Investigating mechanisms of immune evasion by HIV-1 Vpr

By

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Dedication

To Olivia, Allison, Mom, Dad, and Peewee

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Table of Contents

Dedication		ii
Acknowledgements		iii
List of Figures		v
Abstract		viii
Chapter 1	Introduction	1
Chapter 2	APOBEC3G enhances natural killer cell recognition of HIV-infected primary T cells	53
Chapter 3	Vpr utilizes DCAF1-DDB1-CUL4 E3 ubiquitin ligase to overcome a macrophage-specific restriction of Env expression and virion production	96
Chapter 4	Characterization of a novel primary macrophage cell culture system and description of a possible role for C-type lectins	139
Chapter 5	Discussion and Future directions	170
Appendix	Vpr may protect uracilated HIV-1 DNA in macrophages	205

List of Figures

1.1	Genomic organization of HIV-1/SIVcpz, SIVagm and HIV-2/SIVsm	31
1.2	Schematic of the HIV-1 viral particle and structural proteins	32
1.3	Graphical representation of Env synthesis and trafficking	33
1.4	Vpr enhances the proteasome-mediated degradation of UNG2	35
1.5	Mechanisms in myeloid cells that inhibit HIV-1 reverse transcription	36
2.1	NKG2D ligand expression in HIV infected cells	75
2.2	Upregulation of A3G correlates with NKG2D ligand upregulation	77
2.3	Activation of the DNA damage response in HIV infected primary T cells	78
2.4	HIV-infected T cells are resistant to NK cell recognition unless an NKG2D ligand is overexpressed	80
2.5	The effect of Vif on NK cell lysis	82
2.6	Vif and Vpr limit uridine incorporation in primary T cells	84
2.7	UNG2 binding by Vpr induces NKG2D ligand expression	85
2.8	Proposed model for A3G-induced DNA damage response activation and NKG2D ligands expression	86
3.1	Vpr increases p24 levels in the supernatant during a spreading infection in MDM	118

3.2	Vpr does not increase the first round of infection in primary human MDM cultures	120
3.3	Calculation of normalized *gag* DNA in HIV-infected MDM	121
3.4	Vpr increases virion production in HIV infected primary MDM	122
3.5	HIV 89.6 Vpr counteracts a macrophage restriction of Env expression	124
3.6	Vpr increases Env incorporation into virions	126
3.7	Vpr counteracts a restriction pathway that targets HIV Env for lysosomal degradation in primary human MDM	127
3.8	Vpr causes accumulation of PLK and turnover of MUS81, DCAF1, and UNG2 in MDM and primary cell targets of HIV-1	129
3.9	DCAF1-dependent functional activities correlate with Vpr-dependent virion production and Env expression	130
3.10	Cell cycle analysis of 293T cells expressing Vpr mutants	131
3.11	DCAF1 is required for Vpr to counteract the macrophage-specific restriction to virion production and Env expression	132
3.12	Proposed model for Env-dependent virion production by Vpr	133
4.1	AMD3100 and Maraviroc inhibit HIV-infection of CEMx174 cells	156
4.2	MDM pretreated with AMD3100 and Maraviroc exhibit a potent restriction to HIV-1 89.6 infection that is counteracted by Vpr	157
4.3	Vpr-mediated infection of MDM treated with AMD3100 and Maraviroc is dependent on the virus inoculum	158
4.4	Vpr partially overcomes a restriction to HIV-infection in MDM treated with reagents blocking entry, but not reverse transcription or integration	159
4.5	Mutants defective at DCAF1 binding are also unable to overcome a restriction to HIV-infection of MDM treated with AMD3100 and Maraviroc	160
4.6	Mannose receptor blocking antibody relieves HIV restriction in MDM treated with AMD3100 and Maraviroc	161

4.7 Vpr and DCAF1 downregulate mannose receptor and DC-SIGN in MDM 162

4.8 Proposed model for restrictions to HIV infection overcome by Vpr in primary MDM 164

5.1 Proposed model for cell type-specific Vpr phenotypes 191

5.2 Proposed implications of Vpr activation of the SLX4 complex for genomic DNA synthesis and HIV reverse transcription 193

5.3 Proposed model for Vpr, Env and C-type lectin receptors 196

A1 Deoxynucleoside treatment overcomes the negative effect of deoxyuridine on the HIV infection of macrophages 228

A2 Increasing the cellular pool of canonical dNTPs partially restores virus production and spread of HIV-1 89.6*vpr*$^{-}$ in macrophages 229

A3 Vpr inhibits A3B-mediated, Rev-independent Gag expression 231

A4 Vpr enhances degradation of UNG2 in primary macrophages 232

A5 Vpr in viral particles is sufficient to mediate degradation of DCAF1 but not DDB1 233

A6 Possible roles for UNG2 in the early stages of HIV-1 replication 234

A7 An elevated dUTP/dTTP ratio and APOBEC3B reveal a requirement for Vpr 235

Abstract

Human immunodeficiency virus (HIV) causes significant morbidity and mortality worldwide, yet several HIV proteins have not been fully characterized. The development of cell culture models to study retroviruses has accelerated discovery of basic steps in the HIV lifecycle. Examination of the HIV proteins reverse transcriptase, integrase, protease, Gag and Env in cell lines has led to life-saving pharmacological therapies. In contrast, lentiviral accessory proteins such as viral infectivity factor (Vif) and viral protein R (Vpr) are not yet targeted therapeutically, and are not required for replication in many cell lines. However, accessory proteins are required for HIV to counteract the innate immune system *in vivo*. Vpr is conserved among lentiviruses, but its function is unknown. In this dissertation, we examine the role of Vpr during HIV-1 infection of primary macrophages, primary $CD4^+$ T cells and cell lines.

We report that uracilation of HIV DNA by the host cytidine deaminase APOBEC3G (A3G) leads to upregulation of natural killer (NK) cell-activating ligands in $CD4^+$ T cells. HIV limits NK cell-activating ligand upregulation by targeting A3G for degradation through Vif. Additionally, we propose that Vpr counteracts uracilation of HIV DNA by recruiting the

host uracil DNA glycosylase UNG2. We also explore the ability of Vpr to suppress the antiviral response to HIV, and identify novel restrictions to HIV infection in primary macrophages counteracted by Vpr. Vpr enhances the spread of HIV-1 in macrophages by increasing virus production per infected cell. Surprisingly, Vpr also increases the intracellular level and virion incorporation of Env by preventing Env lysosomal degradation. Importantly, these novel Vpr activities are dependent on the DCAF1-DDB1-Cul4 E3 ubiquitin ligase complex. We also identify novel cell culture conditions that increase the requirement for Vpr to infect macrophages with HIV. Mechanisms of action of eight of the nine HIV-1 genes have been determined. However, *vpr* is unique because its function has not yet been characterized. This dissertation describes a systematic study of the mechanism through which Vpr enhances HIV replication in primary macrophages. Mechanistic insight into the function of Vpr in HIV-infected macrophages may lead to novel strategies to cure HIV.

Chapter 1:

Introduction[1]

Acquired immunodeficiency syndrome (AIDS) was first described in 1981 in four homosexual men, all of whom died rapidly from opportunistic infections or rare neoplasms (Siegal et al., 1981). It is now established that the causative agent of AIDS is human immunodeficiency virus type 1 [HIV-1,(Knipe and Howley, 2013)]. Existing treatment options for HIV-1 infection can inhibit viral replication by targeting several stages of the replication cycle, including entry, reverse transcription and integration. Yet, the prevalence of HIV-1 is increasing (Yehia and Frank, 2011). The expanding scale of the HIV-1 pandemic has led to the emergence of strains that have acquired resistance to one or more of these agents.

To maintain effective antiretroviral therapy for future generations, it is imperative to uncover novel molecular targets for pharmacological intervention. The human immune system has evolved innate antiviral responses that decrease the efficiency of HIV-1 infection, but these are not sufficient to halt progression to AIDS. HIV-1 encodes proteins to counteract these antiviral mechanisms, but these "accessory proteins" have not all been characterized. This dissertation examines the interaction between innate immunity and

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HIV accessory proteins, focusing on HIV-1 viral protein R (Vpr). Emphasis is placed on primary cell model systems with intact innate immune mechanisms. A better understanding of the interaction between HIV-1 and the human immune system represents the next frontier in the discovery of molecular targets for therapeutic intervention.

This chapter will introduce the HIV-1 replication cycle including entry, reverse transcription, and assembly. Then, evidence will be presented that lentiviral accessory proteins counteract host innate immune mechanisms in the major targets of HIV-1, $CD4^+$ T cells and macrophages.

The global impact of HIV

An estimated 34 million people are infected with HIV-1 worldwide, and one to two million are in the US (Demberg and Robert-Guroff, 2012). The incidence of HIV-1 in the US is almost 50,000 people per year, and the global prevalence is increasing at an alarming rate (Yehia and Frank, 2011). While HIV-1 infection has been cured in a patient with HIV and leukemia by chemotherapy followed by bone marrow transplantation using cells from a donor with a genetic deletion in an HIV receptor (Allers et al., 2011), this therapy is too risky to be implemented widely. The current standard of care is lifelong pharmacological therapy, called highly active antiretroviral treatment (HAART). Although HAART is effective at suppressing viral replication, treated individuals confront a multitude of disorders, including lipodistrophy, hepatotoxicity, nephrotoxicity cardiovascular disease, and neurological disease (Llibre et al., 2009). The cost of treating a person varies from $30,000 to $60,000 per year in the US (Fleishman et al., 2010). Thus, HIV-1 is a significant economic burden in addition to causing widespread morbidity and mortality.

Major target cells of HIV-1

CD4⁺ T lymphocytes

CD4⁺ T cells are a clinically significant target of HIV-1 (Stites et al., 1986; Ziegler and Stites, 1986) because CD4⁺ T cell counts correlate inversely with HIV-related disease progression (Detels et al., 1988). A CD4⁺ T cell count lower than 200 cells per microliter is one of the defining characteristics of AIDS (Yeni et al., 2004). Several AIDS-defining lesions have been identified since the emergence of the pandemic, including opportunistic infections such as Pneumocystis jiroveci pneumonia (PCP), cytomegalovirus (CMV), and *Candida albicans*, as well as neoplasms such as Kaposi's sarcoma (Siegal et al., 1981). Because CD4⁺ T cells play a crucial role in the mobilization of the adaptive immune system, it is hypothesized that CD4⁺ T cell decline is responsible for immune dysfunction in AIDS (Ziegler and Stites, 1986).

CD4⁺ T cell activation is necessary for an adaptive immune response to a specific pathogenic epitope. Antigen presenting cells (APCs) initiate this process by activating the T cell receptor (TCR) and a costimulatory receptor such as CD28. T cell activation dramatically increases the permissivity of CD4⁺ T cells to HIV-1 (Carr et al., 1999; Zack et al., 1990). Activation-dependent permissivity has been proposed to follow a decrease in the expression of a number of host restriction factors (2010; Baldauf et al., 2012; Chiu et al., 2005; Wu, 2012; Yan and Lieberman, 2012). Interestingly, although activated cell models are frequently used to study HIV-1 *in vitro*, the majority of the CD4⁺ T cells circulating *in vivo* are quiescent (Stevenson et al., 1990).

One of the most significant barriers to curing HIV infection is the ability of the virus to establish a latent infection in quiescent CD4⁺ T cells (Siliciano and Greene, 2011).

Resting memory T cells from HIV-infected individuals have been found to contain integrated provirus (Chun et al., 1995). It is believed that HIV-infected, resting memory T cells persist after surviving active infection or become latently infected after reverting from an activated to a quiescent state (Chomont et al., 2009). These latently infected cells cannot be targeted by current pharmacological agents and are subject to minimal immunodetection and cytotoxicity in the absence of viral gene expression.

Although $CD4^+$ T cells are an important cell type for the propagation and persistence of HIV-1 *in vivo*, infected cells may be targeted for lysis by natural killer (NK) cells(Richard et al., 2010). NK cells recognize infected cells through a combination of inhibitory and activating signals(Lanier, 2008). Because viruses such as HIV-1, adenovirus, and human cytomegalovirus (HCMV) interfere with presentation of viral peptides to Cytotoxic T lymphocytes (CTL) by downmodulating major histocompatibility complex class I (MHC-I) molecules(Collins and Baltimore, 1999), the presence of these proteins on the cell surface inhibits NK cell lysis(Petersen et al., 2003). Virus-infected cells upregulate NK-cell activating signals, such as ligands for the NK cell natural-killer group 2, member D (NKG2D) receptor(Lanier, 2008). HIV infection leads to upregulation of NKG2D ligands through the DNA damage repair pathway(Gasser et al., 2005; Richard et al., 2010). Thus, HIV-infected $CD4^+$ T cells can be lysed by NK cells.

