependent upregulation of NK cell activating ligands and the DNA damage response also requires HIV viral protein R (Vpr). Our results reveal that A3G acts not only as an antiviral factor but also as an activator of the NK cell response.

Introduction

Following exposure to HIV, the acute phase of infection is characterized by high viral loads, which are counteracted by a rapid immune response[1]. The first line of defense against viral infections is the innate immune system, which uses pattern recognition receptors to detect pathogen associated molecular patterns. This response leads to cytokine secretion, which increases the expression of innate immune factors such as A3G that limit viral replication and spread[2]. Some studies have reported that induction of A3G expression can be regulated by IFN-α, IFN-γ and IL-2[2-4].

A3G is a member of a family of cytidine deaminases known as apolipoprotein B editing complex (APOBEC) proteins. One of these family members, activation-induced cytidine deaminase (AID) has important functions in both innate and adaptive immunity. AID edits B cell receptors and promotes class switching[5]. AID expression is upregulated in virally infected cells and has been shown to promote the expression of natural killer (NK) cell activating ligands by inducing the cellular DNA damage response[6, 7]. In addition to inhibiting HIV, members of the APOBEC3 family inhibit DNA viruses, endogenous retroviruses, and LINE-1 retrotransposition[8-13].

The mechanism by which A3G inhibits HIV replication is probably twofold. Firstly, A3G is packaged into HIV particles and deaminates C residues to U in the minus strand of the virus during first strand cDNA synthesis[14-16]. This editing leads to G to A hypermutation in the plus strand, which inactivates the virus. Secondly, a deaminase-independent mechanism of A3G inhibition has been revealed by the finding that A3G deaminase domain mutants also inhibit HIV infection[17]. This mechanism has recently been attributed to an ability of A3G and A3F to inhibit the translocation of reverse transcriptase along template RNA[18].

The viral infectivity factor (Vif) from HIV-1 counteracts the inhibition of viral replication by targeting A3G for proteasomal degradation, thus reducing the amount of A3G incorporated into virus particles[19, 20]. However, the action of Vif is not absolute and hypermutated viral genomes have been isolated from individuals harboring full-length

HIV-1[21, 22]. These sub-lethal mutations may influence HIV-1 evolution and promote the acquisition of drug resistance[23].

HIV-1–specific CD8$^+$ cytotoxic T lymphocytes (CTLs) are involved in the decrease of viremia during acute infection and chronic stages of the disease[24]. Despite this CTL response, most infected individuals control viremia poorly in the absence of antiviral treatment. Several cellular and viral factors contribute to the failure of the immune system to control HIV-1[1]. CTLs recognize peptides derived from exogenous or endogenous antigens presented by MHC class I molecules (MHC-I). One key factor, HIV Nef, protects HIV-infected cells from CTL recognition by disrupting antigen presentation by MHC-I[25]. Viral effects on MHC-I antigen presentation however may lead to enhanced recognition by NK cells, which are normally inhibited by MHC-I via the signaling of NK cell inhibitory receptors (reviewed in [26]). Additionally, viral infection can upregulate expression of NK cell activating ligands, which enhance NK cell recognition and target cell lysis. Upregulation of NKG2D ligands has been linked to expression of the HIV Vpr protein via its capacity to activate the DNA damage response pathway as measured by activation of the ATR kinase[27]. However, HIV infected cells are not efficiently recognized by NK cells[28, 29].

Here, we examined the role of A3G in recognition of HIV-infected cells by NK cells. We first studied NK cell recognition of full-length and Vif mutant HIV isolates. We then analyzed the role of A3G in the upregulation of NK cell activating ligands. Finally, we examined the role of the DNA damage response in linking viral infection to the cellular immune response. Altogether, our results uncover a capacity of A3G to activate the DNA damage response pathway and to upregulate NKG2D ligands. A3G expression enhances the ability of HIV-infected cells to activate NK cells. Therefore, A3G cytidine deaminase acts as an intrinsic antiviral factor not only by disrupting viral genome synthesis but also by regulating the recognition of HIV-infected cells by the immune system.

HIV-infected primary T cells are resistant to NK cell recognition unless NKG2D ligand is overexpressed.

HIV-1 Nef protects infected primary T cells from CTL recognition by downmodulating surface MHC-I expression[25, 30]. Although this activity of Nef provides an explanation for how HIV evades CTLs, the presence of low MHC-I levels also raises questions about whether infected cells become targets of natural killer (NK) cells. Normally, MHC-I molecules send inhibitory signals to NK cells that block the release of cytotoxic factors such as perforin and granzymes. A reduction in inhibitory signals through Nef-mediated MHC-I downmodulation may increase the sensitivity of infected cells to NK cell killing. To examine this possibility, we used a previously described flow cytometric killing assay[25, 31] to assess recognition of HIV-infected T lymphocytes by autologous NK cells.

In this assay system, target cells pre-labeled with CFSE are incubated with media or unlabeled, highly purified, autologous NK cells. Immediately afterward, the cell mixture is stained with antibodies directed against the NK cell markers, CD16 and CD56 as well as the vital dye 7-AAD, to discriminate live and dead cells. The number of 7AAD-negative, CFSE-positive cells is then scored as a function of a defined number of inert counting beads that are added just prior to flow cytometric analysis. When this assay system was tested using the highly sensitive K562 cells, which lack MHC-I and express high levels of NK cell activating ligands, we were able to demonstrate very efficient loss of living CFSE$^+$ target cells with the addition of primary NK cells (Figure 2.1).

To assess NK cell lysis of HIV-infected primary T lymphocytes, we infected cells with HIV expressing a GFP reporter inserted within *env* to distinguish infected from uninfected cells (Figure 2.2a). On day two of infection, autologous NK cells were mixed with the infected cells for a four-hour incubation and then analyzed to detect NK cell lysis as described for Figure 2.1. We found that a high fraction of HIV-infected primary

T lymphocytes survived treatment with autologous NK cells, whereas K562 cells were completely killed (Figure 2.2b and c). At the highest effector to target cell ratio tested, 47% of the HIV-infected primary T lymphocytes survived, compared with <1% survival of K562 cells (Figure 2.2c).

Because NK cell lysis depends on a balance of inhibitory and activating signals, we determined the relative contributions of NK cell activating ligand expression and residual MHC-I expression on the recognition of HIV-infected T lymphocytes by NK cells. To test this, we constructed an HIV genome that overexpressed the NKG2D activating ligand, ULBP1 (NL-GI*ulbp1*$^+$) plus or minus a full-length *nef* open reading frame (Figure 2.2a). Primary T cells infected with NL-GI*ulbp1*$^+$ expressed roughly 40-fold more ULBP1 on their surface compared to control infected cells (Figure 2.2d). We found that ULBP1 overexpression decreased the survival of HIV-infected cells approximately ten-fold suggesting that NKG2D activating factors were normally a limiting factor for NK cell lysis of HIV-infected primary T cells (47% survival versus 4.8% survival at the highest effector:target cell ratio; Figure 2.2b, right panels and quantified in Figure 2.2c).

We also examined NK cell recognition of infected, Nef-negative cells that had normal MHC-I expression levels. Interestingly, these cells were substantially more sensitive to NK cell lysis than mock treated cells (45% survival compared to 85% at a 5:1 effector to target cell ratio), which suggested that HIV infection did activate NK cell lysis even without MHC-I downmodulation (Figure 2.3a and b). These data are consistent with prior reports that HIV infection upregulates NKG2D ligands[32, 33]. However, overexpression of NK cell activating ligands by infection with NL-GI*ulbp1*$^+$*nef*$^-$ further increased killing and resulted in loss of most of the remaining cells.

Vif downmodulates NKG2D activating ligands.

To better understand what determines the levels of NKG2D activating ligands on HIV-infected primary T cells, we measured NKG2D activating ligand expression on cells infected with full-length HIV (NL-PI) or HIV mutated to disrupt the expression of each accessory protein (Figure 2.4a). To assay for total NKG2D ligand upregulation, cells

were stained with recombinant human NKG2Dfc. As shown in Figure 2.4b and c, HIV that encoded all of the accessory proteins (NL-PI, black histogram) induced the expression of NKG2D ligands in primary T cells by an average of 3.3-fold compared to mock-treated controls. However, expression was further increased in the absence of Vif (4.1-fold, $p < 0.02$) or Nef (3.9-fold, $p < 0.02$), demonstrating that Vif limited NKG2D ligand expression and confirming a previous report that Nef reduced NKG2D ligand expression[34]. This effect was not additive in the double mutant, *vif⁻nef⁻* (mean 3.9-fold expression relative to mock treated; $p < 0.05$ compared to NL-PI), suggesting that Vif and Nef were acting on the same pathway.

In contrast, NKG2D ligand expression was nearly the same as mock–infected cells in the absence of Vpr expression and was 3.4-fold less than NL-PI-infected cells (*p* < 0.002 compared to NL-PI, Figure 2.4b and c), confirming that Vpr expression was required for HIV-mediated NKG2D ligand upregulation[27]. Furthermore, examination of doubly mutated genomes revealed that all of the additional ligand upregulation observed in the absence of Vif and Nef was also dependent on Vpr expression (Figure 2.5a and b). Thus, both Vif and Nef counteracted Vpr-dependent NKG2D ligand upregulation, again suggesting that Vif and Nef were acting on the same pathway.

NKG2D ligand modulation correlates with NK cell susceptibility.

To determine whether the ability of Vif to counteract NKG2D ligand upregulation was functionally relevant, we asked whether Vif expression affected NK cell lysis of infected cells. As shown in Figure 2.6, an analysis of infected cells from five independent donors revealed that cells lacking Vif were significantly more sensitive to NK cell recognition than full-length HIV controls in a four hour cytotoxicity assay (mean survival 60% and 66% for full-length compared with 38% and 45% for infected cells lacking Vif, at effector to target ratios 5:1 and 2:1, respectively).   Thus, the ability of Vif to limit NKG2D ligand expression resulted in increased survival of HIV infected T cells.

NK cell lysis of HIV infected primary T cells depends on both MHC-I and NKG2D activating ligands.

MHC-I allotypes unaffected by Nef (HLA-C and HLA-E) have been shown to inhibit NK cell lysis[28]. To separate the inhibitory effects of residual MHC–I on NK cell recognition from the activating effects of ligand expression, we developed an assay system where surface expression of all MHC-I allotypes was reduced by knocking down β2-microglobulin (β_2M). In cells in which β_2M expression has been knocked-down, the MHC-I heavy chains are expressed poorly and accumulate in the endoplasmic reticulum[35]. As shown in Figure 2.7, β_2M knockdown reduced the surface expression of HLA-A2, HLA-B57, HLA-Cw4 and HLA-E molecules in a T cell line. In primary T lymphocytes, β_2M knockdown resulted in a 10-fold reduction of total MHC-I surface expression levels. We used these cells to assess NK cell lysis of T cells with moderately reduced MHC-I levels (Figure 2.8a and b, gray boxes). We found that non-selective reduction of MHC-I surface expression by β_2M knockdown alone resulted in a decrease in cell survival compared to control shRNA infected cells and was similar to that observed in full-length HIV infected cells (Figure 2.8c, compare black triangles 100%, black squares 54% and white triangles 64%). Non-selective reduction of MHC-I in full-length HIV infected cells led to a 22% decrease in survival at the highest E:T tested when compared to HIV infection alone (Figure 2.8c, from 64% to 42%, compare white triangles and white squares), which is consistent with previous reports that residual MHC-I inhibits NK cell lysis[28]. However, the survival decreased an additional 22% at the highest E:T tested in the absence of Nef when compared to full-length HIV (Figure 2.8c, from 42% to 20%, compare gray squares to white squares), consistent with the effect of Nef on NKG2D ligands (Figure 2.4c).

We then examined whether expression of Vpr was responsible for the increased sensitivity of infected versus uninfected cells, as NKG2D ligand expression is Vpr-dependent in HIV infection (Figure 2.4c). For these experiments, MHC-I was reduced using the shβ_2M system so that NK cell killing could be assessed in the absence of Nef-mediated selective MHC-I downmodulation and without Nef's effects on NKG2D ligand expression. Furthermore, cells were infected with NL-PI *vif*⁻*vpr*⁺*nef*⁻ to yield maximal

NKG2D ligand expression. These were compared to cells infected with NL-PI vif^+vpr^- nef^-, which expressed minimum NKG2D ligands and whose MHC-I was reduced by $\beta_2 M$ knockdown alone. As shown in Figure 2.9a and b, we did indeed note decreased target cell survival of infected (PLAP$^+$) versus uninfected (PLAP$^-$) cells with reduced MHC-I when Vpr (but not Vif or Nef) was expressed (Figure 2.9b, from 51% to 35%, compare black squares to white squares). In contrast, there was no significant decrease in cell survival in the absence of Vpr compared with uninfected cells with low MHC-I (Figure 2.9a and b, compare black squares and gray squares, 51% and 59%, respectively).

To determine whether the increased susceptibility to NK cells observed in infected cells expressing Vpr was due to NKG2D ligand upregulation, we asked whether this effect could be blocked by an anti-NKG2D antibody. As shown in Figure 2.9c, Vpr-expressing infected cells were more susceptible to NK cell lysis than uninfected cells with similarly reduced MHC-I (32% and 70% survival for infected and uninfected with IgG control antibody, respectively). However, in the presence of anti-NKG2D antibody, survival of Vpr-expressing, infected cells increased to that of uninfected cells (70% survival, Figure 2.9c, upper panel). In contrast, infected cells lacking Vpr survived better when co-incubated with NK cells (55% versus 32% survival for Vpr-expressing, Figure 2.9c, upper panel). Anti-NKG2D antibody further increased the survival of Vpr-negative infected cells (81% survival, Figure 2.9c, upper panel). This antibody also increased the survival of uninfected PHA-activated primary T lymphocyte control cells, which we and others have noted express low levels of NKG2D ligands [36 and data not shown]. As expected, for all conditions, the susceptibility of the cells to NK cell lysis was dependent on reduction of MHC-I expression via expression of sh$\beta_2 M$ and only minimal amounts of lysis were observed when cells were treated with the negative control shRNA (Figure 2.9c, lower panel).

A3G upregulates NKG2D ligands.