Myeloid cells

Myeloid cells, including dendritic cells and macrophages, play an important role in the innate and adaptive immune response against viral pathogens like HIV. Myeloid cells are also important targets of HIV and simian immunodeficiency virus (SIV) (Cameron et al.,

1992a; Kaushik et al., 2009). Macrophages and dendritic cells (DCs) express the necessary receptors for HIV-1 entry and, like $CD4^+$ T cells (Gupta et al., 2002; Zhang et al., 1999), are among the earliest targets for HIV-1 and SIV *in vivo* (Hladik et al., 2007; Hu et al., 2000), reviewed in (Cohen et al., 2011). HIV and SIV have been detected in macrophages in secondary lymphoid tissue by *in situ* hybridization *in vivo* (Reinhart et al., 1997; Schacker et al., 2001). In later stages of pathogenesis, HIV infected macrophages are thought to be the cause of AIDS related encephalopathy (Koenig et al., 1986), and SIV infected macrophages cause an analogous central nervous system pathology in the rhesus macaque model (Desrosiers et al., 1991). HIV-1 infected macrophages have been reported to secrete paracrine factors, including soluble CD23, that increase the permissivity of resting $CD4^+$ T cells (Swingler et al., 2003). However, myeloid cells are somewhat resistant to HIV and SIV infection because they express high levels of host restriction factors that represent significant post-entry blocks to HIV-1 infection (Berger et al., 2011; Laguette et al., 2012).

DCs propagate HIV-1 primarily by *trans* infection, a pathway in which DCs capture and transmit internalized viral particles by Ca^{2+}-dependent C-type lectin receptors (CLR), including DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and human mannose receptor [hMR, (Geijtenbeek et al., 2000; Saifuddin et al., 2000)]. A heparin sulfate proteoglycan dependent pathway and a cholesterol-dependent pathway for internalization of intact viral particles have also been described (Bobardt et al., 2003; Gummuluru et al., 2003). More recently, it was shown that sialyllactose is a molecular recognition pattern in gangliosides in the HIV-1 membrane that allows DCs to capture viral particles (Izquierdo-Useros et al., 2012; Puryear et al., 2012). In addition, galactosyl

ceramide can mediate cell-to-cell transfer of HIV-1 from dendritic cells to T lymphocytes (Magerus-Chatinet et al., 2007).

Productive infection of DCs with HIV-1 has also been reported *in vitro* (de Silva et al., 2012). However, only a small percentage of DCs have been found to be infected *in vivo* (Wu and KewalRamani, 2006) and most evidence indicates that DCs do not support efficient HIV replication. Therefore, the contribution of HIV infection of DCs to pathogenesis requires further study. It is possible that the main role of DCs in HIV disease is to transmit internalized viral particles to $CD4^+$ T cells rather than to directly support productive infection (Cameron et al., 1992b; Geijtenbeek et al., 2000) reviewed in (Altfeld et al., 2011; Coleman and Wu, 2009).

Within the myeloid lineage, macrophages are thought to be the most permissive to HIV-1. Macrophages become permissive to HIV-1 following differentiation because of a decrease in expression of the host restriction factors SAM domain and HD domain-containing protein 1 (SAMHD1) and APOBEC3A (Berger et al., 2011; Laguette et al., 2012). In addition to being infected, there is also evidence that macrophages archive HIV-1 virions for transfer to $CD4^+$ T lymphocytes via the virological synapse, an HIV-induced interface between two cells that facilitates cell-to-cell infection (Sharova et al., 2005). Infection of macrophages may be particularly important because, compared to HIV-infected T cells, infected macrophages have a relatively long half-life (Sharova et al., 2005). The monocyte-derived macrophage (MDM) model is commonly used to study HIV-1 replication in macrophages *in vitro* (Waki and Freed, 2010). HIV-1-infected MDMs, like macrophages infected *in vivo*, persist for weeks in culture and mediate cell-to-cell infection of $CD4^+$ T cells (Sharova et al., 2005). In sum, current data indicates that myeloid cells play an

important role in the pathogenesis of HIV-1 infection as a relatively long-lived target of HIV and as a viral conduit to $CD4^+$ T cells.

Lentiviral genes and replication

HIV-1, HIV-2 and SIV are lentiviruses within the family *retroviridae* (Coffin et al., 1997). Long terminal repeats (LTRs), 640 base pairs of DNA containing repetitive sequences, flank viral genes, mediate integration into host genome, and contain promoter and enhancer elements that increase the transcription of viral genes (**Figure 1.1**, (Cullen, 1991). Lentiviral genomes contain the conserved retroviral genes, *gag*, *pol* and *env* (**Figure 1.1**). Proteins encoded by these genes accomplish the basic steps required for retroviral replication, including entry, uncoating, reverse transcription, integration, and formation of virus-like particles (VLPs), as described below.

HIV-1 Assembly

The HIV-1 viral particle is the infectious unit responsible for the propagation of the replication cycle in permissive cells (Montagnier et al., 1984). These 100-200 nm, spherical particles (**Figures 1.2C and D**) were initially called lymphadenopathy-associated virus (LAV) because they were discovered in the cultured T lymphocytes of a patient with lymphadenopathy (Gelderblom et al., 1987; Montagnier, 1984). The HIV-1 Gag pr55 precursor peptide is the structural protein that is necessary and sufficient for the formation of VLPs (**Figure 1.2B**, (Freed, 1998; Gheysen et al., 1989). Gag pr55 multimerizes into spherical structures at the plasma membrane that bud from the surface of the cell (**Figure 1.3C**). Several domains within Gag, including matrix (MA), capsid (CA), nucleocapsid (NC),

and p6 direct the assembly process (Freed, 1998). MA targets Gag to the plasma membrane and is necessary for the incorporation of the HIV envelope (Env) into viral particles (Freed and Martin, 1995). NC recruits two copies of the viral genome into budding virions. Finally, p6 facilitates release of viral particles from the plasma membrane by recruiting host factors involved in the endogenous vacuolar sorting pathway such as TSG101 and endosomal sorting complexes required for transport (ESCRT) proteins (Garrus et al., 2001).

Following release of the immature particle, the HIV-1 protease (PR) cleaves Gag pr55 into the structural components of the mature particle, p17 (MA), p24 (CA), p7 (NC), and p6. The mature particle forms an electron-dense, cone-shaped capsid (**Figures 1.2C and D**). Particle maturation inverts the function of Gag. Whereas pr55 mediates formation and release of viral particles from the plasma membrane of the producer cell, processed Gag proteins mediate the controlled process of uncoating following entry into the target cell.

Env synthesis and trafficking

Env is synthesized as a 160 kDa precursor protein in the rough endoplasmic reticulum (RER, **Figure 1.2A**, (Hunter and Swanstrom, 1990). This precursor contains a transmembrane domain and a surface domain inserted into the RER lumen during translation (**Figure 1.3A**). Cotranslational glycosylation of Env with N and O-linked glycans has been demonstrated to limit immunodetection of infected cells and neutralization of viral particles (Allan et al., 1985). The mannose structures on Env vary between producer cell types and facilitate the capture of viral particles by cells bearing C-

type lectin receptors (Lin et al., 2003). The majority of gp160 oligomerizes into trimers, although tetramers have also been reported (Checkley et al., 2011; Schawaller et al., 1989).

In the Golgi, gp160 is subsequently cleaved into surface gp120 and transmembrane gp41 regions by furin, a host endoprotease that also activates the hemagglutinin protein of fowl plague virus (FPV, **Figure 1.3B**, (Checkley et al., 2011; Hallenberger et al., 1992). These domains associate non-covalently in heterotrimeric spikes that can be visualized by electron microscopy (Zhu et al., 2003). The weak interaction between gp41 and gp120 can allow shedding of gp120 from the surface of the cell (**Figure 1.3F**).

Env gp120 contains variable (V1-V5) regions that vary significantly between molecular clones and constant (C1-C5) regions that are relatively conserved (Starcich et al., 1986). While C1-C3 mediate binding of Env to CD4, polymorphisms within the V3 sequence determine binding to host coreceptors CCR5 or CXCR4 (Fouchier et al., 1992). Env gp41 contains a cytoplasmic tail (CT), transmembrane domain (TMD), and an extracellular domain (Brasseur et al., 1990). Classical models of gp41 plasma membrane topology suggest that the TMD anchors gp41 into the plasma membrane and the CT is entirely cytoplasmic. Interestingly, one study suggests the CT, which contains two hydrophobic sequences, may itself span the plasma membrane twice(Cleveland et al., 2003). For example, neutralizing antibodies can be directed against a sequence within the CT, implying that this region is actually extracellular (Cleveland et al., 2003). Finally, the extracellular region of gp41 contains a sequence called the fusion peptide, which mediates fusion of the viral envelope with the plasma membrane following Env binding to CD4 (Bosch et al., 1989). Therefore, gp120 mediates attachment and host cell tropism, and gp41 facilitates fusion and anchoring into the plasma membrane.

Env incorporation into viral particles

Viral particles have been reported to contain eight to ten Env trimers per virion (Zhu et al., 2003). The process through which Env is incorporated into viral particles is complex and still under investigation. Surprisingly, determinants of Env incorporation appear to be cell-type dependent. Many cell lines, including HeLa, 293T and MT-4 do not require the Env cytoplasmic tail for incorporation into viral particles (Freed and Martin, 1996). Envelopes from other viruses such as vesicular stomatitis virus G glycoprotein (VSV-G) can be efficiently incorporated into HIV-1 particles, in addition to many cellular proteins (Ott, 2008; Page et al., 1990). Thus, Env incorporation appears to occur passively in some cell lines.

In CEM T cells and primary cells, however, the cytoplasmic tail is necessary for Env incorporation into viral particles and replication beyond the first round (Murakami and Freed, 2000). Several gp41 CT point mutants are incorporated less efficiently than a CT truncation mutant, suggesting that the CT sequence can also actively exclude Env from viral particles (Gabuzda et al., 1992). In addition, Gag MA is located just under Env in the viral particle (**Figure 1.2C**) and the two viral proteins are thought to associate because they can be chemically crosslinked (Gebhardt et al., 1984). MA, like the gp41 CT, is necessary for Env incorporation in primary cells, and mutations in MA can eliminate virion incorporation of Env (Dorfman et al., 1994). Interestingly, virion incorporation of Env is functional in molecular clones that harbor mutations in both the gp41 CT and MA, suggesting that Env is incorporated passively in the absence of signals within MA (Freed and Martin, 1995). Thus,

in primary cells, Env is actively recruited to sites of assembly through a mechanism dependent on MA and the gp41 CT (**Figure 1.3D**).

Recent studies have uncovered an increasing number of cellular and viral factors that are necessary for the incorporation of Env into viral particles. Although the gp41 CT and MA are necessary for Env incorporation into virions in primary cells, they are not sufficient (Rousso et al., 2000). HIV-1 assembly and budding occurs in detergent-resistant lipid rafts in the plasma membrane (Nguyen and Hildreth, 2000). Palmitoylation of two cysteine residues on gp160 targets Env to lipid rafts. Mutation of these cysteine residues inhibits the incorporation of Env into viral particles and results in production of non-infectious virus (Rousso et al., 2000). The gp41 CT also associates with the host clathrin-associated adaptor complexes AP-1 and AP-2 (Wyss et al., 2001). These complexes mediate endocytosis of Env from the cell surface and Env subcellular localization. Env is rapidly internalized outside of sites of assembly to avoid immunodetection of HIV-infected cells (**Figure 1.3E**,(Rowell et al., 1995). The identification of novel factors involved in Env trafficking and virion incorporation may facilitate the development of therapies to target virion assembly.

Entry

Despite the severity of symptoms associated with HIV-1 infection, only a subset of the cells in the human body is susceptible to the virus. Permissive cells, including macrophages and $CD4^+$ T cells, express the CD4 receptor and at least one of the co-receptors CXCR4 or CCR5 (Berger, 1997). Env mediates binding of viral particles to CD4 and co-receptors (Blumenthal et al., 2012). Viral particles lacking Env are non-infectious

because they cannot enter cells. Env proteins from molecular clones that bind CXCR4 preferentially are designated X4-tropic, and others that bind CCR5 are called R5-tropic. Dual-tropic envelopes bind efficiently to both CXCR4 and CCR5. $CD4^+$ T cells are permissive to both X4 and R5 tropic viruses because they express both CXCR4 and CCR5 (Alkhatib et al., 1996). Macrophages, however, are non-permissive to X4-tropic viruses because they express only CCR5 (Ayinde et al., 2010). Thus, polymorphisms within *env* determine host cell tropism.

Permissive cells can be infected with HIV-1 through cell-free or cell-to-cell mechanisms. Infections established by the cell-free mode occur when virions not associated with a producer cell infect a target cell (Sigal et al., 2011). This mode of infection is thought to be relatively inefficient *in vivo* because it is sensitive to neutralizing antibodies and antiretroviral drug therapy (Abela et al., 2012; Sigal et al., 2011). In contrast, cell-to-cell spread describes a contact-dependent process in which virions are transferred from one cell to another through the virological synapse, a structure dependent on binding of HIV-1 Env molecules on the donor cell to CD4 molecules on the target cell (Abela et al., 2012; Sigal et al., 2011). Macrophages can infect both quiescent and phytohemagglutinin (PHA)-stimulated $CD4^+$ T cells by a cell-to-cell spread mechanism (Waki and Freed, 2010). This mode of infection is thought to predominate *in vivo*, given that most circulating $CD4^+$ T cells are quiescent under physiological conditions (Carr et al., 1999).

Reverse transcription, integration and viral gene expression

Pol activity is essential for the formation of infectious virus. The UAG stop codon at the end of *gag* is read through about five percent of the time and the resulting Gag-Pol polyprotein supplies viral particles with Pol activity (Wills et al., 1991). Pol contains reverse transcriptase (RT) and integrase (IN) in addition to PR as described above. Retroviruses insert their genomes into the DNA of the target cell, where viral genes may be expressed using host cell transcriptional machinery. However, because the HIV-1 genome is encoded on two positive-sense molecules of RNA, the virus requires an activity that is not encoded by cells: The requirement to synthesize DNA from an RNA template is fulfilled by RT (Baltimore, 1970). In the cytoplasm of the target cell, RT generates double stranded DNA from the RNA genome. Viral cDNA translocates into the host cell nucleus associated with a number of viral proteins that comprise the preintegration complex (PIC), including MA, RT, IN and Vpr (Bukrinsky et al., 1993). IN catalyzes the insertion of the viral genome into the DNA of the target cell (Bukrinsky et al., 1992).

Gene expression in eukaryotic cells is regulated by a large number of transcription factors and *cis*-acting nucleic acid sequences (Pereira et al., 2000). After HIV-1 has integrated, the provirus must ensure the transcription and translation of viral genes in a complex nuclear environment. Lentiviruses meet this requirement by encoding the regulatory proteins, transactivator of transcription (Tat) and regulator of expression of virion proteins (Rev,(Cohen et al., 1990a; Cohen et al., 1990b; Malim and Emerman, 2008). The Tat protein enhances transcription of viral genes from the LTR by binding to the transactivating response element (TAR) on initiated viral transcripts and recruiting positive transcription elongation factor b (P-TEFb) to the integrated provirus(Kashanchi et al., 1994; Wei et al., 1998). Although the expression of viral genes is low immediately

following integration, Tat accumulates in the nucleus by positive feedback and accelerates transcription of the viral RNA.

Lentiviruses encode multiple genes by alternative splicing of a single molecule of RNA. This full length RNA is the viral genome that is packaged during assembly, but is also spliced into mRNA encoding viral proteins. Several viral transcripts, including *tat*, *rev* and *nef* are spliced and exported from the nucleus by the cellular CRM1 pathway (Fornerod et al., 1997). These genes are translated early after integration. Late genes, however, including *gag*, *pol*, *env*, *vpr*, *vpx*, *vpu* and *vif* are unspliced or incompletely spliced and contain introns that prevent their export by host mechanisms (Bohne et al., 2005). For these late genes, nuclear export is dependent on the binding of Rev to the Rev response element (RRE) on their unspliced transcripts (Cullen and Malim, 1991). Therefore, the expression of late genes is low before Rev accumulates in the nucleus. In this way, both Rev and Tat regulate the timing and magnitude of lentiviral gene expression.

Lentiviral accessory proteins counteract innate immunity

Unlike Gag, Gag-Pol, Env, Tat, and Rev, the lentiviral accessory proteins, viral protein R (Vpr), viral protein X (Vpx), viral protein unique (Vpu), viral infectivity factor (Vif) and negative factor (Nef) are not required for replication in many cell lines, but permit immune evasion *in vivo* (Malim and Emerman, 2008). Accessory proteins lack any known intrinsic enzymatic activity, but serve as multifunctional adaptor proteins that recruit or redistribute host proteins.

As will be discussed below, the many cell lines and primary cell types used to study lentiviruses express different levels of innate immune factors. Several discoveries have

been made by comparing the phenotypes of lentiviral accessory proteins in different cell types (Malim and Emerman, 2008). It was found, for example, that Vif is crucial for the production of infectious HIV-1 from CEM-CCRF cells, but not from CEM-SS cells (Sheehy et al., 2002). This observation led to the discovery that Vif facilitates the proteasomal degradation of the host restriction factor, APOBEC3G (A3G), in CEM-CCRF cells (Sheehy et al., 2003). In the absence of Vif, A3G is packaged within viral particles and renders them non-infectious in the next round of replication through hypermutation of viral cDNA or inhibition of reverse transcription (Bishop et al., 2008). A3G is expressed in a wide range of cell types that includes the primary targets of HIV-1, $CD4^+$ T lymphocytes and macrophages (Koning et al., 2009).

Vpu also demonstrates a variable phenotype in different cell lines. It has been shown that Vpu significantly enhances viral particle release from HeLa cells and primary cells, but not from several other cell lines (HOS, 293T or HT1080, (Neil et al., 2008). Furthermore, a Vpu requirement for viral particle release can be induced in 293T cells by interferon-α treatment. The hypothesis that a restriction factor limiting viral particle release and counteracted by Vpu is expressed in HeLa cells and is inducible in 293T cells led to the discovery of BST-2 (tetherin). Vpu induces the proteasomal degradation of tetherin in 293T cells by recruiting the substrate adaptor β-TrCP[62]. Vpu has also been reported to recruit β-TrCP to promote proteasomal degradation of CD4 (Fujita et al., 1997). This latter activity prevents non-productive associations of the surface domain of Env with CD4 in the producer cell (Chen et al., 1993). Recent studies suggest Vpu can enhance viral particle release without altering the steady state level of tetherin protein by altering

tetherin localization (Dube et al., 2010; Mangeat et al., 2009). Therefore, the mechanism through which Vpu counteracts tetherin is still under investigation.

Nef has a variable effect on HIV-1 infectivity in different cell types, but the mechanism for this phenotype has not been determined (Dorfman et al., 2002; Vermeire et al., 2011). In addition, it has been demonstrated that Nef induces macrophages to secrete paracrine factors that increase the permissivity of $CD4^+$ T cells to HIV-1 (Swingler et al., 2003). Nef is also cytotoxic, and may contribute to $CD4^+$ T cell death via Nef-containing exosomes in addition to direct infection (Lenassi et al., 2010). Finally, Nef alters the trafficking of several host proteins, including major histocompatibility complex class I (MHC-I)(Collins et al., 1998), CD4(Chen et al., 1996), CD8 (Leonard et al., 2011) and hMR(Vigerust et al., 2005). Downmodulation of MHC-I prevents immunodetection of HIV-infected cells by cytotoxic T lymphocytes (CTLs, (Collins et al., 1998). Downmodulation of CD4 prevents re-infection (Chen et al., 1996). Nef activity against CD8 is intriguing because $CD8^+$ T lymphocytes are not thought to be a major target of HIV-1. While it is clear that the ability of Nef to downmodulate CD8 is conserved among multiple lentiviral isolates, the physiological significance of Nef-mediated CD8 downmodulation is still under investigation (Heigele et al., 2012).

Interestingly, lentiviruses encode a variable repertoire of accessory genes (**Figure 1.1**, reviewed in (Ayinde et al., 2010; Le Rouzic and Benichou, 2005; Malim and Emerman, 2008; Peeters and Courgnaud, 2002). HIV-1 contains *vpu*, but not *vpx* (Ayinde et al., 2010). Human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus of sooty mangabeys (SIVsm) encode *vpx* but not *vpu*. Simian immunodeficiency virus of African green monkeys (SIVagm) encodes neither *vpu* nor *vpx*. Because lentiviruses co-

evolve with their hosts, variability in accessory protein usage may reflect fundamental differences in the immune systems of different primate species (Lim and Emerman, 2011; Lim et al., 2012). Two accessory proteins that enhance infection of myeloid cells, Vpx and Vpr, are discussed in greater detail below.

The role of Vpx in SIV pathogenesis

SIVs related to HIV-2/SIVsm lacking Vpx (Δv*px*) are significantly less efficient than wildtype SIVs at establishing an infection in the pigtail macaque model by intrarectal or intravenous inoculation (Belshan et al., 2012; Hirsch et al., 1998). Animals infected with SIV Δ*vpx* maintain lower viral loads and higher $CD4^+$ T cell counts than animals infected with wildtype SIV. However, Rhesus monkeys infected with SIV Δ*vpx* eventually acquire AIDS related symptoms, indicating that while Vpx promotes infection, it is not absolutely required for pathogenesis *in vivo* (Gibbs et al., 1995).

In vivo studies of Vpx function

A number of studies have been performed to better define the activities of Vpx in vivo. One such study examined SIVmne027 infection in pigtail macaques with or without expression of a functional Vpx protein (Belshan et al., 2012). Seven to ten days following intrarectal inoculation, wildtype SIVmne027 infected macaques had viral loads almost 100 fold higher than SIVmne027 Δ*vpx* infected animals. $CD4^+$ T cell counts were reduced from baseline in wildtype SIVmne027 infected animals, but not in SIVmne Δ*vpx* infected animals. Based on *in situ* hybridization, 98% of infected cells found in secondary lymphoid tissue were lymphocytes, whereas infected myeloid cells were rare [(fewer than 2% of infected

cells were HAM56+ macrophages and no infected DCs were found, (Belshan et al., 2012)]. The dramatic effect of Vpx on infection of $CD4^+$ T cells *in vivo* seems to contrast with the *in vitro* results that indicate a primary role for Vpx in the infection of myeloid cells [see below, (Belshan et al., 2012)]. Further studies are clearly needed to better understand this apparent enigma.

In vitro studies of Vpx function

In cell culture models, Vpx has only a minimal effect on infection of primary T cells whereas Vpx dramatically enhances lentiviral infection of macrophages. The effect of Vpx on infectivity correlates with enhanced accumulation of 2-LTR circles [dead-end HIV-1 cDNAs measured because they are proportional to the amount of nuclear HIV-1 cDNA (Mandal and Prasad, 2009; Sharova et al., 2008)]. This activity of Vpx requires expression of the host protein damaged DNA binding protein 1 (DDB1), the E3 ubiquitin ligase complex scaffolding factor previously shown to be required for Vpr to cause cell cycle arrest (Sharova et al., 2008). It was proposed that the interaction of Vpx with DDB1 leads to the degradation of host proteins that are detrimental to viral replication (Kaushik et al., 2009; Sharova et al., 2008).

Vpx targets the host factor SAMHD1

Recent studies have now demonstrated that Vpx promotes macrophage and DC infection by targeting the cellular factor SAMHD1 (Hrecka et al., 2011);(Laguette et al., 2011), also reviewed in (Lim and Emerman, 2011); (Planelles, 2012)). SAMHD1 was initially identified as a host protein that bound to Vpx (Hrecka et al., 2011; Laguette et al.,

2011). Subsequent studies demonstrated that silencing SAMHD1 enhanced HIV-1 and SIV Δ*vpx* infection of myeloid cells (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1 silencing also enhances HIV-1 infection of resting $CD4^+$ T cells, suggesting that SAMHD1 also restricts HIV-1 infection in quiescent T cells (Baldauf et al., 2012; Descours et al., 2012). Conversely, ectopic overexpression of SAMHD1 reduced HIV-1 and SIV Δ*vpx* infection of otherwise permissive cell lines (Laguette et al., 2011). Based on these data, SAMHD1 is sufficient to inhibit infection of myeloid cells and quiescent $CD4^+$ T cells by lentiviruses not expressing Vpx (Hrecka et al., 2011; Laguette et al., 2011).

SAMHD1 contains a putative HD domain that provided investigators with some clues as to the role of SAMHD1 in lentiviral infection (Laguette et al., 2011). HD domains contain conserved histidine and aspartate catalytic residues and are found within a superfamily of metalloenzymes with known or predicted phosphohydrolase activity (Aravind and Koonin, 1998). Studies by Goldstone *et al.* and Lahouassa *et al.* have recently provided evidence that SAMHD1 inhibits HIV-1 infection in myeloid cells by restricting the intracellular pool of dNTPs (Goldstone et al., 2011; (Lahouassa et al., 2012) highlighted in (Jermy, 2012) and (Schaller et al., 2012), respectively, **Figure 1.5A**. Recombinant SAMHD1 reduces the concentration of all four dNTPs by direct hydrolysis in vitro (Goldstone et al., 2011; Lahouassa et al., 2012) and expression of SAMHD1 in myeloid cells reduces dNTP concentrations to a level that is suboptimal for reverse transcription (Lahouassa et al., 2012). Silencing SAMHD1, providing Vpx in trans and provision of exogenous deoxynucleosides all increase the amount of available dNTP (Lahouassa et al., 2012) and dramatically increase myeloid cell permissivity to HIV-1 and SIVΔ*vpx*. Thus, the main mechanism employed by SAMHD1 to restrict lentiviral infection is dNTP hydrolysis.

Given the striking ability of SIV Vpx to stimulate HIV-1 infection of myeloid cells by inhibiting SAMHD1, it is puzzling as to why HIV-1 appears to lack an equivalent activity against SAMHD1. Some have suggested that myeloid cells play a limited role in the propagation of HIV-1 *in vivo* because $CD4^+$ T cells are more permissive to HIV (Schaller et al., 2012). Others predict that infection of some types of myeloid cells by HIV would be detrimental to the overall infection (Schaller et al., 2012). This hypothesis is supported by experiments in which coerced infection of dendritic cells treated with Vpx containing VLPs was found to trigger an IFN response that inhibited the infection of other cell populations (Manel et al., 2010). Despite this hypothesis, HIV-1 infected $CD11c^+$ macrophages are frequently found in lymph node biopsies from chronically infected individuals (Xu et al., 2009), and any anti-viral cytokines secreted by these cells are insufficient to contain the infection. Thus, additional experiments will be needed to better understand whether SAMHD1 has an effect on the infection of myeloid cells during HIV-1 infection *in vivo*.