The cytidine deaminase A3G is an intrinsic inhibitor of HIV infection, causing hypermutation in the viral genome. The HIV Vif protein counteracts these protective effects by targeting A3G for degradation. Given Vif's ability to limit NKG2D ligand

expression and its role in degrading A3G, we asked whether A3G expression influenced NKG2D ligands. A3G expression has been shown to be upregulated by IFN-α, IFN-γ and IL-2[3, 4]. However, treatment of PBMCs with IFN did not result in increased NKG2D ligand expression (data not shown). Interestingly, we observed that when CD56⁻CD8⁻ PBMCs were incubated with conditioned supernatant from CD8⁻ PBMC cultures the surface expression of NKG2D ligands increased (Figure 2.10). Mock-infected cells treated with conditioned supernatant for 2 days expressed 2.3-fold higher NKG2D ligands than control treated cells (As shown in Figure 2.10b, left panels). Further analysis of the mock-infected supernatant treated cells revealed that there was a range of total A3G expression among donors and that incubation with conditioned supernatant increased A3G expression within each donor (Figure 2.10c). Interestingly, the relative difference in A3G among donors correlated strongly with increased NKG2D ligand expression (Figure 2.10d).

Furthermore, cells infected with a Vif-deficient HIV also expressed increased NKG2D ligands expression when cultured in conditioned supernatant (Figure 2.10b, 40% increase compared with control media and 7-fold increase compared with mock–infected cells with control media). Western blot analysis revealed that HIV containing 293T cell supernatant dramatically stimulated expression of A3G and that this increase was not secondary to expression of Vpr (Figure 2.11a). The magnitude of the increase varied across donors but was usually at least two-fold and was sometimes greater than ten-fold, especially in viruses lacking Vif (Figure 2.11b) or containing a mutant Vif (Vif $Y_{44}A$) that was unable to bind A3G[37].

Overall, we noted a strong correlation between the induction of NKG2D ligand expression and A3G levels for both infected and uninfected cells and a significant additional effect of infection (Figure 2.11c, mock $R^2 = 0.66$, NL-PI*vif*⁻ $R^2 = 0.84$, regression lines significantly different $p < 0.0001$). These data support the model that A3G expression determines the extent to which NKG2D ligands are upregulated in infected cells.

Cells infected with a Vif $Y_{44}A$ HIV, had similar NKG2D ligand expression as Vif-deficient virus, indicating that Vif's ability to limit NKG2D ligands was dependent

on A3G binding (Figure 2.11d, donors independent of Figure 2.4, NL-PI*vif⁻* $p = 0.003$, NL-PI Vif $Y_{44}A$ $p = 0.001$ when compared to NL-PI). These data provide direct evidence that A3G expression affects NKG2D ligand expression.

In a second cohort of donors we again observed that Vpr expression was also required for HIV-induced NKG2D ligand expression because levels of ligand were similar to mock-infected in NL-PI*vif⁻vpr⁻* infected cells (Figure 2.11d and Figure 2.5b, NL-PI*vif⁻vpr⁻* $p = 0.008$ when compared to NL-PI). Thus both A3G and Vpr contribute to maximal ligand expression in HIV-infected primary T lymphocytes.

A3G activates the DNA damage response in HIV infected primary T cells.

The cellular response to DNA damage results in phosphorylation of the cyclin-dependent kinases, ATR or ATM, leading to cell cycle arrest and repair of the damaged DNA (reviewed by [38]). Phosphorylated ATR and ATM phosphorylate the histone H2AX, which serves as a scaffold to recruit signaling molecules to the site of DNA damage and amplify the repair response. The signaling cascades initiated by ATR and ATM lead to phosphorylation of the checkpoint kinases, Chk1 and Chk2, respectively, which inhibit cell cycle progession and promote DNA repair. Recent studies have demonstrated a link between activation of the DNA damage response and upregulation of NKG2D ligands[39]. Therefore, we asked whether the DNA damage response pathway was activated in HIV-infected cells and whether this pathway could be linked to A3G expression and/or upregulation NKG2D ligands in HIV-infected cells. Indeed we observed that HIV infection increased phosphorylated Chk2 several fold and we noted further increases in Vif-deficient virus or Vif $Y_{44}A$ containing HIV (Figure 2.12a and b, NL–PI*vif⁻* $p = 0.045$ when compared to NL–PI). HIV infection and Vif expression also influenced Chk1 phosphorylation in some donors, although to a lesser degree than Chk2 (Figure 2.12a and c). Chk1 and Chk2 phosphorylation were affected by HIV (Figure 2.12a, b, and c) in a manner that mirrored the induction of NKG2D ligands in that it was Vpr-dependent and attenuated by Vif expression (compare Figure 2.12a to Figure 2.11d).

We also demonstrated that the magnitude of Chk2 phosphorylation correlated with A3G expression in both Vif-deficient HIV infected cells and mock infected controls

but was substantially higher in HIV-infected cells (Figure 2.12d, mock $R^2 = 0.75$, NL-PI*vif* $R^2 = 0.92$, lines significantly different $p = 0.01$). In contrast, when A3G expression levels were compared to Chk1 phosphorylation there was no significant correlation and NL-PI*vif* infected cells were indistinguishable from mock infected (Figure 2.12e, $R^2 = 0.02$, compare white and gray symbols).

Phosphorylated H2AX (γH2AX) is specifically associated with sites of DNA damage[40]. Therefore we determined whether HIV-infected primary T cells specifically upregulated the DNA damage response by analyzing γH2AX expression by western blot. Indeed, primary T cells infected with full-length HIV expressed γH2AX while mock-infected controls did not (Figure 2.13a, compare lanes 1 and 2). γH2AX expression was also determined for T cells infected with Vif-, Vpr- and Nef-deficient HIVs. There was little change in γH2AX expression in the absence of Vif or Nef compared to full-length HIV-infected cells (Figure 2.13a, compare lanes 2 through 5). However, NL-PI*vpr* infected cells expressed roughly 10-fold less γH2AX than NL-PI-infected (Figure 2.13a, compare lanes 2 and 4), suggesting that Vpr stimulates γH2AX. γH2AX expression in Vpr-deficient HIV infected cells remained 5-fold higher than in mock-infected cells (Figure 2.13a, compare lanes 1 and 4), indicating that Vpr-independent DNA damage response activation occurred in HIV-infected primary T cells.

To further determine whether A3G directly induced DNA damage in HIV-infected cells, CEM-SS CD4$^+$ T cells stably expressing A3G or a negative control cell line were infected with HIV and γH2AX was assayed by western blot. As shown in Figure 2.13b, mock-infected CEM-SS cells expressing A3G expressed 4-fold higher γH2AX than control CEM-SS cells, indicating that A3G expression alone activated the DNA damage response. In CEM-SS cells, HIV infection upregulated γH2AX expression roughly 11-fold compared to mock-infected controls (Figure 2.13b and c). However, HIV-induced γH2AX was roughly 3-fold higher in A3G expressing CEM-SS cells compared to negative control cells (Figure 2.13b and c). In some experiments, γH2AX expression was higher in Vpr-expressing HIV infected CEM-SS cells than in Vpr-deficient infected cells, although this was less consistent than in primary T cells.

Together these data indicated that A3G expression activated the DNA damage response and that it was amplified in HIV-infected cells.

Discussion

The APOBEC/AID family of cytidine deaminases are host factors that defend against pathogens. In B cells, AID is necessary for antibody class–switch recombination and somatic hypermutation. The "DNA damage" response that results leads to upregulation of NK cell activating ligands that transiently mark these cells as potentially dangerous[6]. APOBEC3 proteins offer a strong intrinsic antiviral defense, however whether these deaminases also alert the cellular immune system to viral attack by upregulation of NK cell activating ligands was unknown.

Recently it has been shown that there may be some overlap between adaptive and innate immune functions of the APOBEC3/AID family members. AID has been shown to be protective against a number of viral pathogens, including Abelson murine leukemia virus, hepatitis B virus, and Epstein–Barr virus[5], although the mechanism is unclear[7]. Whereas, mouse APOBEC3 is needed to efficiently generate neutralizing antibodies against Friend virus[41]. In these studies, it was proposed that mouse APOBEC3 might play a role similar to that of AID in shaping the antibody repertoire. Additionally, A3G has been shown to stimulate CTL recognition through the generation of defective translation products[42]. Here, we provide evidence for a new function for A3G. In addition to its protective effects on viral replication, we found that like AID[6], A3G sensitized cells to NK cell recognition by the upregulation of NKG2D ligand expression. The effect of A3G on NKG2D ligand expression was partially counteracted by the HIV Vif protein in a manner that depended on the A3G-interaction domain of Vif. It is intriguing to speculate that these effects on NKG2D ligands may also contribute to the A3G-dependent increase in CTL recognition[42], as NKG2D serves as a co-stimulatory molecule in CTLs[43, 44]. It will be interesting to determine whether there is a role of NKG2D in CTL recognition of HIV-infected cells.

Maximal upregulation of NK ligands also required HIV Vpr. This effect of Vpr did not appear to be secondary to increased A3G expression levels because induction of A3G expression was independent of Vpr expression. Upregulation of A3G expression by HIV containing cell supernatant has not previously been reported and the mechanism is unknown. It is interesting to speculate that HIV-1 ssRNA, which is recognized by TLR7/8 [45], induces the expression of A3G. A3G expression is upregulated by Influenza virus ssRNA[46], although it is unclear whether the upregulation of A3G involves PRR detection of virus. Further study is required to explore this and other potential mechanisms.

In addition, we found that both Vpr and A3G were required for maximal activation of the DNA damage response in HIV infected T cells. The effect of A3G was direct because it was inhibited by wild type Vif but not by Vif mutants defective at binding and degrading A3G. Furthermore, A3G expression in cell lines directly stimulated expression of the DNA damage marker γH2AX. One model that explains the synergistic effects of Vpr and A3G is that Vpr expression stabilizes A3G-generated mutations through its previously reported effects on DNA repair enzymes UNG2, SMUG1 and HHR23A[47, 48] (Figure 2.14). Further study will be needed to investigate these and other possibilities.

Interestingly, it has been shown that genetic variation at the A3G locus may predict disease progression in HIV infected people[49]. It will now be of interest to determine whether these genetic alterations influence the effectiveness of the NK response. Furthermore, the development of small-molecule inhibitors of the Vif-A3G interaction may help augment NK cell activity [50-52].

In conclusion, a number of host and viral factors likely affect the efficiency of NK cell killing and the role of NK cells in controlling HIV infection[53]. Studies of *KIR* and *HLA* allelic frequencies among cohorts of HIV seropositive individuals have provided strong evidence for a role of NK cells in limiting HIV infection. For instance, HIV-infected individuals that encode both the NK cell activating *KIR3DS1* allele and *HLA-Bw4-80Ile* progress to AIDS at a slower rate than individuals encoding one or neither of the alleles[54], which was later attributed to increased KIR3DS1 expression among *HLA-*

Bw4[+] donors[55]. Also, a study of female sex workers revealed that there was a higher incidence of *KIR* gene heterozygotes that lacked the cognate inhibitory *HLA* ligand among HIV-exposed seronegative individuals than seropositive, which may be due to a lower threshold of NK cell activation with reduced inhibitory signals[56]. These studies suggest that NK cell activity influences HIV infection and disease progression. The data presented here demonstrate that A3G expression enhances recognition of HIV-infected cells by natural killer cells through the activating receptor NKG2D. Thus, therapeutic strategies aimed at augmenting the anti-viral activity of A3G may have the added benefit of promoting immune clearance of virally infected cells.

Methods

Cell lines and culture conditions.

K562 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to their specifications. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from HIV seronegative donors (American Red Cross, Lansing, MI and New York Blood Center, New York, NY) by Ficoll gradient centrifugation. For NK cell isolation, whole PBMCs were cultured in R10 media and depleted of adherent cells for 90 minutes followed by immunomagnetic CD56$^+$ selection (EasySep, StemCell Technologies). NK cell cultures were at least 98% CD56$^+$ as determined by flow cytometry (data not shown). CD56$^+$ NK cells were cultured in RPMI 1640 supplemented with 10% human type AB serum (Fisher scientific) plus penicillin, streptomycin, L-glutamine and 500 IU/ml rhIL-2[57]. For viral infections, non-adherent PBMCs were depleted of CD56$^+$ and CD8$^+$ cells by magnetic sorting (CD56$^+$ EasySep, StemCell Technologies and CD8$^+$ Dynabeads, Invitrogen). Non–adherent, CD56$^-$ CD8$^-$ PBMCs were stimulated with phytohemagglutinin lectin (PHA 50μg/ml) and rhIL-2 (50 IU/ml) in R10 media for 48 hours prior to HIV-1 infection as previously described[58]. CD56 and CD8 depleted PBMCs were greater than 95% CD56$^-$ and 99% CD8$^-$ as determined by flow cytometry (data not shown). CEM-SS cells stably expressing A3G and negative control cells were cultured as described previously[59].

Lentivirus construction.

The PLAP reporter HIVs, HXBePLAP, NL-PI and *nef*$^-$, *vpr*$^-$, *vpu*$^-$, *vpr*$^-$*nef*$^-$ accessory protein mutants have been reported previously[25, 60]. NL-PI*vif*$^-$ and NL-PI*vif*$^-$ *vpr*$^-$ were generated by inserting a frame shift mutation in *vif* at M29 of NL-PI and NL-PI*vpr*$^-$, respectively. Briefly, the SpeI to SalI fragments of NL-PI and NL-PI*vpr*$^-$ were subcloned into pLitmus38 (New England Biolabs), the NdeI site at position 83 of *vif* was filled-in by Klenow polymerization, and the SpeI to SalI fragments were re-ligated into NL-PI and NL-PI*vpr*$^-$. This mutation generated a premature stop codon and blocked viral

infection of non-permissive CCRF-CEM cells but had no effect on the infectivity of permissive CEM–SS cells (data not shown). NL-PI*vif⁻nef⁻* was generated by inserting a frameshift mutation in NL-PI*vif⁻* by Klenow fill-in of the XhoI site at position 101 of *nef*. This Nef mutation eliminated MHC-I downmodulation as previously described[25].

The Y44A amino acid substitution of NL-PI Vif was generated by site directed mutagenesis. In brief, the SpeI-SalI fragment of NL-PI was cloned into pLitmus38. A point mutation was then introduced to generate an amino acid substitution at Tyr44 to Ala by PCR using Phusion HF polymerase (New England Biolabs) and the corresponding primers (Table 2.1, underlined sequence indicates mutation), producing a 701bp fragment from the NdeI site within *vif* to the SalI site within *vpr*. The mutated product was then cloned into the NdeI to SalI sites of pLitmus38 NL-PI and sequenced through the entire amplified region. The correct Y44A clone was then reintroduced into NL-PI using SpeI and SalI restriction sites.

FG12 shβ_2M, a short hairpin RNA (shRNA) expression construct targeting beta 2-microglobulin (β_2M) was cloned into the lentiviral vector FG12[61] as described previously[62]. Complimentary primers were annealed together and ligated into BglII and HindIII digested pRNAi[63]. The primer sequences used are indicated in Table S1 where the target sequence is underlined. The non-targeting FG12 shNC was used as a negative control[62]. To confirm knockdown of β_2M, CEM-SS cells stably expressing HA tagged MHC-I HLA-A*0201, HLA-B*5701, HLA-C*0401 and HLA-E allotypes[58, 64] were transduced with FG12 shβ_2M and assayed for HA surface expression at 5 days post transduction by flow cytometry (Figure 2.7).

pNL-GI*ulbp1*⁺ was constructed by two-step PCR amplification of IRES*ulbp1* (see below) and ligation into NheI and BglII sites within the *env* open reading frame of pNL-G (pNL4-3-deltaE-EGFP)[65].

Ulbp1 DNA was isolated from a human cDNA library (MegaMan Human Transcriptome Library, Stratagene) by nested PCR amplification and cloned into XbaI and XhoI sites of pLitmus29 (New England Biolabs) using *ulbp1* UTR forward 5'-TGTCCACGGAGCTCCAGGTCTA-3' and reverse 5'-AATGAGTGGCCTGA-

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GATGTTGAGC-3' primers followed by a second round PCR with *ulbp1* nested forward 5'-AGCTCTAGAGCCACCATGGCAGCGGCCGCCAGCCCCGC-3' and nested reverse 5'-CCGCTCGAGTCATCTGCCAGCTAGAATGAAGCAGA-3' primers. The IRES*ulbp1* cassette was then amplified by PCR using pLitmus29*ulbp1* and NL-PI as templates for *ulbp1* and IRES, respectively, and the primers indicated in Table 2.1. The final construct was confirmed by sequence analysis and by direct detection of ULBP1 protein using antibody staining and flow cytometric analysis of infected cells. To generate pNL-GI*ulbp1*$^+$ *nef*$^-$ and pNL-G*nef*$^-$ constructs, a frameshift mutation was inserted by Klenow fill-in of the XhoI site within *nef*. The absence of Nef was confirmed by the inability of the construct to downmodulate MHC-I alleles HLA-A and HLA-B as determined by flow cytometric analysis.

Virus production and T cell transduction.

HIV-1 molecular clones were produced in 293T cells by transfection using Lipofectamine 2000 (Invitrogen) or linear polyetheleneimine, M_T 25,000 (Polysciences, Inc.) as previously described[66]. VSV-G pseudotyped shRNA expressing lentiviruses were produced in 293T cells as previously described[62]. PHA stimulated primary T lymphocytes and CEM-SS cells were infected with virus in the presence of 8 μg/ml Polybrene as previously described[25]. In some experiments, the infected PHA-stimulated primary T lymphocytes were cultured in conditioned media isolated from mock-infected, non-adherent, CD8-depleted PBMCs. Conditioned media was added either 16 hours prior to spin infection or immediately after two hour spin infection and incubated for the duration of the experiment.

Flow cytometric analysis.

With the exception of NKG2D ligand staining, cells were stained in FACS buffer (PBS supplemented with 2% FBS, 1% human type AB serum plus 1% HEPES and 1% NaN$_3$) and fixed in 2% paraformaldehyde as previously described[58]. Antibodies to the following proteins were used for flow cytometry; PLAP (Sigma Aldrich and Serotec), ULBP1 (R&D Systems), CD3 (BD Pharmingen), CD56-PE-Cy5, CD16-PE-Cy5, CD8-

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Cy-Chrome (BD Pharmingen), CD4 (Serotec), CD4 (clone OKT4 [67]), CD4-AlexaFluor 488 (Caltag), MHC-I (clone W632), HLA-A2 (clone BB7.2 [68]), HA (Covance), HLA-Bw4, A9, A32 and HLA-Bw6, B77 anti-MHC–I (One Lambda). Isotype specific mouse immunoglobulin was used as negative control (BD Pharmingen). Following unconjugated primary antibody staining, cells were incubated with fluorescently conjugated isotype specific secondary antibodies (Invitrogen or Caltag).

For NKG2D ligand staining human serum was omitted from all buffers. Cells were incubated with soluble rhNKG2Dfc (R&D Systems) and anti-CD3 antibody diluted in FACS buffer for 20 min at 4°C, washed once in cold FACS buffer, incubated with fluorescently conjugated goat anti-human Fc F(ab')$_2$ fragments (Jackson Immunoresearch) and isotype specific goat anti-mouse secondary antibody diluted in FACS buffer for 20 min at 4°C then washed once in cold FACS buffer. Cells were then fixed in 2% paraformaldehyde and permeabilized with 0.1% TritonX-100 followed by incubation with fluorescently conjugated anti-Gag antibody (clone KC57, Beckman Coulter). NKG2D ligand expression was determined for CD3 positive PBMCs. All data was collected using FACScan or FACSCanto (BD Biosciences) instruments followed by analysis with FlowJo software (Tree Star, Inc.).

NK cell cytotoxicity assay.

NK cell cytotoxicity was determined by flow cytometry in a manner that was similar to a previously described method[25, 31]. Briefly, K562 cells, HIV-1 infected and mock treated primary T lymphocytes were labeled with 500 μM CFSE (Invitrogen) one-day prior to the NK cell assay and allowed to recover overnight at 37°C. For shRNA and NL-G experiments, the target cells were not CFSE-labeled due to GFP expression by these viruses. CFSE-labeled target cells were then co-incubated with purified, autologous CD56$^+$ NK cells at increasing effector:target ratios for 4 hours at 37°C. Cells were then washed in FACS buffer and stained for surface MHC-I, PLAP, CD56, CD16 and 7-amino actinomycin D (7-AAD, Calbiochem) and analyzed by flow cytometry. Live target cells were determined by light scatter parameters, 7-AAD exclusion, CD56$^-$/CD16$^-$/CFSE$^+$ staining. A constant number of fluorescent counting beads (Countbright

beads, Invitrogen) were added to each tube and counted during cytometric acquisition. The normalized number of live cells was calculated as the number of cells counted times the fraction of beads counted. The percent target cell survival was determined as the percent normalized target cells remaining after NK cell incubation. For some experiments, NK cells were incubated with 30 μg/ml anti-NKG2D (clone MAB149810, R&D Systems) or an IgG1 isotype control antibody for 30 minutes at room temperature and used in a 4 hr cytotoxicity assay, as previously described[36].

Western Blot Analysis.

48 hours post HIV infection, PBMCs or CEM-SS cells were washed in PBS, lysed in RIPA buffer, and sonicated as previously described[69]. Whole cell lysates were then normalized for total protein concentration, run on SDS–PAGE and analyzed by western blot using the following: phospho-Histone H2AX (S139), phospho-Chk1 (S345) and phospho-Chk2 (T68) polyclonal antibodies (Cell Signaling Technology), α-tubulin monoclonal antibody (Sigma), APOBEC3G antiserum[70], anti-Vif (#319) monoclonal antibody[71] and HIV-1$_{SF2}$ p24 antiserum. Membranes were then incubated with rat anti-mouse IgG$_1$-HRP or goat anti-rabbit IgG-HRP secondary antibodies (Invitrogen) and developed with the ECL Plus Western Blotting Detection kit (Amersham). Background subtracted median band intensities were determined using Adobe Photoshop software and were normalized to tubulin levels for quantification.

Statistical Analysis

Statistically significant differences were determined by paired *t* test. Linear and non-linear regressions were determined and compared using GraphPad Prism software.

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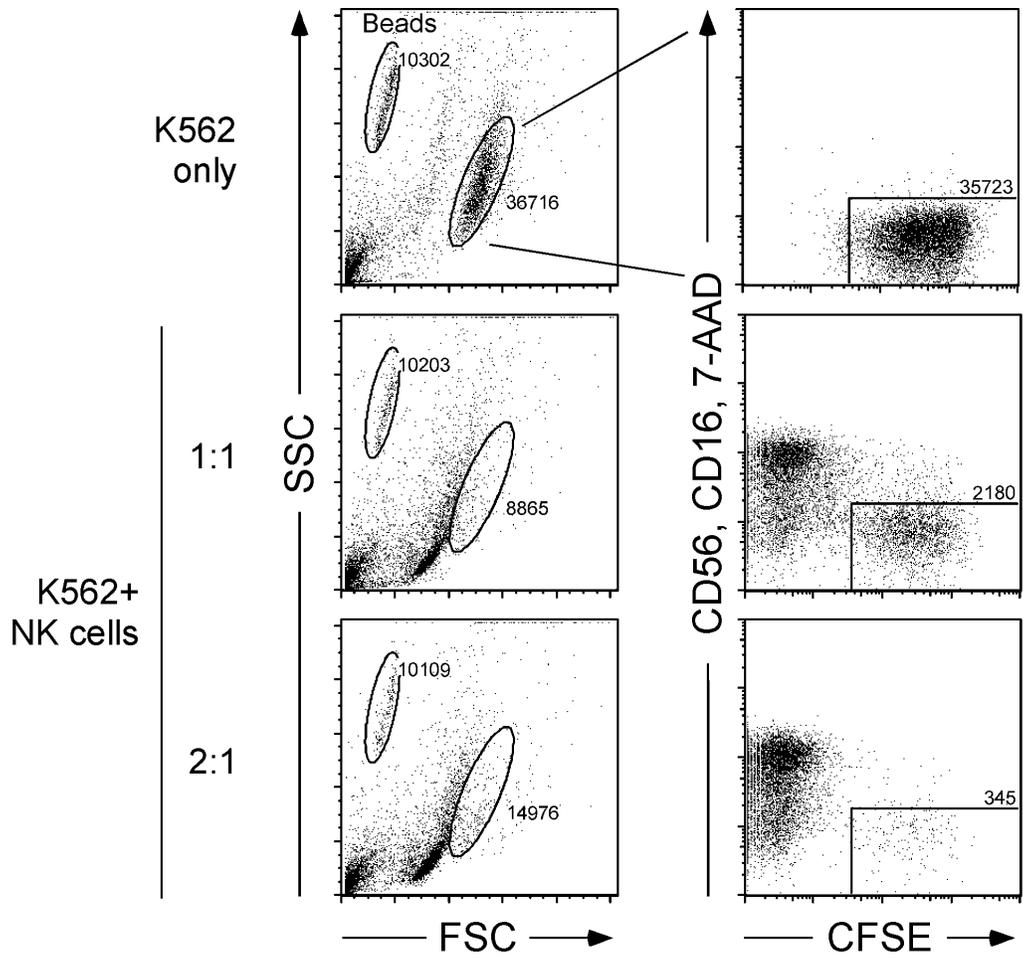
Figure 2.1: K562 target cell recognition via a flow cytometric NK cell cytotoxicity assay

CFSE labeled K562 target cells were co-incubated with IL-2 stimulated primary NK cells at increasing effector to target cell ratios in a 4 hr cytotoxicity assay. After co-incubation, cells were stained with 7-AAD, anti-CD56 and anti-CD16 (both PE-Cy5 conjugated antibodies) and assayed for FL3 and CFSE expression. Live CFSE$^+$, CD56-CD16-7-AAD-negative target cells were normalized to a constant number of inert counting beads added to each tube prior to FACS analysis.
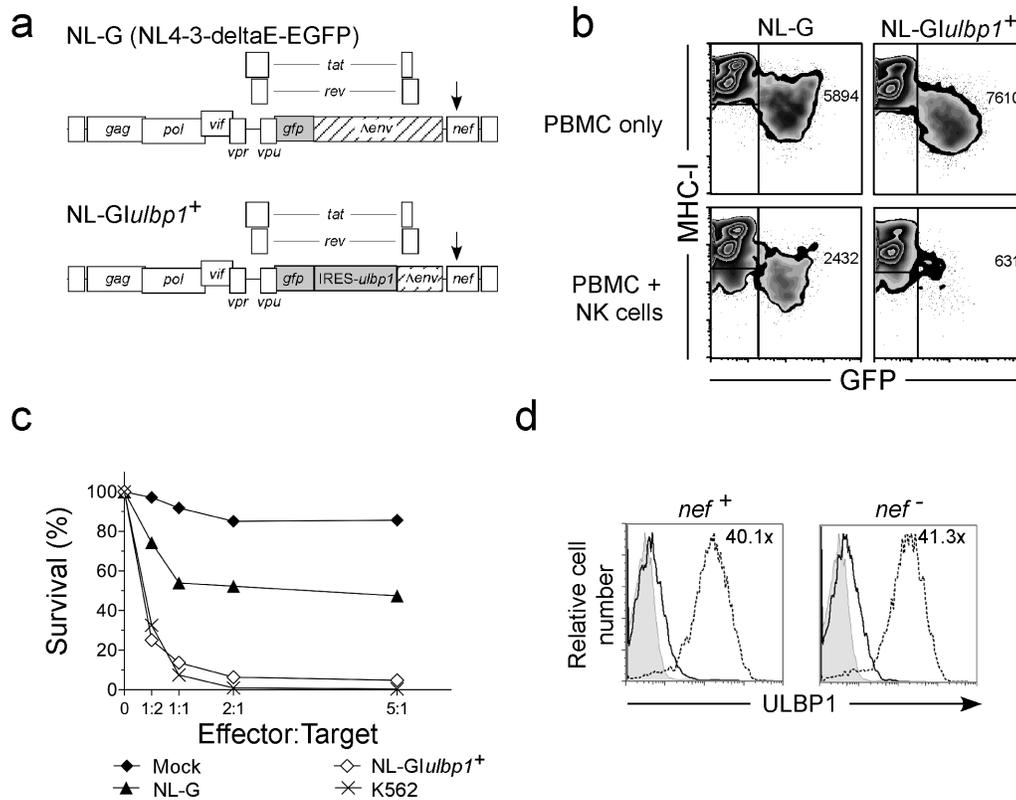
Figure 2.2: HIV–infected T cells are resistant to NK cell recognition unless an NKG2D ligand is overexpressed