Vpx counteracts SAMHD1 via the ubiquitin ligase complex DDB1 and CUL4A.

Recent studies have demonstrated that Vpx counteracts SAMHD1 by targeting the restriction factor for degradation (Hrecka et al., 2011; Laguette et al., 2011). In Vpx expressing cells, SAMHD1 interacts with the DCAF-DDB1-CUL4A E3 ubiquitin ligase complex (Hrecka et al., 2011; Laguette et al., 2011). Vpx-dependent degradation of SAMHD1 is prevented by silencing DCAF1 or by inhibiting proteasomal degradation (Hrecka et al., 2011; Laguette et al., 2011). These studies support the model that Vpx ubiquitylates and degrades SAMHD1 via the DDB1-CUL4-DCAF1 E3 ubiquitin ligase complex.

Interestingly, Vpx stimulates infectivity in primary human macrophages to a greater extent than SAMHD1 knockdown (Lahouassa et al., 2012). While this observation might be explained by partial knockdown of SAMHD1, it is also possible that SAMHD1 is not the only target of Vpx that is relevant in myeloid cells. Indeed, it has been reported that Vpx causes partial degradation APOBEC3A, another potential host restriction factor in macrophages (Berger et al., 2011).

The role of Vpr in HIV and SIV infection

In comparison to Vpx, which is encoded by only two lentiviral lineages, Vpr is conserved amongst all primate lentiviruses (Lim et al., 2012). However, a requirement for Vpr is more difficult to demonstrate *in vitro* and is more prominent at low multiplicity of infection (MOI, (Ogawa et al., 1989). Some SIV Vpr alleles are able to degrade SAMHD1 in a species-specific manner. However all HIV-1 Vpr alleles that have been tested lack activity against human SAMHD1 (Lim et al., 2012). While the major effect of Vpr seems to be to enhance infection of macrophages, there is also evidence that Vpr can enhance the infection of activated $CD4^+$ T lymphocytes under some conditions (Balliet et al., 1994; de Silva et al., 2012; Heinzinger et al., 1994; Rey et al., 1998).

In vivo studies of Vpr function in SIV-infected macaques

To examine the role of Vpr, investigators have utilized rhesus macaques infected with SIVmac239, which causes AIDS like symptoms. In these studies, an SIV containing a mutation in the Vpr start codon (SIVmac239 *vpr*$^-$) reverted to wild type in 3 of 5 animals (Lang et al., 1993). Low viral load was observed in the two animals infected with virus that

did not revert. However, in a subsequent study, using an SIVmac239 *vpr*⁻ deletion mutant that cannot revert *in vivo*, two of four animals infected with SIV Δ*vpr* developed AIDS like symptoms (Hoch et al., 1995). Therefore, Vpr is not absolutely required for the development of AIDS in this model system.

The role of Vpr in HIV pathogenesis

Unlike SIV, HIV does not contain Vpx and does not require Vpx for infection *in vivo*. Vpr is contained within HIV and is more conserved among primate lentiviruses than Vpx (Lim et al., 2012). However, Vpr has a comparably modest effect on macrophage and dendritic cell infection (Balliet et al., 1994; de Silva et al., 2012; Heinzinger et al., 1994). While *in vivo* studies have not been able to directly address the contribution of Vpr to HIV-1 pathogenesis, a Vpr allele with attenuated cytopathogenicity *in vitro* has been isolated from a long-term non-progressor (Somasundaran et al., 2002). These findings suggest that Vpr is important for HIV replication *in vivo*.

In vitro studies of Vpr function

Further investigation is needed to understand how Vpr stimulates infection. Vpr is a 96 amino acid, 14 kDa protein containing three alpha α-helices that mediate interaction with several host proteins, as well as with HIV-1 Gag (Morellet et al., 2003). Vpr is expressed late in the replication cycle, but is present during early events because it is packaged within virions (Lu et al., 1993; Selig et al., 1999). Packaging is mediated by an interaction between the amino-terminal alpha helix of Vpr with an $(LXX)_4$ motif within the p6 region of Gag (Paxton et al., 1993). Vpr is incorporated into virions at a molar ratio of

1:7 to capsid (CAp24), or 275 molecules per virion (Muller et al., 2000). The discovery of Vpr in virions has led to the hypothesis that, like Vpx, Vpr acts on an early step in the viral replication cycle (Cohen et al., 1990b; Lim et al., 2012). For example, Vpr exhibits nuclear localization and was thought to mediate nuclear import of the preintegration complex in non-dividing cells such as MDMs (Chen et al., 2004; Heinzinger et al., 1994; Vodicka et al., 1998), but this model is not universally accepted (Yamashita and Emerman, 2005). Transcription of lentiviral genes from the LTR is also increased by Vpr's direct interaction with p300/CBP coactivators (Cohen et al., 1990b; Cui et al., 2006; Felzien et al., 1998; Forget et al., 1998; Kino et al., 2002). Despite *vpr* conservation and a clear phenotype *in vivo*, mutation of *vpr* results in a subtler phenotype in existing cell culture models than mutation of *vpx*, *vif* or *vpu* (Ayinde et al., 2010).

Vpr also associates with a Cul4 E3 ubiquitin ligase containing DDB1 and DDB1 and Cul4-associated factor 1 (DCAF1, **Figure 1.4B**). DCAF1 is a substrate adaptor that provides specificity for the DDB1-Cul4 E3 ubiquitin ligase (Wen et al., 2012). The interaction of Vpr with DCAF1 is responsible for the observation that Vpr arrests cells in the G_2 phase of the cell cycle (Hrecka et al., 2007). G_2 cell cycle arrest has been reported to increase the expression of HIV-1 genes (Goh et al., 1998; Li et al., 2010). If it is true that Vpr-induced cell cycle arrest confers some replication advantage, however, it is puzzling that the most dramatic *vpr* replication phenotypes have been observed in non-dividing MDMs. A recent study has provided new insight into the mechanism through which Vpr induces cell cycle arrest(Laguette et al., 2014). This study demonstrated that Vpr activates the structure-specific endonuclease (SSE) regulator SLX4 to process HIV DNA, also resulting in cell cycle arrest. These data suggest that rather than being a primary function of Vpr, cell cycle arrest

is a side effect of processing of HIV DNA to avoid triggering a type I interferon response to HIV infection(Laguette et al., 2014). However, it is not known if Vpr targets a host restriction factor to recruit SLX4 to HIV DNA.

The only known target of Vpr is the host uracil DNA glycosylase, UNG2, which excises uracil from DNA in the base excision repair pathway (Sire et al., 2008). Vpr has been observed to associate with and increase the ubiquitin-mediated proteasomal degradation of UNG2 (Schrofelbauer et al., 2007) by recruiting the Cul4-DDB1-DCAF1 E3 ubiquitin ligase (**Figures 1.4A, B and C**, (Ahn et al., 2010). DCAF1 has a weak intrinsic affinity for UNG2, and in the absence of Vpr, this ubiquitin ligase regulates the steady state level of UNG2 in different periods of the cell cycle (Wen et al., 2012). Silencing of DCAF1 prevents Vpr-mediated UNG2 degradation. Although Vpr-mediated UNG2 degradation initially implicated this host protein as a restriction factor(Weil et al., 2013), it has been repeatedly observed that UNG2 knockdown results in the production of non-infectious virus (Guenzel et al., 2012; Priet et al., 2005). Vpr has been reported to recruit UNG2 into virions to repair HIV-1 cDNA during reverse transcription (Chen et al., 2004). Nevertheless, the benefits of UNG2 degradation and virion incorporation to HIV-1 replication remain controversial, as discussed below. Thus, Vpr may also target an additional unknown cellular factor to mediate its full complement of functions.

Uracil as a restriction factor

Non-dividing cells, such as macrophages, have been reported to have a high dUTP/dTTP ratio relative to $CD4^+$ T lymphocytes (Kennedy et al., 2011). In macrophages, the intracellular ratio of dUTP to dTTP is sufficient to allow uracil incorporation into viral

cDNA, while the dUTP to dTTP ratio in PBMCs is not [**Figure 1.5B,** (Kennedy et al., 2011)]. If uracil incorporation into viral DNA restricts viral replication (reviewed in Sire et al., 2008), the dUTP/dTTP ratio in macrophages predicts this restriction would be more efficient in macrophages than in $CD4^+$ T lymphocytes.

In a separate study that measured incorporation of uracil into HIV DNA (uracilation) in primary T cells 48 hours post infection, uracil incorporation was only detected in the absence of both Vpr and Vif expression (Norman et al., 2011), highlighted in (Croxford and Gasser, 2011) and was not apparent in wild type virus. To detect uracil incorporation, this study utilized uracil DNA glycosylase (UDG), which removes the uracil base from uridine-containing DNA rendering uracilated templates inactive for subsequent PCR amplification. Because uracilation was only detectable in the absence of Vif and Vpr, it was hypothesized that both Vif and Vpr play a role in reducing uracil incorporation. Vif acts by inhibiting A3G, which incorporates uracil into single stranded viral DNA during reverse transcription by deaminating cytosine residues (Harris et al., 2003). Vpr was proposed to reduce uracil incorporation by activating DNA repair pathways (Norman et al., 2011). As described above, Vpr is known to interact with UNG2, a cellular uracil glycosylase, which normally functions to remove uracil incorporated into DNA (Chen et al., 2004; Schrofelbauer et al., 2007). Vpr also activates ATM and ATR DNA damage signaling pathways (Zimmerman et al., 2006), which in turn stimulate repair of DNA (reviewed in (Cimprich and Cortez, 2008; Lavin and Kozlov, 2007). Interestingly, macrophages do not appear to express ATR (Zimmerman et al., 2006). If ATR is necessary for the repair of uracil-containing DNA, then uracilation of the HIV genome may be a more important restriction factor in macrophages than in T cells.

Data from other studies have provided evidence that UNG2 is recruited into the viral particle and is necessary for HIV DNA stability (Priet et al., 2005). The authors of these studies used the differential ability of Taq and Pfu polymerases to demonstrate the presence of uracil-containing templates. These studies revealed that depletion of UNG2 in virus-producing cells substantially increases uracil incorporation into early reverse transcripts in the target cell. In addition, these authors used a synthetic uracil-containing primer-template substrate to demonstrate that recombinant UNG2 and HIV-1 RT can cooperate to repair uracilated DNA in a cell-free system. These data suggest that UNG2 supports viral replication by catalyzing repair of uracilated viral cDNA.

There is also evidence that UNG2 -dependent uracil deglycosylation is detrimental to viral infection (**Figure 1.5D,** reviewed in (Gu and Sundquist, 2003). In support of this model, one study demonstrated that expression of the UNG2 inhibitor Ugi or silencing of UNG2 in virus producing cells partially blocks the antiviral activity of A3G against mutant HIVs that lack *vif* (Yang et al., 2007). Thus, UNG2-dependent deglycosylation of hyperuracilated DNA and subsequent cleavage by apurinic endonucleases may lead to viral cDNA degradation or inhibition of reverse transcription (Yang et al., 2007).

Another recent study observed positive effects of uracilation on overall HIV infection. These authors measured uracil incorporation at ten hours post infection, before integration has occurred and provided evidence that uracilation promotes integration by limiting the nonproductive autointegration pathway that produces two LTR circles (Yan et al., 2011). To measure uracilation, these investigators took advantage of the differential abilities of Taq and Pfu to amplify a uracilated template. In addition, an *in vitro* integration assay was employed to measure the efficiency of integration of DNA containing varying

amounts of uracil. In this assay, less autointegration was detected when templates contained more uracil. Thus, this study concluded that uracil incorporation into HIV cDNA can actually have a beneficial role in HIV infection by inhibiting non-productive auto-integration pathways.

Vpx and Vif counteract APOBEC family-mediated restriction.

As described above, Vif protects the viral genome by degrading the host restriction factor A3G (Sheehy et al., 2002). Increasing evidence suggests that A3G is a member of a family of cytidine deaminases that plays a role in host innate immunity (reviewed in (Malim, 2009). It was recently discovered that APOBEC3A (A3A), one member of this family, is highly expressed in myeloid cells. The high level expression of A3A in immature monocytic cells contributes to their resistance to HIV infection (Berger et al., 2011; Peng et al., 2007). Upon differentiation of monocytic cells into more mature macrophages, A3A expression decreases (Peng et al., 2007). Remarkably, it was found that silencing A3A in monocytes infected with HIV-1 GFP reporter increases virus particle production 3 to 4 fold (Peng et al., 2007). Similarly, it was reported that A3A silencing increases HIV infection of primary macrophages, dendritic cells, and the monocytic cell line, THP-1, by 5-7 fold (Berger et al., 2011). In the same study, the authors demonstrate that Vpx reduces A3A expression in 293T cells co-transfected with plasmids expressing A3A and SIV_{MAC} Vpx. Based on this data it appears that A3A may be an additional target of Vpx that leads to increased permissivity of myeloid cells to lentiviruses.

While A3A expression decreases during macrophage differentiation, APOBEC3B (A3B) expression increases (Peng et al., 2007). In cell lines overexpressing A3B, HIV-1

replication is weakly inhibited (Pak et al., 2011). It remains controversial whether A3B, which is predominantly nuclear, inhibits retroviral replication when expressed at physiological levels (Harris and Liddament, 2004). Further study is needed to determine if A3B has more potent antiviral activity in differentiated macrophages.

There is a great deal of evidence that most A3 family members act within the viral particle such that A3 expression in the viral producer cells determines the requirement for Vif. However, recent studies have provided evidence that in some cases A3 expression in target primary macrophages may also play a role (Koning et al., 2011). Because A3 proteins favor certain target sequences for deamination, it is possible to link patterns of mutation to particular A3 family member activity. While A3G targets 5'-C<u>C</u> sequences for deamination on the (-) strand, leading to 5'-<u>A</u>G hypermutations on the (+) strand, the other APOBEC proteins preferentially deaminate the 5'-T<u>C</u> dinucleotide sequence (reviewed in (Chiu and Greene, 2008). Because an analysis of HIV DNA amplified from macrophages using PCR revealed G to A hypermutation with a strong 5'-T<u>C</u> (-) strand bias, the authors concluded that a non-A3G APOBEC protein was playing a role. In sum, these studies indicate that A3 family members are capable of acting on HIV in the target cell and that A3A is a strong candidate for this role in macrophages (Berger et al., 2011). However, because only 1% to 6% of HIV-1 sequences amplified contained G to A hypermutations, this study also concluded it is unlikely that A3A mediated hypermutation accounts for A3A mediated lentiviral restriction in myeloid cells and propose that A3A has other mechanisms to inhibit HIV (**Figure 1.5C**, (Koning et al., 2011). These studies may be only beginning to uncover the antiviral properties of A3 proteins.

Emerging model for HIV-infection of myeloid cells

Macrophages and DCs are important cell types in the pathogenesis of SIV and HIV because they are early targets for infection (Hladik et al., 2007) and long-lived virus-producing cells capable of promoting viral transfer to $CD4^+$ T cells (Sharova et al., 2005). Thus, it is important to understand innate immunity to lentiviral infection in this cell type. Recent evidence suggests that myeloid cells have unique innate immune factors that counteract lentiviral infection, including high expression of the myeloid-specific host restriction factors (SAMHD1 and A3A) and a high intracellular dUTP/dTTP ratio. It is still unclear whether or how these mechanisms cooperate: Does SAMHD1 nucleotide hydrolysis elevate the dUTP/dTTP ratio? What is the relative impact of uracil incorporation by A3A deamination compared with misincorporation of dUTP by reverse transcriptase? Does the increased time required for reverse transcription in the presence of SAMHD1 and low concentrations of dNTPs make the preintegration complex more susceptible to deamination by A3A? A better understanding of the relative importance of each of these pathways and their interplay is crucial to understand innate immunity to HIV infection in myeloid cells.

Summary of dissertation

The following work improves our understanding of the molecular mechanisms behind diverse consequences of Vpr expression. We also examine host and HIV-1 factors that impact HIV-1 replication in different cell types. It has been well established that SIV Vpx enhances lentiviral replication in myeloid cells by counteracting SAMHD1 and A3A. However, HIV-1 encodes Vpr, a homologous protein that counteracts neither of those

proteins. Instead, Vpr enhances the infection of macrophages through an unknown mechanism. While macrophages express a multitude of antiviral factors, these are not sufficient to prevent infection. A better understanding of the unique interplay between antiviral factors and HIV accessory proteins in macrophages may lead to the identification of novel molecular targets for therapeutic intervention.

This dissertation is organized into five chapters and an appendix. In Chapter 2, we demonstrate that that A3G deamination of HIV DNA leads to the upregulation of NKG2D ligands. That work led to the hypothesis that Vpr promotes the repair of uracilated HIV DNA by recruiting UNG2, an idea that we continue to explore in in the Appendix. Because Vpr has recently been proposed to limit the host antiviral response to HIV DNA(Laguette et al., 2014), we speculate that uracil may be a pathogen-associated molecular pattern (PAMP) shielded by Vpr from immunodetection. Chapter 3 describes novel effects of Vpr on virion production and Env expression in MDM that we propose are downstream of innate immune detection of HIV. In Chapter 4, I present a novel cell culture system that amplifies the effect of Vpr on infection of primary MDM, leading to the discovery of hMR as a host restriction factor counteracted by Vpr. Finally, in Chapter 5, I discuss data and future experiments to support an emerging molecular model for Vpr in cell lines, primary $CD4^+$ T cells and primary macrophages.

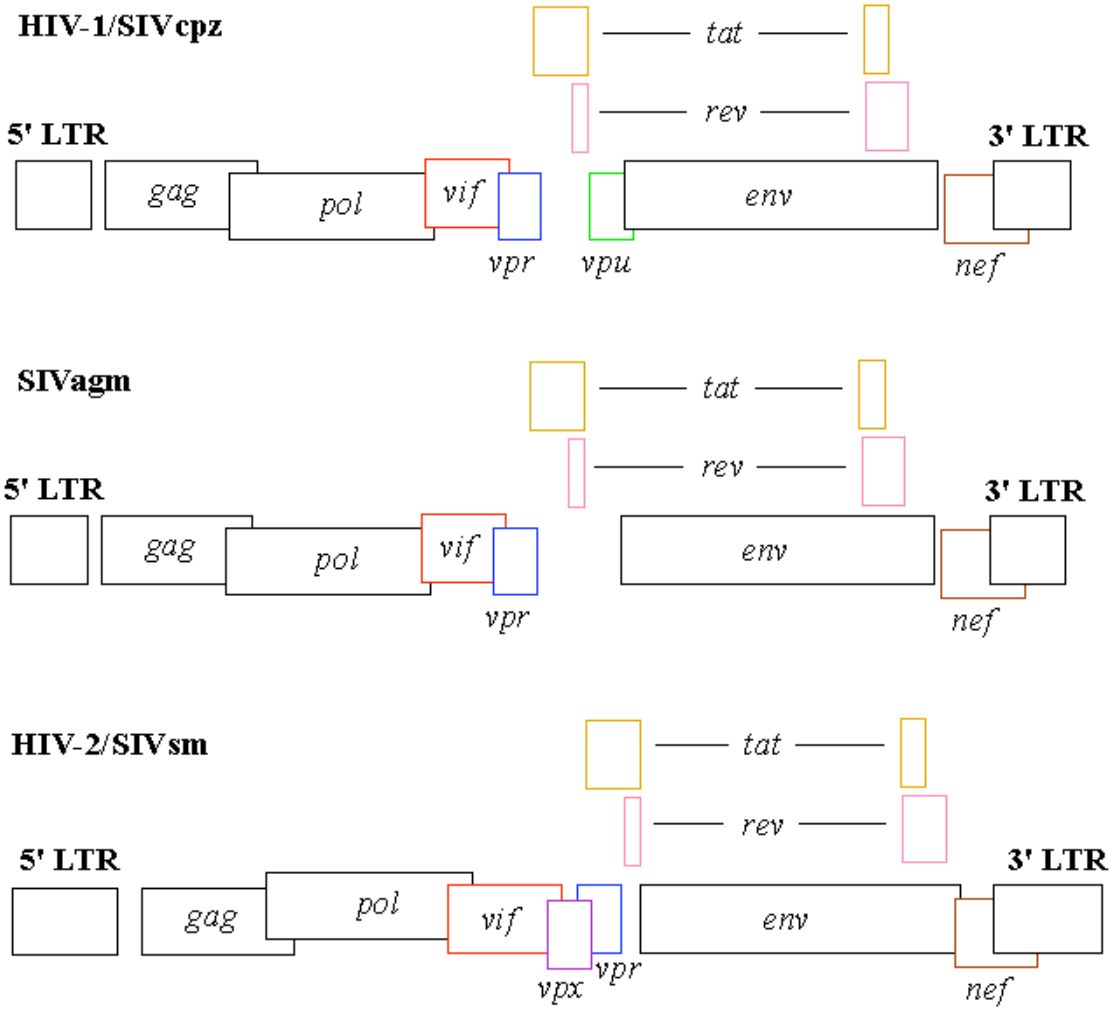

Figure 1.1. Genomic organization of HIV-1/SIVcpz, SIVagm and HIV-2/SIVsm. HIV-1 contains the *vpu* accessory gene, but not the vpx accessory gene. HIV-2 and SIVsm encode *vpx* but not *vpu*. SIVagm encodes neither *vpu* nor *vpx*. Modified from (Mashiba and Collins, 2013).[2]

[2] This figure was created by Michael Mashiba.

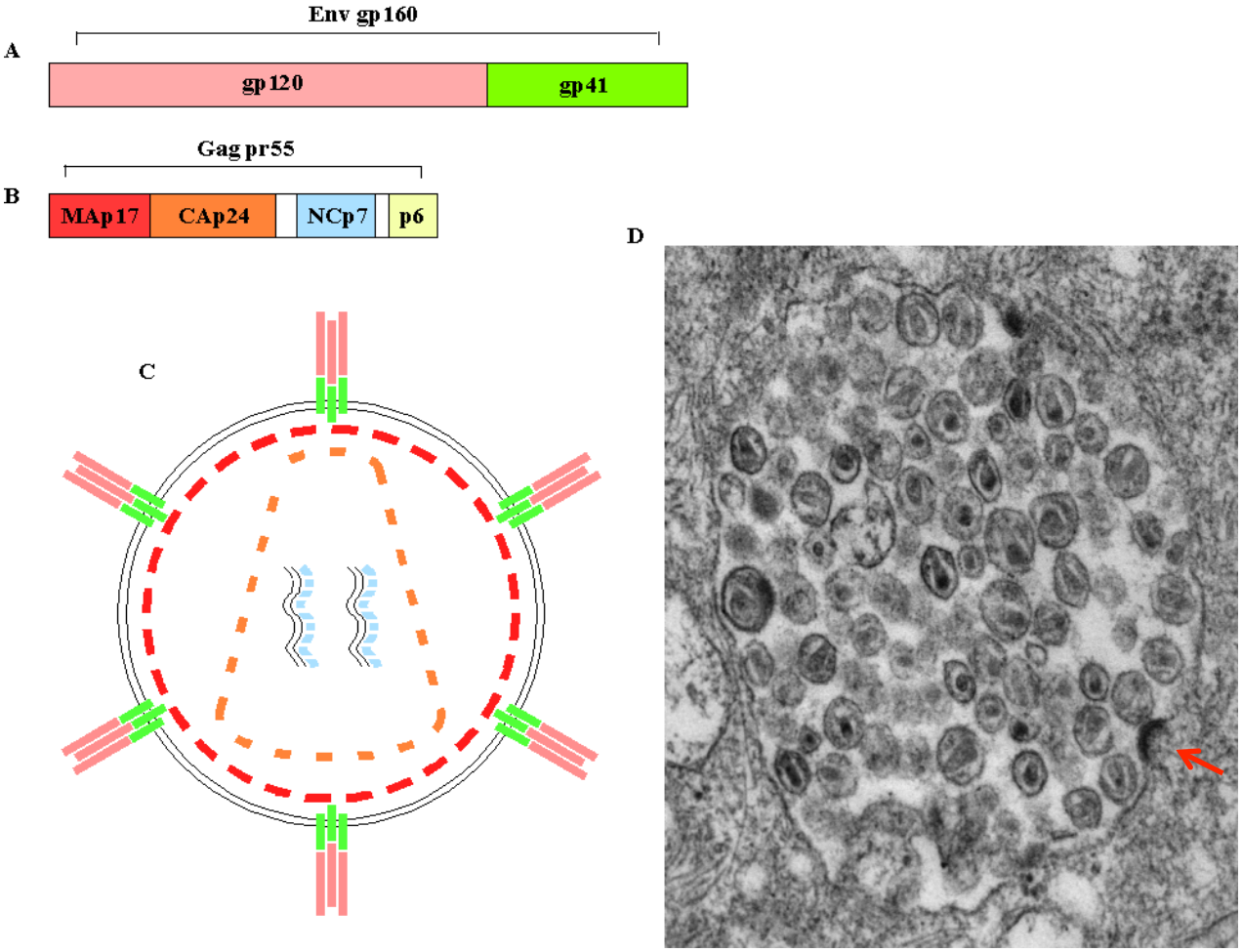

Figure 1.2. Schematic of the HIV-1 viral particle and structural proteins. Domains of Env **(A)** and Gag **(B)**. **(C)** Diagram of the structure of the HIV-1 viral particle. Proteins depicted include gp120 (pink), gp41 (blue), MA (red), CA (orange) and NC (blue). **(D)** Transmission electron micrograph taken of a virus containing compartment (VCC) within an HIV-infected macrophage. A particle in the process of budding is shown at the lower right (Red arrow).[3]

[3] This figure was created by Michael Mashiba.