(a) The NL4–3–deltaE–EGFP (NL–G) has been described previously[65]. NL–GI*ulbp1*+ was created by inserting an IRES*ulbp1* cassette within NL–G *env*. In some experiments, *nef*⁻ viruses were used, which contained a frame shift mutation in *nef* (indicated by the arrow). (b) PHA activated primary T cells were infected with the indicated virus and co–incubated with IL–2 stimulated autologous NK cells at a 2:1 effector to target ratio in a 4 hour cytotoxicity assay. Plots show MHC–I and GFP expression of 7–AAD⁻ CD56⁻ CD16⁻, live target cells. The total number of GFP⁺ cells normalized to counting beads is indicated. (c) Percent cell survival at increasing E:T ratios of HIV–infected cells, K562 and mock infected controls from part b. Data are representative of three independent donors. (d) PHA activated primary T cells were transduced with NL–GI*ulbp1*+, +/– *nef* or NL–G +/– *nef* control viruses. ULBP1 expression of the GFP positive cells transduced with NL–G control virus (solid black line), NL–GI*ulbp1*+ virus (dashed black line), and isotype control stained cells (solid gray histogram) were determined by flow cytometry. The fold change in ULBP1 expression relative to control virus is indicated. Data are representative of two independent donors.
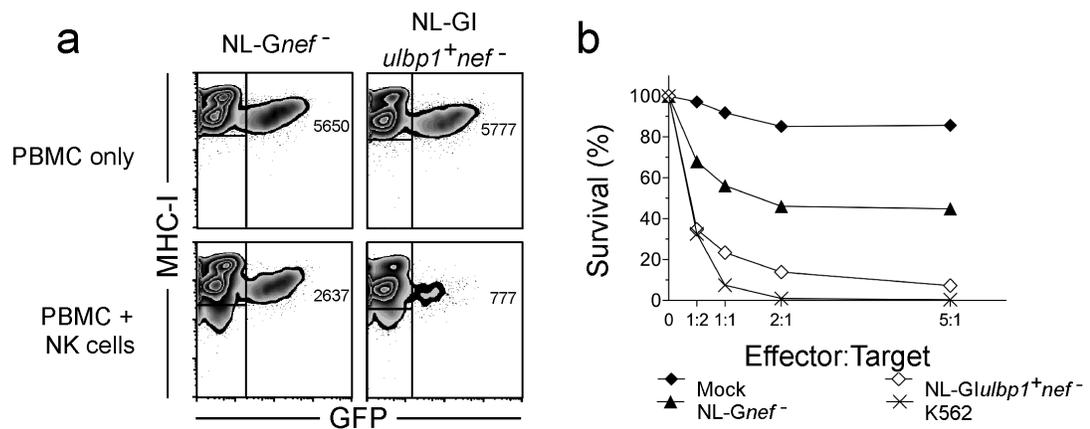
Figure 2.3: NKG2D ligand overexpression induces NK cell lysis in cells independent of reduced MHC-I.

(a) PHA activated primary T cells were infected with the indicated viruses and co-incubated with autologous NK cells as in Figure 2.2b. Plots show MHC–I and GFP expression of 7–AAD⁻ CD56⁻ CD16⁻, live target cells. The total number of GFP⁺ cells normalized to counting beads is indicated. (b) Percent cell survival at increasing E:T ratios of HIV–infected cells, K562 and mock infected controls from part a. Data are representative of two independent donors.
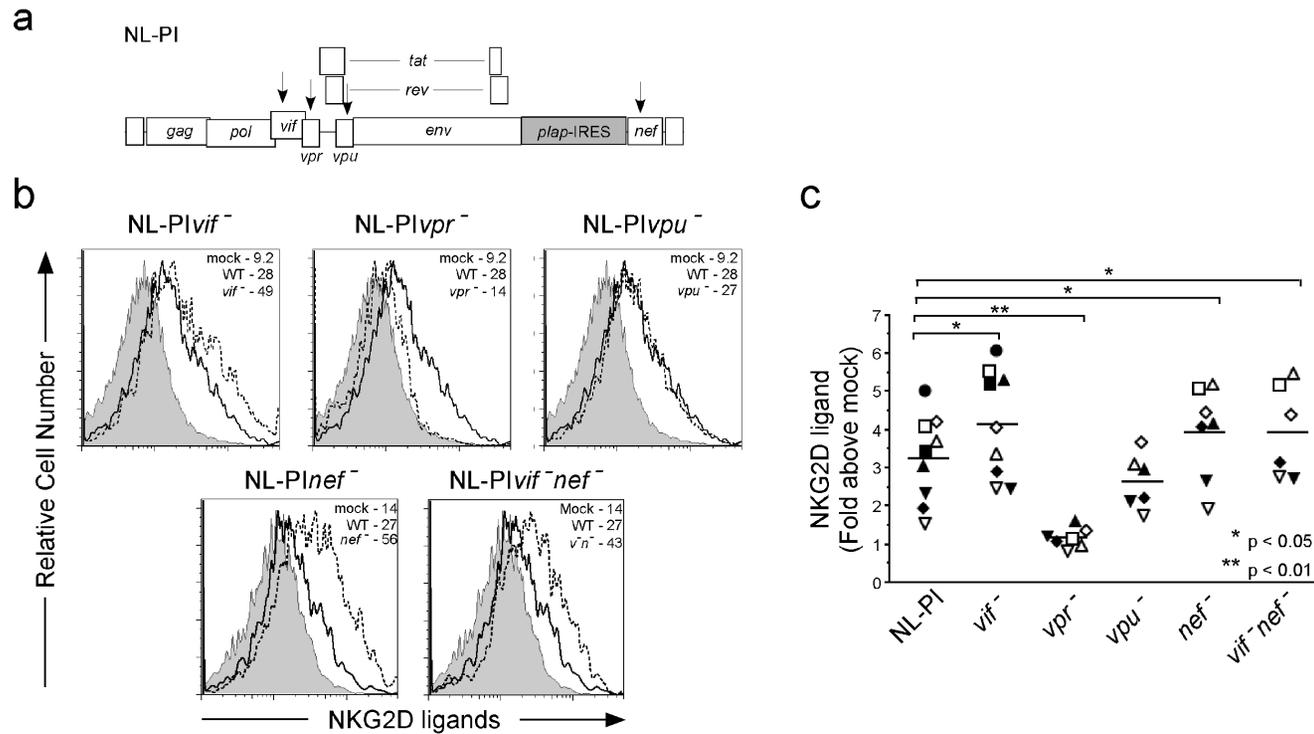
Figure 2.4: NKG2D ligands upregulation by HIV depends on Vpr and is attenuated by Vif.

(a) The NL–PI virus expresses PLAP to mark infected cells; accessory protein mutants were made of these constructs and are indicated by the black arrows[25, 60]. (b) Total NKG2D ligand expression of primary T cells infected with NL–PI (solid black line), NL–PI accessory protein mutants (dashed black line) and mock treated control (shaded gray histogram). Cells were analyzed for rhNKG2Dfc staining and mean fluorescence values are shown for all conditions. For all samples, only alive, CD3$^+$ cells were gated. (c) Fold difference in NKG2D ligand expression relative to mock treated controls for independent donors, each represented by a different character. Black bars represent the mean change in ligand expression for each virus. Statistical analyses were calculated by paired t test of accessory protein mutants compared to NL–PI.
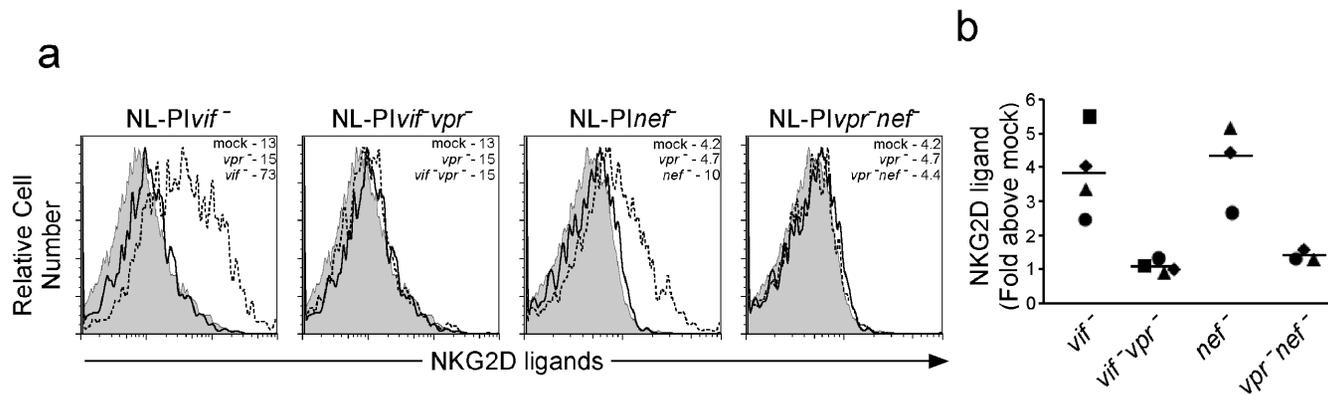
Figure 2.5: Vif and Nef limit NKG2D ligands upregulation by Vpr.

(a) NKG2D ligand expression of primary T cells infected with NL–PI*vpr⁻* (solid black line) or the indicated NL–PI accessory protein mutant (dashed black line) and mock treated controls (shaded gray histogram). Cells were stained with rhNKG2Dfc and mean fluorescence values are shown for all conditions. (b) Fold difference in NKG2D ligand expression relative to mock treated controls for independent donors, each represented by a different character. Black bars represent the mean change in ligand expression for each virus. Statistical analyses were calculated by paired *t* test of accessory protein mutants compared to NL–PI.
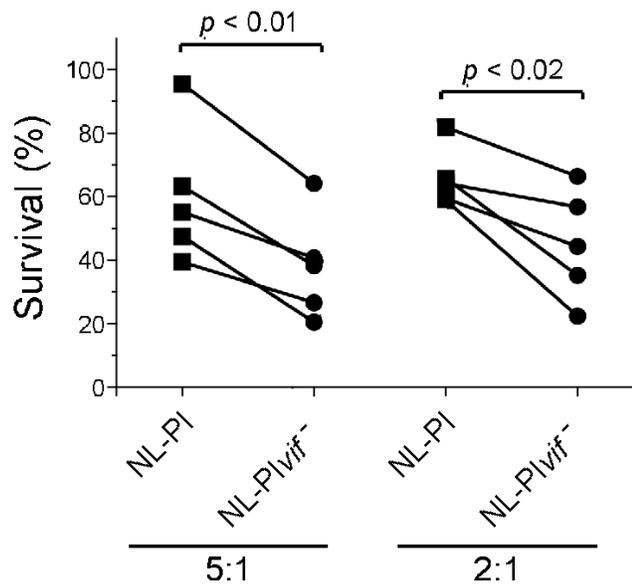
Figure 2.6: Vif limits NK cell recognition of HIV-infected T cells.

Activated, primary T cells were infected with NL–PI +/– *vif*, labeled with CFSE, and co–incubated with IL–2 stimulated autologous NK cells at a 5:1 and 2:1 effector to target cell ratios in a 4 hr cytotoxicity assay. The percentage of cells surviving NK cell co–incubation from five independent donors is shown.
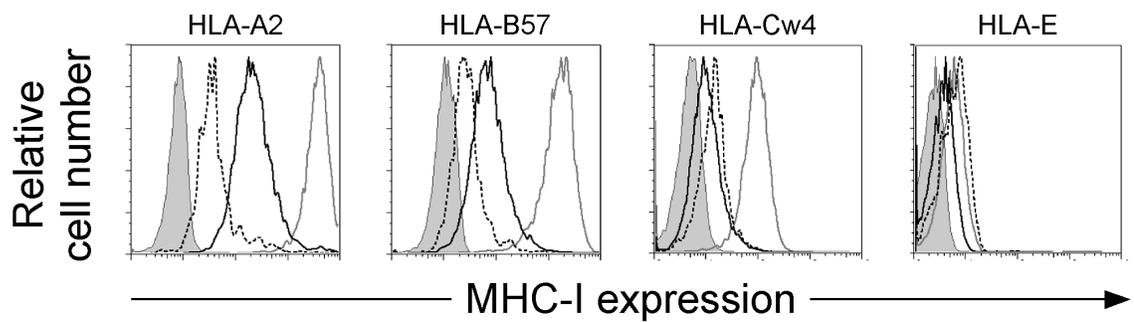
Figure 2.7: β_2M knockdown reduces MHC-I surface expression.

CEM-SS cells stably expressing HA-tagged MHC-I alleles HLA-A2, HLA-B5701, HLA-Cw4 or HLA-E [64, 72] were transduced with FG12 shNC (solid gray line), FG12 shβ_2M (solid black line) or FG12 shβ_2M plus HxBePLAP (dashed black line) and stained for HA surface expression. Isotype control staining is indicated by the filled gray histogram.
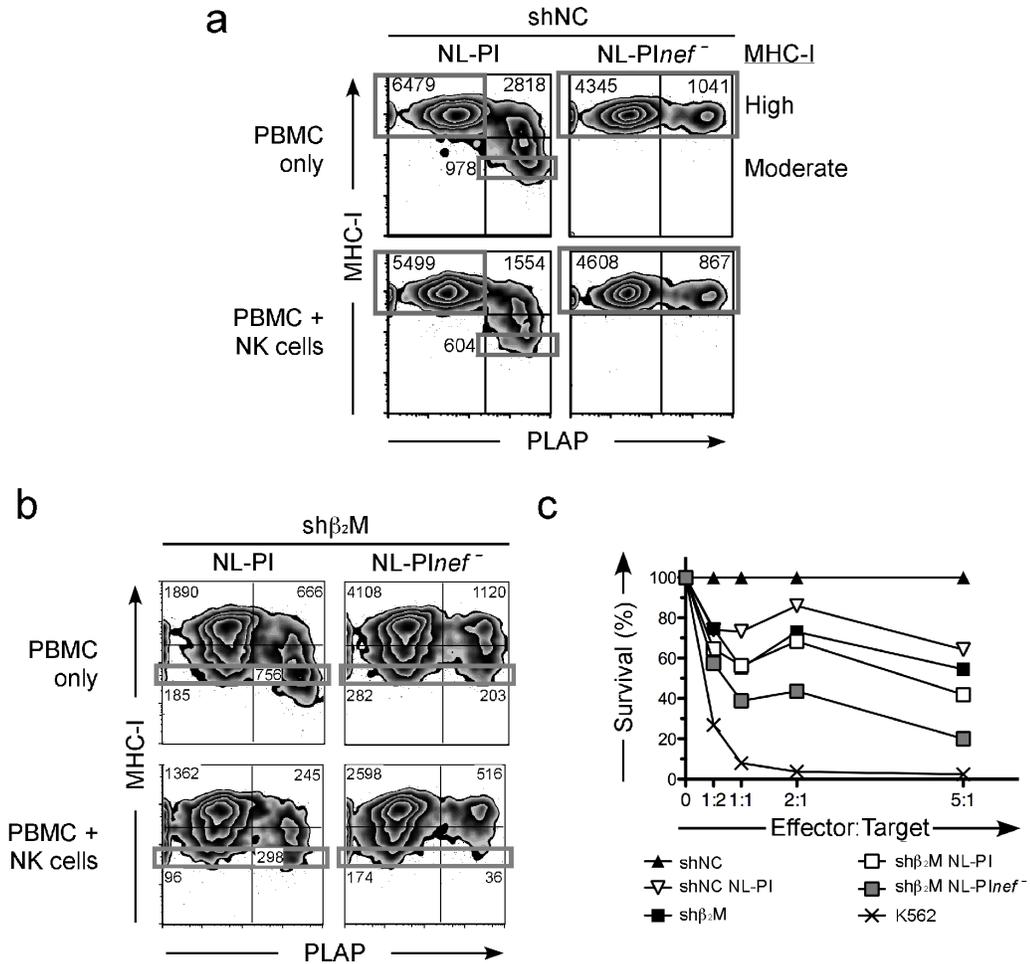
Figure 2.8: Nef limits NK cell recognition of HIV-infected T cells.