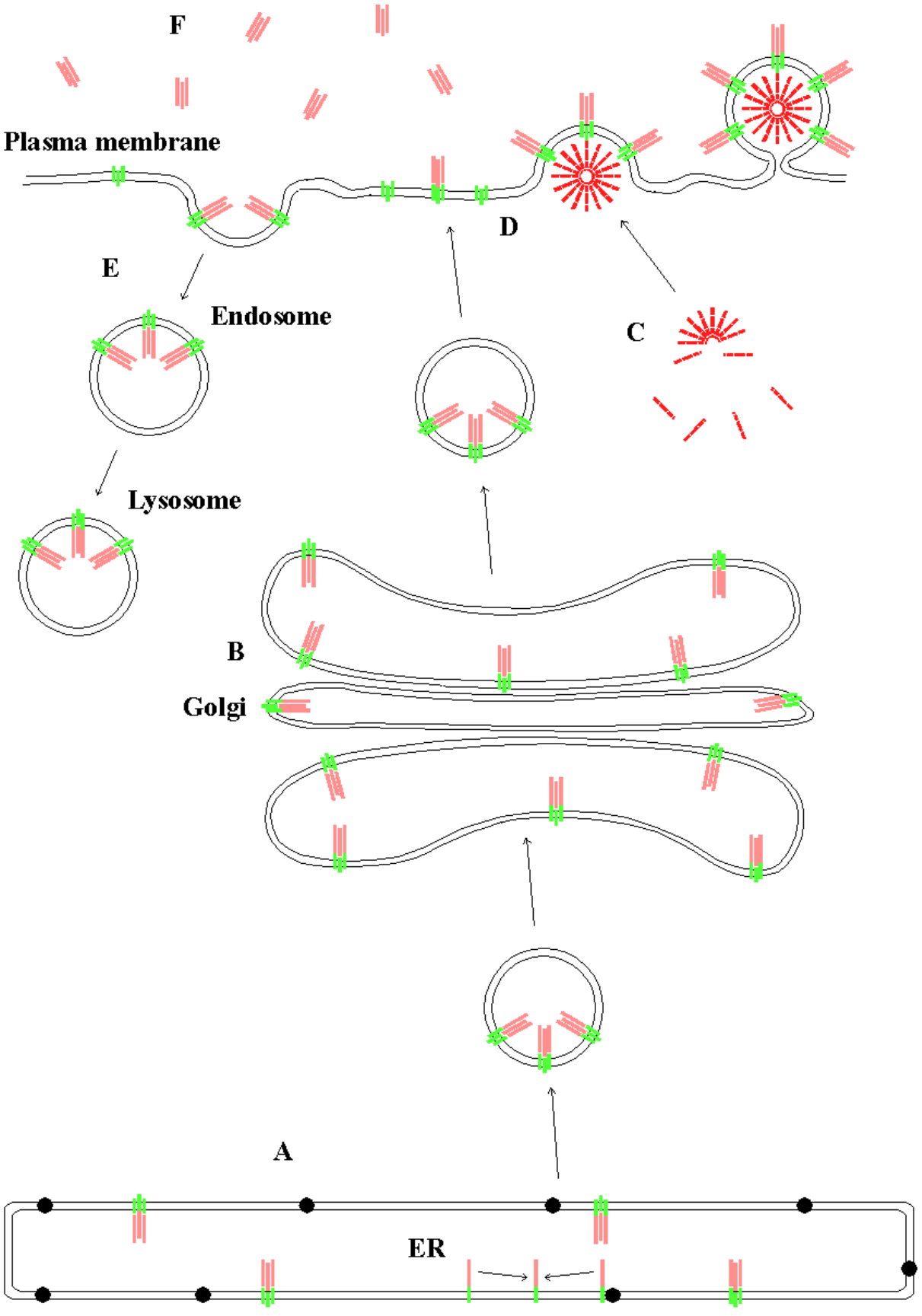
F
Plasma membrane
D
E
Endosome
C
Lysosome
B
Golgi
A
ER

Figure 1.3. Graphical representation of Env synthesis and trafficking. **(A)** Env gp160 is synthesized in the rough endoplasmic reticulum and forms trimers. **(B)** In the Golgi apparatus, a host furin protease cleaves gp160 into gp41 and gp120, which associate non-covalently. **(C)** Gag pr55 forms immature particles at the plasma membrane. **(D)** Env heterotrimers are trafficked to the plasma membrane where they are incorporated into immature viral particles. **(E)** In the absence of Gag, Env is rapidly internalized from the cell surface and degraded in lysosomes. **(F)** The weakness of the non-covalent association between gp41 and gp120 allows shedding of gp120 from the surface of the cell. [4]

[4] This figure was created by Michael Mashiba.

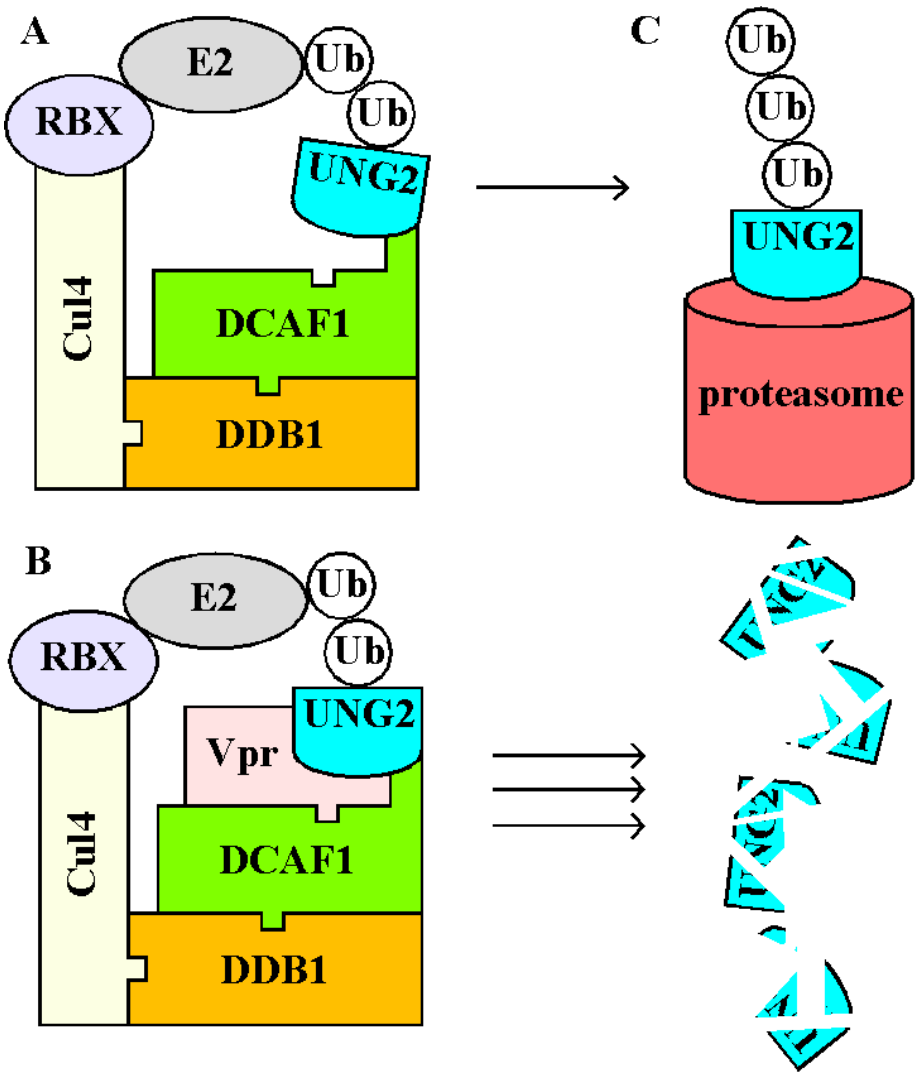

Figure 1.4. Vpr enhances the proteasome-mediated degradation of UNG2. **(A)** UNG2 polyubiquitination via the DCAF1-DDB1-Cul4A E3 ubiquitin ligase regulates the basal rate of UNG2 proteasomal degradation. **(B)** Vpr increases the affinity of the DCAF1-DDB1-Cul4A E3 ubiquitin ligase for UNG2. Vpr-mediated polyubiquitination leads to increased UNG2 proteasomal degradation **(C)**, depleting the steady state level of UNG2.[5]

[5] This figure was created by Michael Mashiba.

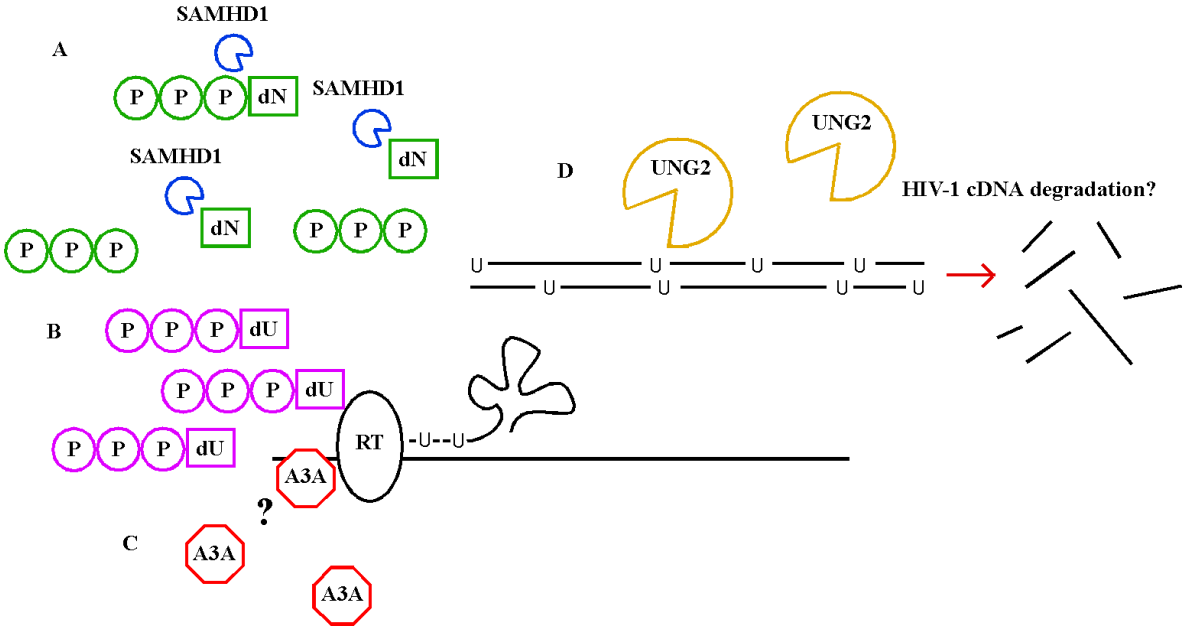

Figure 1.5. Mechanisms in myeloid cells that inhibit HIV-1 reverse transcription. dNTPs are dephosphorylated by SAMHD1 **(A)**. A high intracellular concentration of dUTP relative to dTTP in myeloid cells increases the incorporation of deoxyuridine into HIV-1 cDNA by reverse transcriptase **(B)**. A3A deaminates cytidine to uridine in HIV-1 cDNA **(C)**. A question mark (?) indicates that A3A may slow reverse transcription through an unknown mechanism. UNG2 catalyzes the removal of uracil from HIV-1 cDNA, and may lead to fragmentation or degradation of HIV-1 cDNA **(D)**. Modified from (Mashiba and Collins, 2013).[6]

[6] This figure was created by Michael Mashiba.

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Chapter 2

APOBEC3G enhances natural killer cell recognition of HIV-infected primary T cells[1]

Abstract

APOBEC3G (A3G) is an intrinsic antiviral factor that inhibits HIV replication by deaminating cytidine residues to uridine. This causes G-to-A hypermutation in the opposite strand and results in viral inactivation. HIV counteracts A3G through the activity of viral infectivity factor (Vif), which promotes A3G degradation. We report that viral protein R (Vpr), which interacts with a uracil glycosylase, also counteracts A3G by reducing uridine incorporation. However, this process results in activation of the DNA damage response pathway and expression of NK cell activating ligands. Our results reveal that pathogen-induced cytidine deamination and the DNA damage response to viral-mediated repair of uridine incorporation enhance recognition of HIV-infected cells by NK cells.

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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 APOBEC3G (A3G) that limit viral replication and spread[2].

A3G is a member of a family of cytidine deaminases known as apolipoprotein B editing complex (APOBEC) proteins. In addition to inhibiting HIV, members of the APOBEC3 family inhibit DNA viruses and retrotransposition[3]. The mechanism by which A3G inhibits HIV replication is twofold. A3G is packaged into HIV particles and deaminates cytosine residues to uracils in exposed stretches of viral ssDNA during cDNA synthesis[4]. This editing leads to G to A hypermutation on the opposite strand, which inactivates the virus. A3G also inhibits the translocation of reverse transcriptase along template RNA, disrupting cDNA synthesis[5]. The importance of A3G in HIV disease pathogenesis is highlighted by the fact that genetic variation at the A3G locus predicts disease progression in HIV-infected people[6].