Activated, primary T cells infected with NL–PI or NL–PI*nef* plus a lentivirus encoding (a) negative control or (b) β_2M–targeting shRNAs were co–incubated with IL–2–stimulated, autologous NK cells at a 5:1 effector to target ratio in a 4–hour cytotoxicity assay. Flow cytometry plots showing PLAP and MHC–I expression of shRNA transduced (GFP^{+}), CD56^{−} CD16^{−} 7–AAD^{−}, live target cells are presented with and without NK cells. The numbers of target cells within the high (upper quadrants) and moderately reduced (bold gray boxes) MHC–I gates normalized to counting beads are indicated. (c) The percentage of cells surviving NK cell treatment at increasing E:T ratios for NL–PI and NL–PI*nef* infected T cells and K562 cells. Values were normalized to the percentage of mock–infected T cells surviving when transduced with the shNC lentivirus alone. Experiments were repeated with two independent donors and representative data are shown.
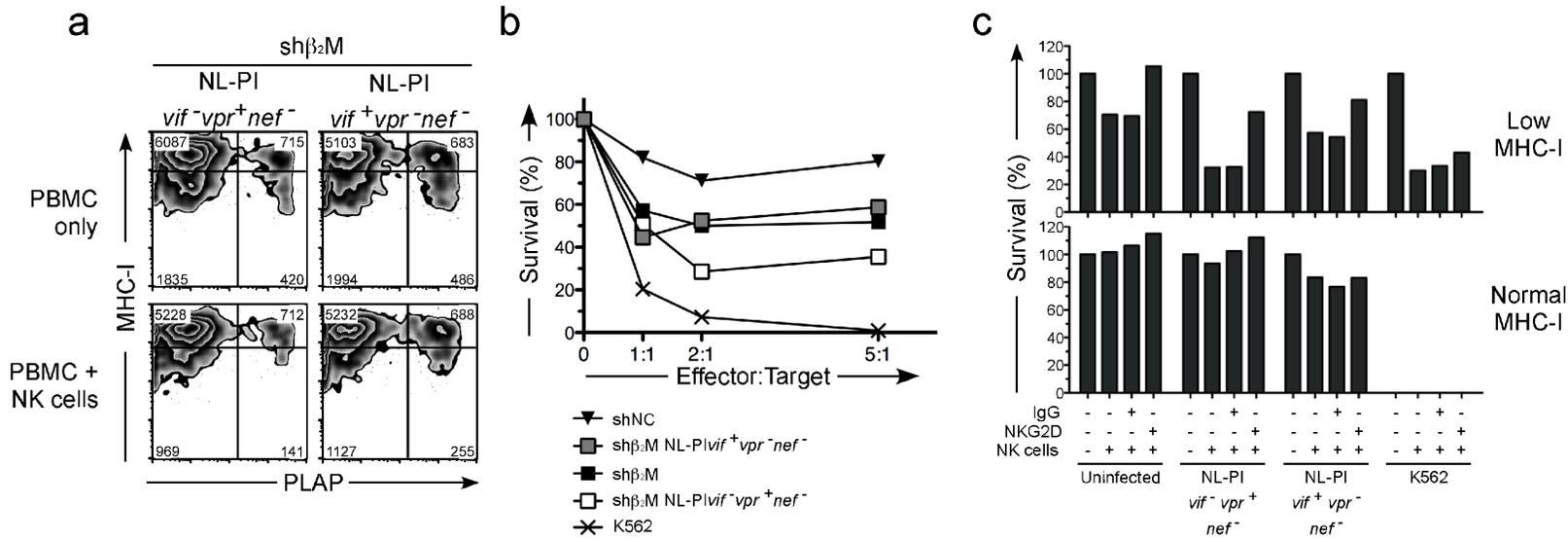
Figure 2.9: Vpr-dependent NKG2D ligand expression induces NK cell recognition of HIV-infected T cells.

(a) Activated, primary T cells were infected with NL–PI*vif⁻vpr⁺nef⁻* or NL–PI*vif⁺vpr⁻nef⁻* plus a lentivirus expressing β2M–targeting or control shRNA and were then co–incubated with autologous NK cells in a 4–hour cytotoxicity assay. PLAP and MHC–I expression of, CD56⁻ CD16⁻ 7–AAD⁻, live target cells incubated with and without NK cells is shown. The values indicate the number of cells normalized to counting beads within each quadrant. (b) The percentage of cells surviving NK cell treatment at increasing E:T ratios. For this analysis, data was compiled only from the shβ2M–expresing cells with low MHC–I or high MHC–I for shNC treated cells. Data are representative of two independent donors. (c) Activated, primary T cells were infected as in part D and then co–incubated with autologous NK cells, which had been pre–incubated with isotype control, anti–NKG2D or no antibodies. The NK cells were added at a 1:1 ratio with the autologous primary T cells. The percentage of cells surviving a 4–hour co–incubation was calculated. Uninfected, CFSE–labeled K562 cells were included as control and the percentage of CFSE⁺, CD56⁻ CD16⁻ 7–AAD⁻, live cells is shown.
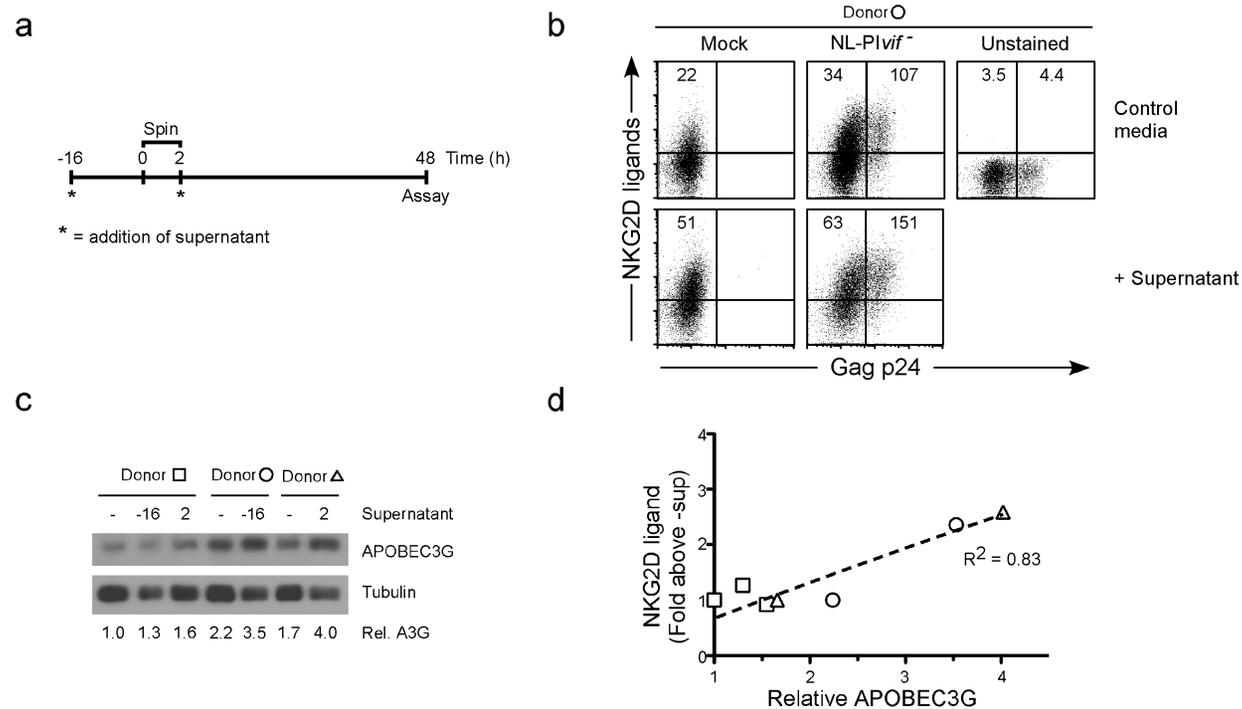
Figure 2.10: Induction of A3G with conditioned supernatant upregulates NKG2D ligands.

(a) Experimental time line. PHA activated CD56⁻CD8⁻ cells were treated with conditioned supernatant from CD8⁻ PBMCs at the times indicated, infected with HIV, and assayed by flow cytometry and western blot at 48 hours post infection. (b) NKG2D ligand and Gag expression of mock or NL–PI*vif⁻* infected CD3$^+$ cells treated with conditioned supernatant or control media. The mean fluorescence intensity is indicated for the total Gag⁻ and Gag$^+$ populations. (c) Western blot analysis of whole cell lysates from three independent donors treated with conditioned supernatant (-16hr or 2hr) or media alone (-). Band intensities were quantified (Adobe Photoshop) and relative A3G expression was determined after normalizing for α-tubulin expression. (d) Plot of relative A3G expression from part c versus NKG2D ligand normalized to control media treated cells. Each symbol represents data from an independent donor and the linear best-fit line is indicated.
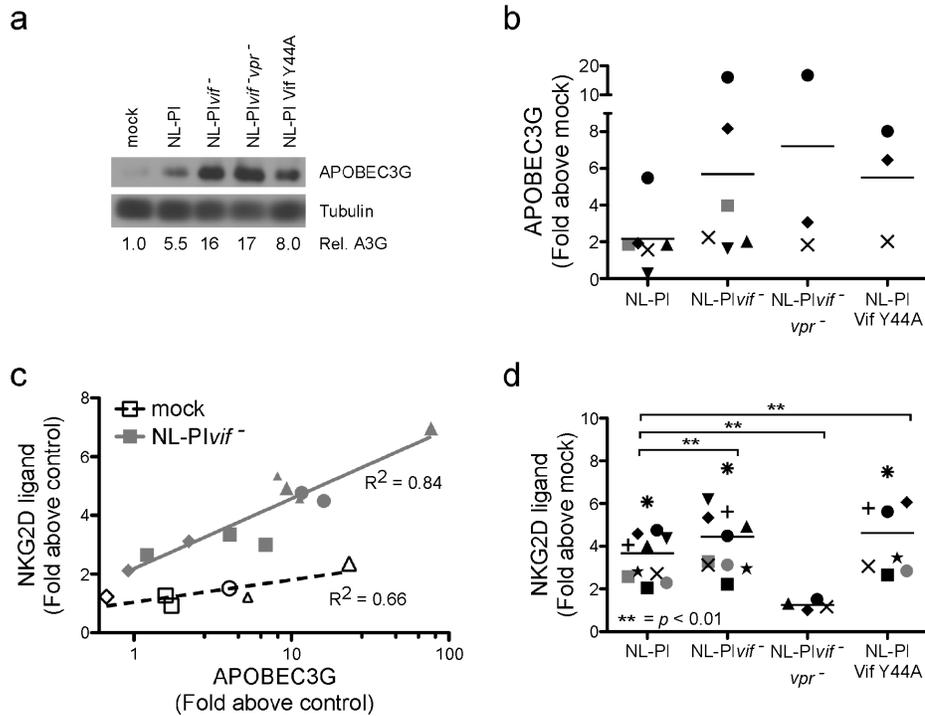
Figure 2.11: Induction of A3G with HIV infection upregulates NKG2D ligands.

(a) Western blot analysis of whole cell lysates from PHA activated primary T cells infected with NL-PI or accessory protein mutants. Band intensities were quantified (Adobe Photoshop) and relative A3G expression was determined after normalizing for α-tubulin. (b) Plot of A3G expression of NL-PI or accessory protein mutant infected cells normalized to mock infected for several independent donors (indicated by different symbols). Mean fold change in A3G expression is indicated by the black bars. (c) Plot of A3G expression versus NKG2D ligand expression of mock infected (white symbols) or NL-PI*vif-* infected (gray symbols) cells for several independent donors (indicated by different symbols). Data are normalized to mock infected, control media treated cells for each donor and linear correlation is indicated. Mock $R^2 = 0.66$, NL-PI*vif-* $R^2 = 0.84$. (d) NKG2D ligand expression of NL-PI or accessory protein mutant infected T cells from several independent donors (indicated by different symbols, all donors are different from those shown in Figure 2) normalized to mock infected controls. Mean change in ligand expression is indicated for each virus by the black bars. Statistical analyses were performed by paired *t* test comparing accessory protein mutant to wild type (NL-PI) infected. ** indicates $p \leq 0.01$.

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Figure 2.12: Activation of the DNA damage response in HIV infected primary T cells depends on A3G expression and is attenuated by Vif.