The viral infectivity factor (Vif) from HIV-1 counteracts A3G by targeting it for proteasomal degradation, thus reducing the amount of A3G incorporated into virus particles[7]. However, the action of Vif is not absolute and hypermutated viral genomes have been isolated from individuals harboring full-length HIV-1[8]. These

sub-lethal mutations may influence HIV-1 evolution and promote the acquisition of drug resistance and immune evasion[9].

HIV-1–specific $CD8^+$ cytotoxic T lymphocytes (CTLs) decrease viremia during acute and chronic stages of the disease[10] but do not prevent the development of AIDS in most infected people. The HIV-1 Nef protein may limit the effectiveness of CTLs by downmodulating surface major histocompatibility complex class I (MHC-I) expression[11, 12]. However, MHC-I inhibits the lytic activity of natural killer (NK) cells and thus MHC-I downmodulation by Nef may increase susceptibility to NK cells.

Studies of NK cell inhibitory receptor allelic frequencies have provided strong evidence for a role of NK cells in limiting HIV infection. For instance, HIV-infected individuals expressing the NK cell inhibitory receptor KIR3DL1 and its cognate ligand, HLA-Bw4, progress to AIDS at a slower rate than individuals without this allelic combination[13]. This delayed progression likely results from a decrease in the activation threshold in the absence of inhibitory signals via HLA-Bw4, which is targeted by Nef in HIV infected individuals[14]. In addition, resistance to HIV-1 among sex workers is associated with a higher incidence of inhibitory *KIR* genes that lack their cognate inhibitory *HLA* ligand, which could lower the threshold for NK cell activation[15].

[NK cell activating signals also influence the progression of HIV disease. Infected individuals who encode the NK cell activating receptor KIR3DS1 and its ligand HLA-Bw4-80Ile progress more slowly to AIDS[16,17].]

NK cell activating signals also influence the progression of HIV disease. Infected individuals who encode the NK cell activating receptor KIR3DS1 and HLA-Bw4-80Ile progress more slowly to AIDS[16], due to increased KIR3DS1 expression by HLA-Bw4$^+$ individuals[17]. Ligands detected by the NKG2D NK cell activating receptor also stimulate NK cell recognition. These ligands represent a diverse family of proteins commonly upregulated in viral infection[18] and can affect NK cell recognition in HIV infected people[19]. In sum, these studies provide strong evidence that NK cells influence HIV disease progression and that strategies aimed at enhancing NK cell activity may benefit HIV-infected people.

Recent reports have linked NKG2D activating ligand expression to viral protein R (Vpr)[20]. Known cellular targets bound by Vpr are DNA repair proteins, including the uracil DNA glycosylases UNG2 and SMUG1 [21, 22]. However, the role of these DNA repair proteins in the normal functioning of Vpr and their potential role in HIV disease pathogenesis is unknown.

Here, we examined the effect of A3G, Vif and Vpr on recognition of HIV-infected cells by NK cells. These studies uncover a role for A3G in alerting NK cells to the presence of viral pathogens and reveal the way HIV evades this response. Specifically, we provide evidence that pathogen-induced cytidine deamination and the DNA damage response to viral-mediated repair of uridine incorporation act as intrinsic antiviral responses by upregulating NK cell activating ligand expression and increasing the sensitivity of HIV infected cells to NK cell lysis.

Results

HIV accessory proteins alter expression of NKG2D ligands

To better understand the factors that influence NK cell recognition of HIV-infected cells, we used a recombinant human NKG2D-Fc fusion protein to directly measure protein expression of NKG2D ligands on primary T cells. Infection with HIVs that encoded all of the viral accessory proteins (Nef, Vif, Vpu and Vpr, **Fig. 2.1a**) resulted in about 3-fold more NKG2D ligand expression in infected cells than in mock-infected cells (**Fig. 2.1b**). This result was consistently observed in nine independent donors with upregulation varying from about 1.5 to 5 fold (**Fig. 2.1c**). However, expression was further increased when cells were infected with an HIV that lacked Vif expression (**Fig. 2.1b**). Data compiled from nine independent donors revealed a consistent and statistically significant increase in ligand expression that was on average 1.5 fold higher than wild type HIV infection (**Fig. 2.1c**). As previously reported, infection with an HIV lacking Nef also yielded higher ligand expression[23] (**Fig. 2.1b and 2.1c**). Infection of T cells with a virus lacking expression of both Vif and Nef resulted in ligand expression similar to that achieved with each single mutant suggesting that Vif and Nef were acting on the same pathway.

In contrast, mutation of Vpr reduced NKG2D ligand expression to that observed in uninfected cells (approximately three-fold lower than wild type virus, **Fig. 2.1b and 2.1c**), confirming that Vpr expression was required for HIV-mediated

NKG2D ligand upregulation[20]. Examination of ligand expression by cells infected with *vif*⁻*vpr*⁻ and *nef*⁻*vpr*⁻ double mutant HIVs revealed that all of the additional ligand upregulation observed in the absence of Vif and Nef depended on Vpr expression (**Fig. 2.1d and 2.1e**).

To confirm that the ligand phenotype we observed was due to alteration in Vif expression by *vif*⁻ HIV, we demonstrated that this phenotype could be rescued by a lentiviral vector expressing wild type *vif* in the HIV-infected target cells (**Fig. 2.1f**, left panel). The expression level of Vif we achieved with lentiviral transduction was less than wild type (**Fig. 2.1g**, left panel) and this difference may explain the partial rescue. In contrast, expression of Vif in the virus producing cells at amounts similar to that achieved with wild type HIV (**Fig. 2.1g**, right panel) did not significantly alter ligand expression of the infected cells (**Fig. 2.1f**, right panel). These data confirm that Vif expression in HIV infected target cells reduces Vpr-dependent NKG2D ligand upregulation.

A3G expression correlates with NKG2D ligand upregulation

Because Vif targets A3G for degradation, we asked whether the effects of Vif on NKG2D ligand expression were related to A3G. Amongst uninfected donors, the amount of baseline A3G protein were highly variable (**Fig. 2.2a**). In addition, we found that we could increase A3G expression by incubating the CD56⁻CD8⁻ target cells with conditioned supernatant from CD8-depleted PBMC cultures (**Fig. 2.2a**) according to the time line shown in **Fig. 2.2b**. We also noted that the surface expression of NKG2D ligands increased with this treatment (**Fig. 2.2c**, left panels).

Variation in the amounts of A3G protein across donors correlated strongly with NKG2D ligand expression (**Fig. 2.2d**).

In some donors, we also observed an increase in the amount of A3G in primary T cells treated with viruses that lacked Vif expression or that contained a mutant Vif (Vif $Y_{44}A$) defective at binding and degrading A3G[24] (**Fig. 2.2e – 2.2g**). The increase in A3G protein with Vif $Y_{44}A$ was less than *vif*$^-$ viruses in some donors, potentially indicating this mutation was incompletely defective. Compared with *vif*$^-$ viruses there was no further effect of mutating *vpr* on A3G levels (**Fig. 2.2g**).

Furthermore, intracellular staining and flow cytometric analysis of a separate set of five donors revealed that A3G expression was higher in *vif*$^-$ HIV infected (Gag^+) cells compared to uninfected cells in the culture or mock infected cells (**Fig. 2.2h**, $p = 0.04$). These data support the conclusion that in the absence of Vif, HIV infection can induce A3G expression in the infected cell for the majority of donors.

Overall, we noted a strong positive correlation across multiple donors between A3G expression, as determined by immunoblotting, and the induction of NKG2D ligand expression for both infected and uninfected cells with a significant additional effect of infection (**Fig. 2.2i**, slope of regression lines significantly different, $p < 0.0001$). These data support the possibility that A3G expression determines the extent to which NKG2D ligands are upregulated.

We then examined NKG2D expression on cells infected with HIV expressing the $Y_{44}A$ mutant *vif*, which is unable to bind and degrade A3G. We found that cells infected with this mutant virus had similar NKG2D ligand protein as Vif-deficient virus in eight independent donors, indicating that Vif's ability to limit NKG2D ligand expression is secondary to its ability to bind A3G (**Fig. 2.2j**).

A3G activates the DNA damage response pathway

Recent reports have indicated that activation of the DNA damage response pathway can lead to increases in NK cell activating ligand expression[25]. DNA damage results in phosphorylation of the cyclin-dependent kinases ATR and ATM and phosphorylation of the checkpoint kinases Chk1 or Chk2 which inhibit cell cycle progression and promote DNA repair. Distinct types of damage activate these pathways. For example, ATR is activated in response to exposed stretches of single stranded DNA whereas ATM is activated in response to double stranded DNA breaks (reviewed in [26]).

To examine whether the DNA damage response pathway could be linked to A3G expression and/or upregulation of NKG2D ligands in HIV-infected cells, we measured the effect of HIV infection on Chk1 and Chk2 phosphorylation. We observed that HIV infection increased Chk2 phosphorylation several fold (**Fig. 2.3a and 2.3b**). Moreover, we noted further increases in phosphorylated Chk2 in cells infected with *vif*⁻ HIVs (**Fig. 2.3a and 2.3b**). HIV infection also influenced Chk1 phosphorylation in some donors, although to a lesser degree than Chk2 (**Fig. 2.3a and 2.3c**). Notably, Chk2 phosphorylation was affected by HIV in a manner that

mirrored the induction of NKG2D ligands in that it was Vpr-dependent and attenuated by Vif expression (compare **Fig. 2.3b** to **Fig. 2.2j**).

We also observed that the magnitude of Chk2 phosphorylation correlated across multiple donors with A3G expression in cells infected with *vif*⁻ HIVs and mock infected control cells, but the slope of the regression line was significantly higher in HIV-infected cells (**Fig. 2.3d**, $p < 0.02$). In contrast, when A3G protein expression across multiple donors were compared to Chk1 phosphorylation there was no significant correlation and NL-PI*vif*⁻ infected cells were indistinguishable from mock infected (**Fig. 2.3e**). While this suggests that Chk2 is more important than Chk1 for this response, higher background expression of Chk1 than Chk2 may limit our ability to detect changes in the infected subpopulation.

Based on the fact that A3G protein expression correlated best with Chk2 phosphorylation, we predicted that A3G was activating an ATM pathway. Consistent with this prediction, we observed an increase in ATM phosphorylation in HIV-infected cells and we found that ATM phosphorylation was enhanced in NL-PI*vif*⁻ infected cells (**Fig. 2.3f**). In addition, we found that the ATM inhibitor KU55933 [27] significantly reduced NKG2D ligand upregulation by wild type and *vif*⁻ HIVs when compared to solvent controls (**Fig. 2.3g**, $p = 0.03$ for NL-PI and $p = 0.02$ for *vif*⁻). Furthermore, cells treated with the ATM inhibitor (KU55933) no longer displayed a significant increase in NKG2D ligands in NL-PI*vif*⁻ infected cells compared to wild type virus infected cells, further indicating that A3G-mediated NKG2D ligand upregulation is ATM-dependent (**Fig. 2.3g**). However, total NKG2D ligand

expression remained 3-fold higher in HIV-infected cells treated with KU55933 than mock-infected cells (**Fig. 2.3g**). The partial inhibition by KU55933 is consistent with the fact that Vpr can also upregulate NKG2D ligands via the ATR pathway[20].

To determine whether the observed increase in Chk2 phosphorylation in *vif*$^-$ HIV infected cells was also ATM-dependent, we measured Chk2 phosphorylation in KU55933 treated primary T cells infected with an HIV lacking Vif expression. As predicted, we found that KU55933 reduced the phosphorylation of Chk2 for *vif*$^-$ HIV infected primary T cells (**Fig. 2.3h**). These data support a model in which A3G upregulates NKG2D ligands through an ATM pathway and in which Vif counteracts this upregulation by reducing A3G expression.

A3G sensitizes HIV infected T cells to NK cell lysis

To assess the relevance of NK cell ligand expression on NK cell recognition of HIV infected cells and to determine whether Vif might protect infected cells from NK cell lysis by degrading A3G, we used a previously described flow cytometric killing assay[12]. In this assay system, target cells pre-labeled with carboxyfluorescein succinimidyl ester (CFSE) were incubated with media or unlabeled, highly purified, autologous NK cells. Immediately afterward, the cell mixture was stained with antibodies directed against the NK cell markers CD16 and CD56 as well as the vital dye 7AAD to discriminate live and dead cells. The number of 7AAD$^-$CD16$^-$CD56$^-$ CFSE$^+$ cells was then scored as a function of a defined number of inert counting beads 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

amounts of NK cell activating ligands, the addition of primary NK cells resulted in a dramatic reduction of living CFSE$^+$ target cells (**Data not shown**).

To assess NK cell lysis of HIV-infected primary T lymphocytes, we infected cells with HIV expressing a GFP reporter inserted within the *env* open reading frame (ORF) to distinguish infected from uninfected cells (**Fig. 2.4a**). On day two of infection, autologous NK cells were co-incubated with the infected cells for four hours and then analyzed to detect NK cell lysis. We found that a high fraction of HIV-infected primary T lymphocytes survived treatment with autologous NK cells, whereas K562 cells were completely killed (**Fig. 2.4b**, left panels and quantified in **Fig. 2.4c**). 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 (**Fig. 2.4c**).