(a) Representative western blot analysis of whole cell lysates from PHA activated primary T cells infected with the indicated virus. (b-e) Summary of western blot analyses. Data were derived from western blot analyses quantified using Adobe Photoshop software and normalized to α-tubulin expression. (b) Chk2 phosphorylation at T_{68} and (c) Chk1 phosphorylation at S_{345} were measured for eight independent donors, each represented by a different symbol. Mean fold change relative to mock for each virus is represented by the black bars. Statistical analyses were performed by paired t test comparing each mutant virus to full-length NL-PI infected. * indicates $p \leq 0.05$. (d and e) A3G expression versus (d) Chk2 phosphorylation at T_{68} or (e) Chk1 phosphorylation at S_{345} for five independent donors each represented by different symbols. Mock infected samples plus conditioned supernatant are represented by open symbols. Infected samples plus or minus conditioned supernatant are represented by gray symbols. Data from each donor were normalized to results from autologous mock-infected cells treated with control media. Separate mock and NL-PI*vif* linear regressions are indicated for part d. Mock $R^2 = 0.75$, NL- PI*vif* $R^2 = 0.92$, $p = 0.011$. Linear regression of the combination of mock and NL- PI*vif* infected is indicated for part e. Total $R^2 = 0.015$.
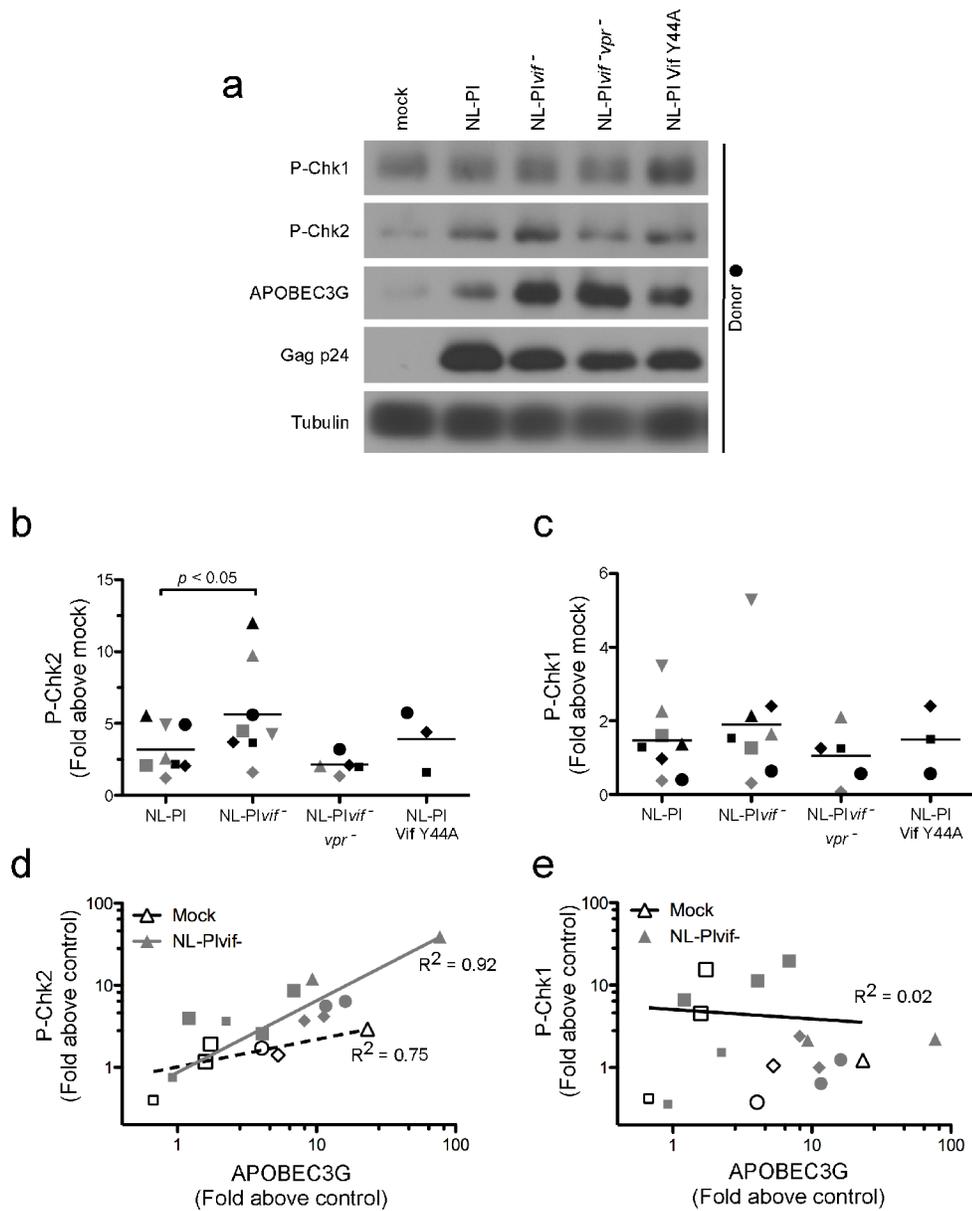
Figure 2.12: Activation of the DNA damage response in HIV infected primary T cells depends on A3G and Vpr expression and is attenuated by Vif.
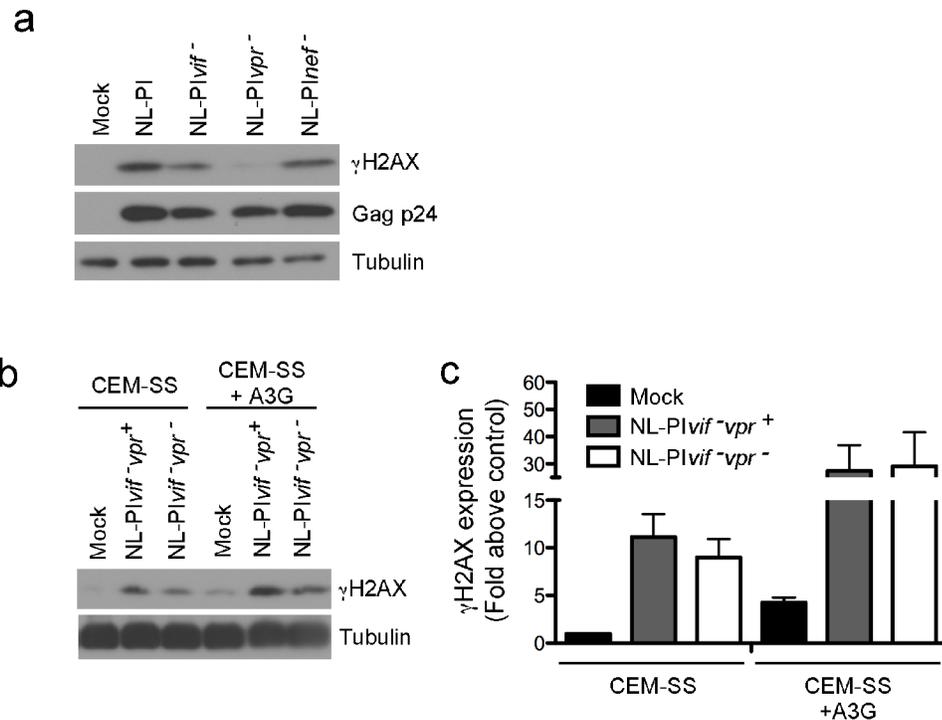
Figure 2.13: Phosphorylation of the DNA damage marker H2AX is induced by A3G and HIV infection.

(a) Representative western blot analysis of whole cell lysates from PHA stimulated primary T cells infected with the indicated viruses. (b) Representative western blot analysis of whole cell lysates from CEM-SS cells expressing A3G or negative control cells infected with the indicated viruses. Data are representative of three independent experiments (c) Summary of western blot analysis from part b. Data were derived from western blot analyses quantified using Adobe Photoshop software and normalized to α-tubulin expression. Mean fold change in expression above mock-infected CEM-SS control cells is represented.
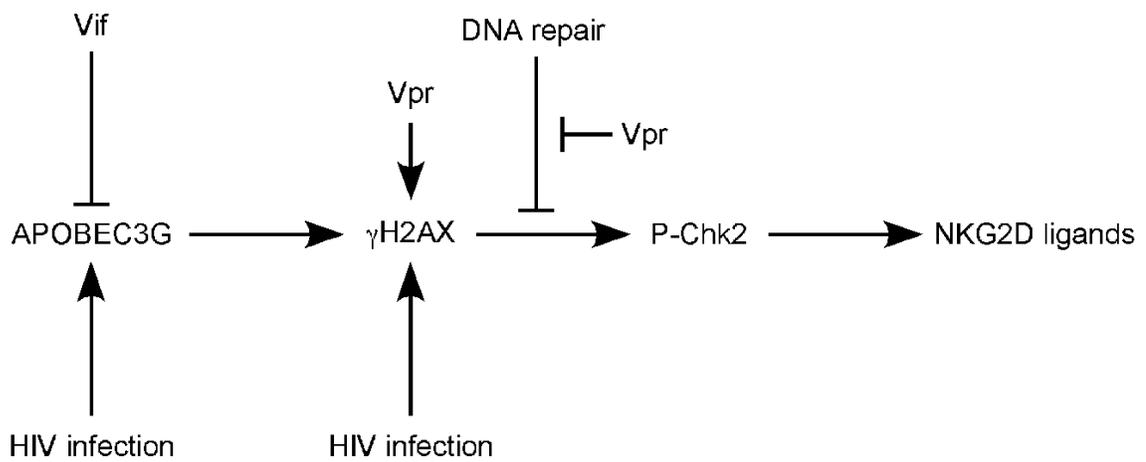
Figure 2.14: Proposed model for A3G-induced DNA damage response activation and NKG2D ligand expression.

Arrows indicate activating effects whereas bars indicate inhibitory effects.

Table 2.1: Primers used in this study

shRNA construct	shRNA Primer sequence (sense strand)	shRNA Primer sequence (antisense strand)	
FG12 shβ₂M	GATCCCCGATTCAGGTTTACTCACGTTTCAAGAGAACGTGAGT AAACCTGAATCTTTTTGGAAA	AGCTTTTCCAAAAAGATTCAGGTTTACTCACGTTCTCTTGAAAC GTGAGTAAACCTGAATCGGG	

HIV-1 construct	5' Primer sequence	3' Primer sequence	Internal Primer sequence
NL-PI Vif Y44A	CACCATATGTATATTTCAAGGAAAGCTA AGGACTGGTTTTATAGACATCACGCTGA AAGTACTA	CACCATATGTATATTTCAAGGAAAGCTA TTTATAGACATCACGCTGAAAGTACTA	
NL-GI*ulbp1*⁺	GGGCTAGCGAATTCCGCCCCTCTCCCTC C	CCAGATCTTCATCTGCCAGCTAGAATGA AGCAGA	TTTGAAAAACACGATGATAATTCTAGA GCCACCATGGCAGCGGCCGCCAGCCCC GC

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Chapter 3

Discussion and future directions

Overview of Dissertation

In this dissertation I have investigated HIV-1 evasion of NK cell recognition. I initially characterized the NK cell activating ligand expression and lysis of primary T cells infected with a panel of HIV-1 accessory protein mutants. These studies led to a more detailed analysis of the mechanism of Vif-mediated ligand inhibition and Vpr-dependent ligand upregulation. The following were the key findings of the work:

- HIV-1 Vif and Nef reduce NK cell recognition of HIV-infected primary T cells by limiting NKG2D ligand upregulation.
- HIV-1 Vpr induces NK cell lysis of HIV-infected primary T cells by upregulating NKG2D ligands.
- Expression of the cytidine deaminase, APOBEC3G, is upregulated in HIV-infected primary T cells.
- APOBEC3G expression activates the cellular DNA damage response indicated by phosphorylation of the histone H2AX.
- APOBEC3G expression correlates with Chk2 phosphorylation and NKG2D ligand expression in HIV-1 Vpr-expressing primary T cells.

I. NKG2D ligand expression in HIV-infected T cells

Discussion

NK cell recognition of a target cell depends on a balance of inhibitory and activating signals[1]. NK cell inhibitory receptors detect MHC-I molecules expressed on the surface of target cells[1]. However, sufficient activating ligand expression on target cells will induce NK cell lysis even in the presence of inhibitory signals[2]. Thus viruses commonly reduce the expression of NK cell activating ligands to evade recognition by NK cells[3-9]. This dissertation analyzes the relative contributions of inhibitory and activating signals in the NK cell recognition of HIV-infected cells.

In HIV infection, residual expression of the MHC-I allotypes HLA-C and -E send inhibitory signals to KIRs and CD94-NKG2A molecules on the surface of NK cells[10]. Although this contributes to insufficient NK cell recognition, I show that NK cell activating signals are also limiting in HIV infection (Figure 2.2). In HIV-infected primary T cells the Vpr protein upregulates the expression of ligands recognized by the NKG2D activating receptor (Figure 2.4)[11, 12]. However, the expression of NKG2D ligands is limited by the HIV-1 Vif and Nef proteins (Figure 2.4). I also show that the modulation of NKG2D ligands affects NK cell recognition of HIV-infected primary T cells (Figures 2.6, 2.8, 2.9). Therefore, the combination of residual MHC-I and limited NKG2D ligand expression results in insufficient NK cell recognition of HIV-infected primary T cells.

The cellular factors involved in NKG2D ligand upregulation by HIV-1 were also analyzed. NKG2D ligands are upregulated when cells experience genotoxic stress[13]. This signals to NK cells the presence of a potentially tumorigenic cell. Therefore, if the damaged cell is unable to repair the DNA damage, circulating NK cells will respond and lyse the damaged cell. One form of damage cells commonly encounter is the presence of uracil in their DNA, which occurs either via dUTP misincorporation or cytidine deamination[14]. The AID cytidine deaminase is normally expressed by activated lymph node germinal center B cells and mediates antibody somatic hypermutation (SHM) and class switch recombination (CSR)[15, 16]. However, retroviral infection ectopically

upregulates AID expression[17] via a poorly defined mechanism involving the transcription factor NF-κB[18]. The DNA damage as a result of AID expression upregulates NKG2D ligand expression[17]. Since APOBEC3 proteins and AID are members of the same family of cytidine deaminases[19, 20], the data presented in this dissertation showing that APOBEC3G (A3G) also upregulates NKG2D ligand expression are consistent.

A3G inhibits HIV infection via mechanisms both dependent and independent of its enzymatic activity[21] but it is unclear whether the products of these mechanisms provide the DNA damage signal that leads to NKG2D ligand upregulation. The HIV-1 genome is hypermutated by A3G during cDNA synthesis[22-24]. The integration of this hypermutated stretch of cDNA would likely trigger a DNA damage response due to the integration event, which has been shown to activate the ataxia–telangiectasia mutated and RAD3-related (ATR) kinase [25], or due to hyperuracilation of the genome. When uracil incorporation is high, DNA fragmentation can occur due to the removal of the uracil residues in close proximity to one another[26]. Normally stretches of ssDNA induces an ATR response, leading to Chk1 phosphorylation[27]. However, in our system there is no correlation between Chk1 phosphorylation and A3G expression (Figure 2.12e). These data may suggest free dsDNA ends result from A3G expression, which would initiate an ATM response[28]. Alternatively, A3G inhibits the completion of viral cDNA synthesis, which results in the accumulation of an abnormal DNA intermediate that fails to integrate[29]. The presence of this abnormal DNA would likely activate a DNA damage response because of the free DNA ends, although it is hard to predict whether it would activate ATM or ATR. HIV cDNA synthesis is inhibited by A3G prior to the second strand transfer event, resulting in a DNA with both single stranded and double stranded regions[29]. Therefore, both ATM and ATR responses could be activated if this abnormal DNA were the signal.

In the absence of HIV infection, A3G modestly activated the DNA damage response, indicated by γH2AX and low-level NKG2D ligand expression (Figure 2.11c and 2.13). Since A3G resides in the cytoplasm, it is unclear how it is activating the damage response in uninfected cells. One possibility is A3G gains access to the cellular DNA during replication following nuclear envelope breakdown, at which point, stretches

of ssDNA may be accessible for deamination. However, UDG enzymes would likely repair some of the deaminated nucleotides, which may explain the intermediate damage response phenotype. Other A3 isoforms might also activate the DNA damage response and may have greater access to cellular DNA due to their nuclear localization[30]. Further characterization of the cellular response to A3 expression may reveal a global upregulation of the DNA damage response.

Downstream Chk2 phosphorylation and significant NKG2D ligand upregulation required HIV infection and Vpr expression (Figure 2.11 and 2.12). It is well established that A3G packaged in the viral particle deaminates the viral genome during cDNA synthesis[22-24]. However, in our experiments virus was produced in cells lacking A3G; therefore, viral particles would not contain A3G. Deamination of the viral genome would consequently have to result from A3G within the target cell. Since A3G is cytoplasmic, it seems likely that deamination of the HIV genome would occur if A3G localized to the site of active reverse transcription, although further experimentation is required to confirm this.

HIV-1 Vpr has consistently been shown to upregulate NKG2D ligands by our group and others[11, 12], although it is unclear whether this response is intentional. Vpr's role in HIV infection is ill defined, yet it seems likely that NKG2D ligand upregulation is a consequence of Vpr expression rather than the objective. Vpr activates ATM and ATR responses and causes arrest of the cell cycle at the G_2/M transition[31-34]. Vpr targets cellular proteins for proteasomal degradation by linking them to an E3 ubiquitin ligase complex[35-37], which is necessary for cell cycle inhibition[38]. The UNG2 and SMUG1 uracil DNA glycosylase (UDG) enzymes and HHR23A protein are known targets of Vpr[39-43]. Interestingly, these are all involved in the removal of mutated nucleotides from DNA[14, 44]. Furthermore, virion associated UNG2 has been shown to degrade A3G-deaminated HIV cDNA[45]. Therefore, one model is that the degradation of these repair proteins by Vpr would result in stabilization of the DNA damage signal and upregulation of NKG2D ligands (Figure 2.14). In this case, the cell may recognize incorporated uracil residues as the damage signal, although further investigation is required to confirm this. Preliminary evidence presented in Appendix 1 suggests the degradation of UNG2 or

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HHR23A by Vpr induces NKG2D ligand expression (Figure A1.1). However, mutating the UNG2 and HHR23A binding sites of Vpr did not completely inhibit NKG2D ligand expression, implying that other cellular targets are involved. Alternatively, multiple DNA damage signals may be generated during HIV-1 infection that have varying degrees of susceptibility to UDG repair. Further investigation is required to clarify the mechanism of Vpr-mediated NKG2D ligand expression.

Cells detect pathogen associated molecular patterns via pattern recognition receptors (PRRs), such as TLRs, RLHs, and NLRs[46-50]. PRR activation leads to the production of proinflammatory cytokine and type I IFN production. In the case of virus infections, viral nucleic acids typically serve as ligands for PRRs[49]. This is true for HIV-1 infection, as a GU rich region of ssRNA within U5 of the HIV-1 LTR is recognized by TLR8 [51]. Although these studies were performed in dendritic cells, CD4^{+} T cells also express TLR8 in endocytic compartments[52]. Therefore, HIV-1 RNA could be recognized by these TLRs if trafficked to these endosomes. In addition to cytokine production, TLR activation has also been shown to induce NKG2D ligand expression[53-55]. Thus, the HIV-1 RNA genome itself could be sufficient for NKG2D ligand expression, although maximal expression would be Vpr-dependent.

The inhibition of NKG2D ligand expression by Vif can be explained in part by its effects on A3G expression levels. However, we also found that A3G expression varied among donors (**Figure 2.10c**), which may suggest that A3G expression differences influence HIV susceptibility by affecting NK cell recognition of infected cells. Furthermore, *apobec3g* is polymorphic and functional differences in A3G among the human population may affect NK cell recognition of HIV-infected cells[30].

The mechanism by which Nef limits NKG2D ligand expression is unclear. However, Nef reduces Vpr-dependent NKG2D ligand upregulation (Figure 2.5). Since Vpr expression induces NKG2D ligands by activating the DNA damage response (Figure 2.12a and 2.13a)[11, 12], it is likely that Nef affects DNA damage response activation. Nef links cellular targets to adaptor proteins, redirecting the target proteins to lysosomal compartments for degradation[56]. One potential model for Nef-mediated NKG2D ligand inhibition would be that Nef targets one or more proteins involved in DNA damage

response signaling for lysosomal degradation. Data presented in Appendix 2 indicate that Nef limits the upregulation of NKG2D ligands rather than downmodulating pre-existing ligand surface expression, which would fit with this proposed model. Together these data may suggest a novel mechanism of Nef-mediated protein downmodulation. However, further investigation is required to characterize this mechanism.

Future directions

A greater understanding of the mechanism of NK cell inhibition by HIV-1 may lead to new treatment strategies by reversing inhibition of NK cell lysis. Two aspects of the mechanism that require further clarification are identification of the DNA damage signal induced by A3G and the requirement for Vpr in NKG2D ligand upregulation. As stated previously, the HIV-1 ssRNA genome, uracil incorporation, and/or cDNA intermediates may be recognized by the cell as "DNA damage". It would be informative to determine whether induction of the DNA damage response by A3G requires its enzymatic activity, which may help identify the damage signal. Preliminary evidence presented in Appendix 1 suggests that the degradation of UNG2 and HHR23A by Vpr induces NKG2D ligand expression (Figure A1.1). These data suggest uracil incorporation is the damage signal, although this must be confirmed. However, UNG2 and HHR23A binding mutants did not completely reverse ligand upregulation (Figure A1.1). Since UDG enzymes have overlapping function[57], complete reversal may only be observed if Vpr binding and degradation of all cellular targets is disrupted. Alternatively, multiple A3G-induced damage signals may be present that are detected by various DNA repair mechanisms. Thus, identification of the damage signal might lead to the detection of the enzymes involved in the repair, or visa versa.

All of our studies to date have analyzed the role of the A3G enzyme in the activation of the DNA damage response, but there are a total of 7 known APOBEC3 (A3) isoforms that could also activate the DNA damage response. Since NKG2D ligand expression is limited by Vif, it would be informative to determine whether other A3 isoforms that are targeted by Vif activate the DNA damage response in HIV infected cells. Although A3G is the strongest inhibitor of HIV-1 infection, A3B, A3C, A3D/E

and A3F are all degraded by Vif and inhibit HIV infection to varying degrees[30]. Of these, A3B, A3C and A3D/E localize to the nucleus[30], which may make them likely candidates for deaminating the genome.

The studies presented in this dissertation were solely done in primary T cells, although macrophages, dendritic cells (DCs) and hematopoetic progenitor cells (HPCs) remain as targets of HIV-1 infection[58-60]. Therefore, it would be informative to repeat these studies in other relevant cell types. Interestingly, the amount of cytoplasmic dUTP varies from one cell type to another[61]. If uraciliated DNA activates the DNA damage response, one might expect those cells with higher cytoplasmic dUTP to have increased damage response activation. Also, DCs are thought to be one of the first sites of HIV-1 infection and transmit HIV to CD4$^+$ T cells[58]. A greater understanding of how HIV-1 evades immune recognition in DCs may lead to therapies aimed at preventing infection of these early targets.

The ultimate goal of these studies is to translate this knowledge into new therapeutic strategies aimed at reducing HIV-1 infection. A3G is a potent inhibitor of HIV-1 infection and, as shown in this dissertation, induces NK cell activating ligand expression. Thus, blocking the interaction between Vif and A3G is an attractive target for drug development. Small molecule inhibitors of the Vif-A3G interaction have been developed and they have been shown to decrease infectivity of the virus *in vitro*[62, 63]. It would be interesting to determine if these inhibitors also rescue NKG2D ligand expression and NK cell recognition of HIV-infected cells.

While CTLs specifically recognize target cells presenting antigen in the context of MHC-I molecules[64], it was recently shown that NKG2D expressed on CTLs serves as a co-stimulatory molecule[65]. It would be informative to determine whether NKG2D is involved in CTL recognition of HIV-infected cells. HIV-1 Nef reduces both MHC-I and NKG2D ligand surface expression (Figure 2.4 and A2.1)[7, 66]. However, Nef-mediated CTL inhibition has only been attributed to MHC-I downmodulation[67]. Our system of reducing total MHC-I by β$_2$M knockdown may provide a means to separate the effects of Nef on MHC-I and NKG2D ligand expression. These findings may reveal a new strategy of CTL immune evasion employed by HIV-1. A3G enhances CTL recognition of HIV-

infected cells[68], which may provide further evidence that NKG2D stimulation is involved, as we have shown that A3G also enhances NKG2D ligand expression. Finally, domains within Nef have specialized roles in the downmodulation of MHC-I and CD4 molecules[56]. It would be interesting to determine whether NKG2D ligand downmodulation by Nef can be attributed to one of these domains.

II. APOBEC3G upregulation in HIV-infected T cells

Discussion

A3G is an intrinsic inhibitor of virus infection and endogenous retroelement transposition[69-74] that is expressed in the cytoplasm of many cell types, including $CD4^+$ T cells, dendritic cells, monocytes, and hepatocytes[20, 75-77]. As stated previously, proinflammatory cytokines and type I IFNs are produced in response to viral infection. Several of these cytokines, including IFN-α, IFN-γ, IL-2 and IL-15 have been shown to induce A3G expression[76-78]. Additionally, type I IFN expressed in response to the TLR3 ligand poly(I:C) has been shown to induce A3G expression[79]. I show in this dissertation that HIV infection of primary T cells stimulates A3G protein expression, although the mechanism remains unclear. The data suggest that A3G expression is induced within infected cells and not uninfected cells in the culture. This can be explained by the finding that A3G upregulation is predominantly observed in the absence of Vif or in A3G-binding mutants (Figure 2.11). If cytokines produced in response to HIV infection were inducing A3G expression, one would expect to observe A3G upregulation throughout the culture. Only a percentage of cells (upwards of 30%) are infected; therefore, the majority of cells would not express Vif and degrade A3G. Thus, no difference in A3G upregulation would be expected if it were cytokine dependent. These data suggest that the cells recognize the virus and the infected cells upregulate A3G protein expression.

These observations are consistent with a recent study of A3G expression in HIV-infected female sex workers[80]. In this study, A3G mRNA expression was determined both pre- and post-infection within the cohort, where A3G mRNA was significantly higher post-HIV infection[80]. Although the viral signal that induces A3G upregulation is

yet to be determined, recognition of the viral RNA genome by PRRs is a possibility. A recent study indicated that Influenza virus infected cell lines upregulated A3G mRNA expression by a mechanism dependent on viral RNA[81]. Further investigation is required to determine whether the recognition of HIV-1 RNA also induces A3G expression. These data may indicate that the upregulation of A3G is an innate response to RNA virus infection.

Future directions

Several questions remain that must be addressed to understand the mechanism of HIV-induced A3G upregulation. First, it would be informative to determine whether HIV-1 RNA upregulates A3G expression and to determine the cellular receptor that detects the RNA. One likely candidate receptor is TLR8, which has been shown to recognize HIV-1 RNA[51]. In other cell types, TLR3 activation induces A3G expression[79]. Since TLR3 and TLR8 signaling lead to the activation of common transcription factors[49], it is possible that TLR8 recognition of HIV RNA also induced A3G expression. Notably, the transcription factor NFκB is activated by TLR3 and 8 and is required for the retrovirus-dependent upregulation of AID[18, 49]. Therefore, A3G upregulation may also be NFκB dependent. The RLH receptors could also be involved in HIV-1 RNA detection, as they detect cytoplasmic viral ssRNA and activate NFκB, as well as other transcription factors[47, 50]. Further investigation is needed to determine if PRRs are involved in HIV-induced A3G upregulation.

It is also enticing to speculate that A3 upregulation is a general response to virus infection. In the case of HIV-1 infection it would be interesting to determine whether other A3 isoforms are also upregulated. Alternatively, it would be interesting to determine whether other RNA viruses upregulate A3G expression. The recent finding that Influenza virus RNA induces A3G expression may indicate that other RNA viruses would do the same[81]. A3 deaminases are potent inhibitors of viral infection[30]; therefore, upregulating their expression in response to virus may greatly enhance resistance to infection.

HIV-1 has several mechanisms of evading recognition by adaptive and innate immune responses. By counteracting these immune evasion strategies, HIV-infected cells may be rendered susceptible to immune recognition. Thus, a greater understanding of the factors involved in the recognition of HIV-1, such as those presented in this dissertation, may prove vital for the treatment of infection.

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Appendix 1

HIV-1 Vpr-mediated NKG2D ligand upregulation involves UNG2 and HHR23A binding

In this dissertation I investigated the susceptibility of HIV-1 infected primary T cells to NK cell recognition. These studies revealed that ligands recognized by the NKG2D activating receptor are upregulated in HIV-infected cells. Maximal NKG2D ligand upregulation correlated with APOBEC3G (A3G) levels and required HIV-1 Vpr expression. One possible explanation for these synergistic effects is Vpr stabilizes mutations generated by A3G by inhibiting DNA repair (Figure 2.14). Vpr has been shown to interact with several proteins involved in DNA repair including, the uracil DNA glycosylases, UNG2 and SMUG1, and the nucleotide excision repair protein HHR23A[1-5]. Mutating the tryptophan at position 54 (W54) of Vpr to arginine and mutating the histidine at position 45 (H45) of Vpr to glutamine disrupt UNG2 (ref[1, 6]) and HHR23A[3] binding, respectively. The SMUG1 binding site of Vpr has not been characterized. Thus, we determined whether disrupting the association between Vpr and UNG2 or HHR23A affected NKG2D ligand upregulation in HIV-infected primary T cells.

To address this question, W54R and H45Q single amino acid substitutions were generated within *vpr* of the molecular clone NL-PI*nef* ⁻ (Figure A1.1a). These mutations were made in a Nef-deficient background to rule out inhibitory effects of Nef on NKG2D ligand expression. Mutating these amino acids had no effect on protein expression [1, 3] (data not shown). As shown in Figure A1.1b and c, primary T cells infected with HIV expressing wild-type Vpr had an average of 5-fold higher NKG2D ligand expression than cells infected with a Vpr-deficient HIV (compare NL-PI*nef* ⁻*vpr* ⁺and NL-PI*nef* ⁻*vpr* ⁻, $p <$ 0.01). Mutating the UNG2 binding site of Vpr reduced the NKG2D ligand expression by

1.6-fold among the six donors tested (Figure A1.1b and c, compare NL-PI*nef⁻vpr⁺* and NL-PI*nef⁻* Vpr W54R, $p < 0.05$). Primary T cells infected with a Vpr mutant unable to bind HHR23A also expressed less NKG2D ligand than WT Vpr (Figure A1.1b and c, compare NL-PI*nef⁻vpr⁺* and NL-PI*nef⁻* Vpr H45Q). Together these data suggest that Vpr upregulates NKG2D ligands via a mechanism involving UNG2 and HHR23A binding.

NKG2D ligand upregulation was only partially reversed by inhibiting either UNG2 or HHR23A binding. A Vpr double mutant that disrupted binding of both UNG2 and HHR23A was also tested, although its expression was less than WT Vpr (data not shown). Therefore, we cannot rule out an additive effect of binding both DNA repair proteins on NKG2D ligand upregulation. Since UDG enzymes have overlapping functions[7], it is possible that the inability to completely inhibit Vpr-mediated NKG2D ligand upregulation is due to compensatory effects of other UDGs. Alternatively, multiple A3G-mediated DNA damage signals could be present, which may have different susceptibilities to UDG or HHR23A-dependent repair. Further study is needed to characterize the mechanism of Vpr-dependent NKG2D ligand expression to rule out these and other possibilities.

Methods

Lentivirus construction

The NL-PI*nef⁻* and *nef⁻vpr⁻* molecular clones have been reported previously[8, 9]. The H45Q and W54R amino acid substitutions of NL-PI*nef⁻* Vpr were generated by site directed mutagenesis. In brief, the SpeI-SalI fragment of NL-PI was cloned into pLitmus38. A point mutation was then introduced to generate an amino acid substitution at His45 to Gln and Trp54 to Arg by PCR using Phusion HF polymerase (New England Biolabs) and the following primers: NL4-3 Vif NdeI fwd 5'-TTAACACATGGAAAAGATTAGTAAAACACC-3', NL4-3 Vpr SalI rev 5'-CCCGTCGACACCCAATTCTGAAA-3', and the internal NL4-3 Vpr W54R 5'-ACTTACGGGGATACT<u>A</u>GGGCAGGAGTGGA-3' and NL4-3 Vpr H45Q 5'-

ACTTAGGACAACA<u>G</u>ATCTATGAAACTTAC-3' primers plus their reverse compliments (underlined bases indicate mutation sites). The full amplicon is a 701bp fragment from the NdeI site within *vif* to the SalI site within *vpr*. The mutated product was then cloned into the NdeI to SalI sites of pLitmus38 NL-PI and sequenced through the entire amplified region. The correct H45Q and W54R clones were then reintroduced into NL-PI*nef⁻* using SpeI and SalI restriction sites.

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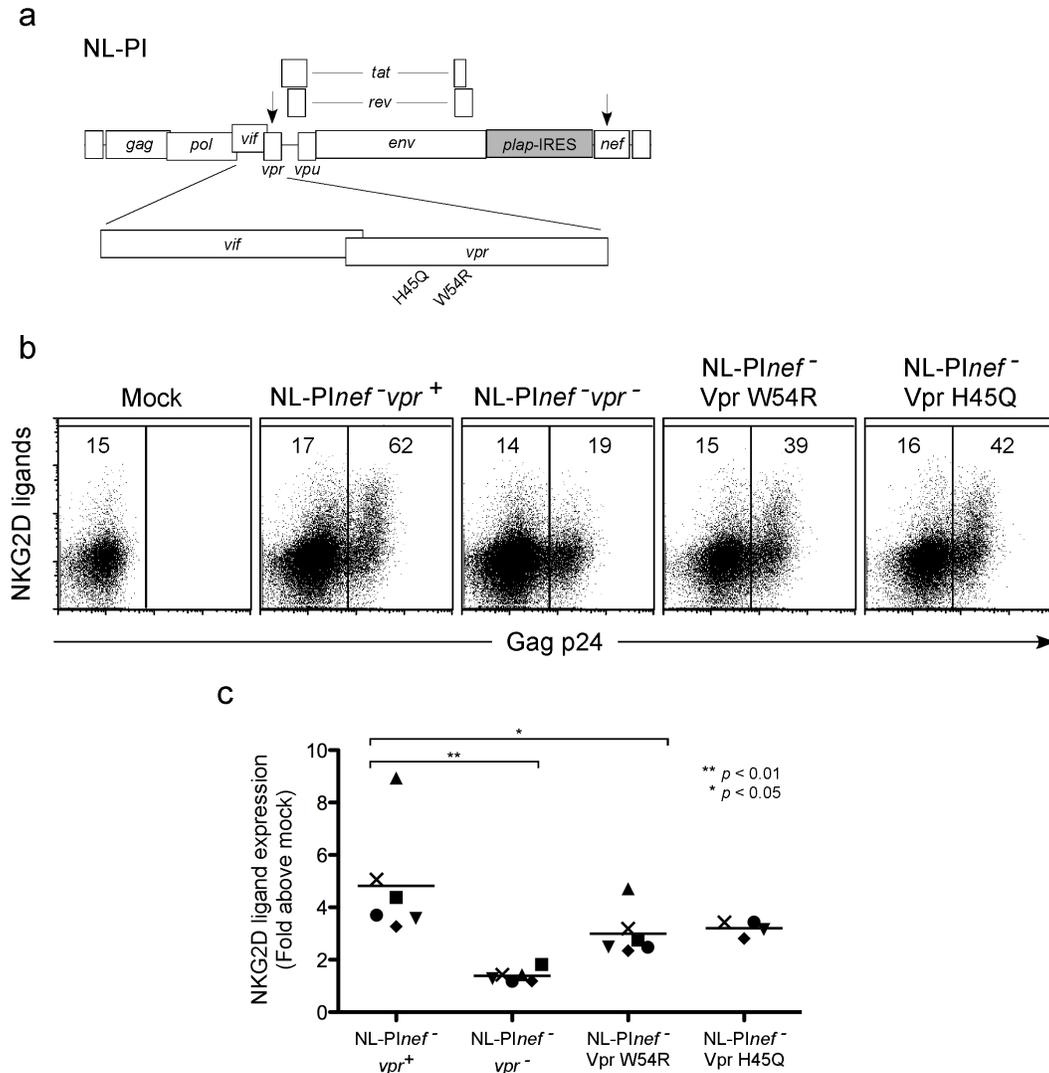
Figure A1.1: Vpr-dependent NKG2D ligand upregulation involves UNG2 and HHR23A binding.

(a) The NL-PI virus expresses PLAP to mark infected cells; accessory protein mutants were made of this construct and are indicated by the black arrows. Sites of amino acid substitution are indicated. (b) NKG2D ligand expression of primary T cells infected with the indicated virus or mock-infected. Mean fluorescence values within the Gag p24+ and Gag p24- populations are shown. Only alive, CD3$^+$ cells were gated. (c) Fold difference in NKG2D ligand expression relative to mock treated controls for independent donors, each represented by a different character. Black bars represent the mean change in ligand expression for each virus. Statistical analyses were calculated by paired t test of accessory protein mutants compared to NL–PI*nef*$^-$*vpr*$^+$.

Appendix 2

HIV-1 Nef-mediated NKG2D ligand inhibition

Much of this dissertation focuses on the synergistic relationship between A3G and Vpr that leads to the upregulation of NKG2D ligands. However, negative factor (Nef) is an integral part of HIV-1 immune evasion. There are two main functions of Nef: CD4 and MHC-I downmodulation. However, we show that Nef also limits the expression of NKG2D ligands on the surface of primary T cells.

CD4 is the major receptor for HIV-1 and limits viral particle budding and release when not removed from the cell surface[1, 2]. In HIV-infected cells, the combined effects of the Nef, Vpu and Env proteins downmodulate CD4, although Nef is sufficient for CD4 downmodulation. While the mechanism is incompletely defined, Nef binds to the CD4 cytoplasmic tail domain, removes it from the cell surface, and targets it for lysosomal degradation (reviewed in [3]). The mechanism of Nef-mediated MHC-I downmodulation is better characterized and serves to limit viral peptide presentation to CTLs[4]. Nef has been shown to selectively bind the cytoplasmic tail domains of HLA-A and -B molecules early in the secretory pathway[3, 5, 6]. This leads to the recruitment of the cellular adaptor protein-1 (AP-1), which redirects MHC-I molecules to early endosomes and then lysosomes for degradation[3, 7, 8]. One obvious theme between these two mechanisms is the binding of the cytoplasmic tail domains of MHC-I and CD4, which leads to the recruitment of cellular adaptor proteins. Further investigation is required to determine whether Nef downmodulates NKG2D ligands by a similar mechanism.

An initial characterization of Nef-mediated NKG2D ligand downmodulation was done in collaboration with a former graduate student, Elizabeth Wonderlich. In these

studies, the downmodulation phenotype of CD4, MHC-I and NKG2D ligands was compared in primary T cells and CEM-SS CD4^{+} T lymphoblastoid cells. Mock-infected, PHA-stimulated primary T cells express low levels of NKG2D ligands and high MHC-I and CD4 (Figure A2.1a, shaded histogram). However, mock-infected CEM-SS cells constitutively express NKG2D ligands, MHC-I and CD4. The differences in baseline NKG2D ligand expression between these cell types allowed us to determine whether Nef removes pre-existing NKG2D ligand surface expression or inhibited the expression of new ligands. As shown in Figure A2.1, NKG2D ligand expression in mock-infected and full-length HIV-infected primary T cells is low and then increases in NL-PI*nef⁻* infected cells (part a, top panel). This may suggest that Nef limits the presentation of NKG2D ligands at the surface of the cell. Alternatively, MHC-I and CD4 expression is downmodulated from the surface in full-length HIV-infected primary T cells compared to Nef-deficient HIV-infected (Figure A2.1a, compare dashed and bold histogram lines), indicating that Nef downmodulates MHC-I and CD4 surface expression on primary T cells.

To compare the downmodulation of NKG2D ligands, MHC-I and CD4 in CEM-SS cells, a previously described Adenoviral system of expressing Nef in CEM-SS cells was used[9, 10]. In this system, CEM-SS cells stably expressing HLA-A2 (CEM-A2) were transduced with increasing MOI of a Nef-expressing or negative control Adenovirus and the surface expression of NKG2D ligands, HLA-A2 and CD4 were determined by flow cytometry (Figure A2.1b). As was seen in primary T cells, Nef reduced the surface expression of HLA-A2 and CD4 in CEM-A2 cells (Figure A2.1b). However, there was no downmodulation of NKG2D ligands from the surface of CEM-A2 cells, even at the highest MOI tested, which expressed a similar amount of Nef as primary T cells (Figure A2.1b and c). These data further suggested that Nef does not downmodulate pre-existing NKG2D ligand from the surface; Nef limits the expression of NKG2D ligands.

Further analysis is required to determine the mechanism of Nef-mediated NKG2D ligand inhibition. The mechanism of Nef-mediated CD4 and MHC-I downmodulation involves binding to the cytoplasmic tail domains of these molecules. However, the NKG2D ligands that are upregulated by HIV, the ULBPs, are GPI-anchored proteins and

lack a cytoplasmic tail domain. Therefore, it would be surprising if Nef affected their expression by a similar mechanism as CD4 and MHC-I. Additional study of the NKG2D ligand inhibition may reveal a novel Nef mechanism of affecting protein surface expression.

Methods

HIV-1 and Adenovirus transduction

PHA-stimulated primary T cells were transduced with NL-PI or NL-PI*nef*⁻ molecular clones as described in Chapter 2. Nef-expressing or control Adenovirus transduction of CEM-SS cells was performed as previously described[9, 10]. Cells were analyzed by flow cytometry as described in Chapter 2.

Western Blot Analysis

72 hours after Adenovirus transduction or 48 hours after HIV infection, cells were washed in PBS (Invitrogen) and lysed in 0.1% SDS 0.3% CHAPS PBS with 1 mM PMSF. Lysates were normalized for both protein concentration and viral transduction and were analyzed by Western blot using an antibody against Nef (AG11 at 1 mg/ml, 1:500, a gift from J. Hoxie, University of Pennsylvania, Philadelphia, PA[11]) followed by an HRP-conjugated secondary antibody. The membranes were developed with ECL Plus Western Blotting Detection kit (Amersham).

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Figure A2.1: Comparative downmodulation of NKG2D ligands, HLA-A2 and CD4 by Nef.

(a) PHA-stimulated primary T cells infected with NL-PI (dashed line) or NL-PI*nef* ⁻ (black line) viruses were analyzed for NKG2D ligand, MHC-I and CD4 surface expression by flow cytometry. Mock-infected cells (shaded histogram) were included as control. (b) CEM-SS cells stably expressing HLA-A2 transduced with increasing MOI of Nef-expressing (dashed line) or control (black line) Adenovirus were analyzed for NKG2D ligand, HLA-A2 and CD4 surface expression by flow cytometry. Isotype antibody staining (shaded histogram) was included as control. (c) Western blot analysis of Nef expression from parts (a) and (b).

Figure A2.1: Comparative downmodulation of NKG2D ligands, HLA-A2 and CD4 by Nef.