To determine whether inefficient killing resulted from deficient NK cell activation, 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 (**Fig. 2.4a**). Primary T cells infected with NL-GI*ulbp1*$^+$ expressed roughly 40-fold more ULBP1 on their surface compared to control infected cells (**Fig. 2.4d**). We found that ULBP1 overexpression enhanced NK cell clearance of HIV-infected cells approximately ten-fold, suggesting that NKG2D activating ligand expression was limiting for NK cell lysis of HIV-infected primary T cells (**Fig. 2.4b**, right panels and quantified in **Fig. 2.4c**).

We then asked whether overexpression of activating ligands would affect NK cell recognition of infected cells that had normal MHC-I expression. To test this, primary T cells were infected with *nef*⁻ HIVs plus or minus ULBP1 overexpression. In the absence of ligand overexpression these cells were substantially more sensitive to NK cell lysis than mock infected cells, indicating that HIV infection activated NK cell lysis even without MHC-I downmodulation (**Fig. 2.4e**, left panels and quantified in **Fig. 2.4f**). These data are consistent with our observations that HIV infection upregulates NKG2D activating ligands. However, overexpression of NK cell activating ligands in infected cells further increased killing and resulted in loss of most of the remaining cells (**Fig. 2.4e**, right panels and **Fig. 2.4f**). These data indicate that at high amounts of NKG2D ligands, MHC-I downmodulation is not essential for efficient NK cell lysis.

To determine whether the inhibitory effect of Vif on NKG2D ligand upregulation was physiologically relevant, we asked whether Vif expression affected NK cell lysis of infected cells. An analysis of infected cells from five independent donors revealed that cells lacking Vif were significantly more sensitive to NK cell recognition than wild type HIV controls in a four hour cytotoxicity assay (**Fig. 2.5a**, mean survival 60% and 66% for wild type compared with 38% and 45% for infected cells lacking Vif, at effector to target ratios 5:1 and 2:1, respectively). Thus, Vif expression resulted in increased survival of HIV-infected T cells.

We then asked whether A3G expression influenced NK cell recognition by knocking down A3G with siRNA. To accomplish this, we constructed a lentiviral

vector expressing an shRNA directed against A3G and GFP, which allowed us to gate on the knock-down cells and assess survival in the presence of NK cells using the flow cytometic killing assay. As a control, we determined that the shRNA containing vector could significantly reduce A3G expression in transfected 293 cells (**Fig. 2.5b**).

When primary T cells were co-transduced with shA3G or shNC expressing lentiviruses and *vif* $^{-}$ HIV, we found that A3G knock down significantly improved survival of infected cells co-incubated with autologous NK cells ($p=0.02$, $n= 3$, **Fig. 2.5c and 2.5d**). These data indicate that A3G sensitizes infected cells to NK lysis and that Vif-dependent A3G degradation likely explains Vif's protective effect (**Fig. 2.5a**).

Vif and Vpr reduce uridine incorporation

Because A3G deaminates cytosine to uracil, it is possible that the presence of uracil in DNA is sufficient to cause upregulation of NK activating ligands. Alternatively, gaps and breaks created by the cellular repair of uridine containing DNA could be responsible. To distinguish between these two possibilities, we directly measured the effect of Vpr and Vif on uridine incorporation. The assay we developed to measure uridine incorporation utilizes the activity of recombinant uracil DNA glycosylase (UDG), which specifically removes uracils, creating an abasic site that no longer serves as a template for PCR. In this assay, uridine incorporation is detected by a relative reduction in amplification of the template following UDG treatment. As a control, we generated uridine-containing and non-uridine

containing PCR products and demonstrated that amplification of templates containing uridine was specifically inhibited by UDG treatment (**Fig. 2.6a**).

When we examined DNA prepared from infected primary T cells, UDG treatment did not affect amplification of wild type (NL-PI) HIV proviral DNA, indicating that there was no detectable uridine in this sample. However, a significant effect of UDG on the amplification of HIV proviral DNA from *vif*⁻ *vpr*⁻ infected cells (**Fig. 2.6b**, compared to theoretical mean of 1.0, $p = 0.03$), indicating the presence of uridine in the DNA from this sample. There was no significant effect of UDG on amplification of proviral DNA from cells infected with either of the single mutants, suggesting that Vif and Vpr can independently limit uridine incorporation.

We noted a similar pattern of sensitivity to UDG when we amplified sequences from a cellular gene (be noted a **Fig. 2.6b**, compared to theoretical mean of 1.0, $p = 0.02$ and $p = 0.0006$ for *vif*⁻ and *vif*⁻*vpr*⁻, respectively) and UDG sensitivity positively correlated with infection rate for DNA isolated from *vif*⁻ *vpr*⁻ HIV infected cells (**Fig. 2.6c**). Thus, these data indicate uridine incorporation can also occur in cellular genomic DNA isolated from infected cells.

We also recovered significantly less HIV DNA from cells infected with *vif*⁻*vpr*⁻ HIV relative to wild type HIV after normalizing for infection rate and cellular DNA content (**Fig. 2.6d**). By comparison, the relative amounts of cellular DNA recovered from each sample, as measured by amplification of b. By comparison, the relative amounts of cellular DNA recover**Fig. 2.6e**). The relatively low recovery of *vif*⁻*vpr*⁻

HIV DNA was not explained by differences in the ability of the polymerase to amplify uridine-containing DNA; control uridine-containing templates were amplified as efficiently as non-uridine containing templates (**Fig. 2.6f**). Thus, *vif*⁻*vpr*⁻ provirus appears to be less stable following infection of primary T cells. The synergistic effect of mutating both Vif and Vpr is consistent with our hypothesis that both proteins act on the same pathway to stabilize provirus.

Vpr-UNG2 interaction and NKG2D ligand upregulation

Based on the fact that uridine was only detectable in cells infected with *vif*⁻*vpr*– HIV and the fact that cells infected with *vif*⁻*vpr*– HIV have low ligand expression, we concluded that uridine itself was not sufficient to induce ligand expression. Thus, we hypothesized that it was the repair of uridine containing DNA that was responsible for Vpr and A3G-dependent NK cell activating ligand upregulation. In this model the effects of Vpr on NKG2D ligand expression would be mediated by interaction with uracil glycosylase, UNG2. To test this, we constructed HIVs that contained a Vpr with a mutation in the UNG2 binding domain (Vpr $W_{54}R$)[28]. The Vpr phenotype is masked in the presence of Vif or Nef (**Fig. 2.1c and 2.1e**). Therefore, to focus on a potential Ung2 binding phenotype, we tested the Vpr mutant in a *nef*– background. As predicted, we found that this mutant was less active at inducing NK activating ligand expression compared with wild type Vpr (**Fig. 2.7a**, $p = 0.0004$), indicating that at least a component of Vpr's effect on ligand expression required UNG2 binding. Mutating the UNG2 binding site of Vpr did not completely eliminate NKG2D ligand upregulation, thus we cannot rule out the

possibility that Vpr also upregulates ligands through interactions with other cellular factors, such as SMUG1.

To confirm a role for UNG2, we knocked down UNG2 expression and assayed for NKG2D ligands in HIV-infected primary T lymphocytes. UNG2 expression was reduced 85% in CEM-SS T cells with UNG2-specific shRNA compared to control treated cells (**Fig. 2.7b**). Consistent with our model, knockdown of UNG2 in primary T cells also reduced the upregulation of NKG2D ligands by wild type HIV compared to control treated (**Fig. 2.7c**, $p = 0.03$). Once again, complete inhibition of NKG2D ligand expression was not observed by knocking down UNG2 expression, potentially implicating other cellular factors. In sum however, these data support a model in which Vpr induces NKG2D ligand upregulation in HIV infected T cells by stimulating UNG2-dependent repair of uridine-containing DNA (**Fig. 2.8**).

Discussion

Members of the APOBEC and AID family of cytidine deaminases are key host factors that aid in the defense against pathogens. There is growing evidence for overlapping roles of these deaminases in alerting killer T cells to infection and DNA damage. For example, A3G has been shown to stimulate CTL recognition through the generation of defective translation products[29]. In addition, the "DNA damage" resulting from AID-mediated somatic hypermutation leads to upregulation of NK cell activating ligands that transiently mark these cells as potentially dangerous[30].

We now show that, like AID, A3G expression sensitizes cells to NK cell recognition through the upregulation of NKG2D ligand expression in HIV infected cells. A3G knockdown increased the survival of cells infected with a Vif-deficient HIV and incubated with autologous NK cells. In addition, wild type Vif inhibited NKG2D ligand expression whereas Vif mutants defective at binding and degrading A3G did not. It is intriguing to speculate that these effects on NKG2D ligands may also contribute to A3G-dependent enhancement of CTL recognition[29], as NKG2D can serve as a co-stimulatory molecule in CTLs[31].

The effect of AID on the DNA damage response is related to its cytidine deaminase activity that results in uridine incorporation into cellular genomic DNA. Removal of the uracil by UNG2 and AP endonucleases generates gaps and breaks that lead to activation of the DNA damage response[32]. In contrast to AID, A3G is a cytoplasmic protein that targets ssDNA and it is less clear how its expression could lead to the incorporation of uridine into double stranded cellular DNA and activate the nuclear DNA damage response. Indeed, a recent publication did not detect an effect of A3G on mutation rate of transfected HEK 293 cells[33]. Thus, the ability of A3G to access cellular DNA appears to be specific to HIV-infected primary T cells lacking Vpr and Vif. The finding that Vif and Vpr specifically inhibit uracilation of cellular DNA suggests this is an important cellular response that HIV has evolved to evade.

Although A3G targets single stranded DNA, and most cellular DNA is double stranded, short stretches of dsDNA have the propensity to temporarily dissociate or

"breathe" [34], and these may be targeted by A3G in infected cells. In addition, it remains possible that other cytidine deaminases, some of which are nuclear and able to target double stranded DNA[33], contribute to uracilation of cellular DNA in the setting of HIV infection. Importantly, in the absence of Vpr and Vif we not only observed uracilation but also instability of HIV DNA. Thus, the in-coming HIV-1 genome is likely the primary target of this response, which results in uracilation followed by degradation of the HIV-1 DNA in the absence of Vpr and Vif.

We also show here that maximal upregulation of NKG2D ligands and the DNA damage response requires Vpr in addition to A3G. One model that explains the combined effects of Vpr and A3G is that Vpr expression is required to promote the repair of A3G-generated mutations through its interaction with UNG2 [28]. This model is consistent with prior studies demonstrating that Vpr reduces the requirement for high Vif expression in the presence of A3G[21]. Moreover, NKG2D ligand upregulation was reduced when the UNG2 binding site of Vpr was mutated or UNG2 expression was knocked down. Complete reversal of NKG2D ligand expression was not observed when UNG2 expression was reduced, either because of incomplete knockdown or because other factors can substitute for UNG2 activity.

Repair of integrated uridines reduces the error rate and improves the infectivity of progeny virus. However, the repair process, which generates nicks, gaps and breaks, has the side effect of activating an ATM response that results in NKG2D ligand upregulation. The link between NKG2D ligand upregulation and deamination reveals broader implications of the intrinsic antiviral response

mediated by APOBEC proteins than had previously been appreciated. Indeed, it appears that the A3G response to infection includes the initiation of a cellular suicide mission meant to contain viral production and spread.

The importance of uridine incorporation as an innate immune response is made clear by the fact that a wide range of viruses encode mechanisms to avoid it[35]. In addition, HIV-1 recruits UNG2 to virions, which has been reported to enhance viral infectivity by limiting dUTP misincorporation during reverse transcription[36] and to excise uridines resulting from A3G-editing[37]. Thus, there may be multiple routes through which HIV limits uridine incorporation.

When prior studies have measured the effect of A3G on viral particle inactivation, it was clear that A3G made in the producer cells and packaged into newly formed virions was required for viral inactivation and that A3G in the target cells was dispensable[7]. However, we provide evidence here that A3G expressed in the target cell also plays a role in the innate immune response. While A3G present in the target cell may be insufficient to inhibit the invading virus from productively infecting the cell, our data indicate that A3G expression in target cells is required for sensitization of HIV-infected cells to NK cell lysis.

We also report here that treatment of primary T cells with Vif-deficient HIV-containing cell supernatants resulted in an increase in A3G expression in HIV infected cells in some donors. This response has not previously been reported and the mechanism is unknown. Influenza virus ssRNA also increases A3G expression[38].

Although the mechanism for this increase is unclear, HIV-1 single stranded RNA binds Toll-like receptors 7 and 8[39]. Pathogen recognition receptor ligation induces type I interferon production, which stimulates A3G expression[2]. Our results highlight the possibility that detection of HIV by the innate immune system could lead to the production of A3G in infected cells.

In sum, the data presented here demonstrate that A3G expression enhances recognition of HIV-infected cells by NK cells through upregulation of NKG2D activating ligands. 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 by NK cells.

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Author Contribution

J.M.N. and K.L.C. designed the experiments and prepared the manuscript. J.M.N., M.M., L.A.M., A.O.N. W.S. and E.C.F. performed experiments. All authors read and edited the manuscript.

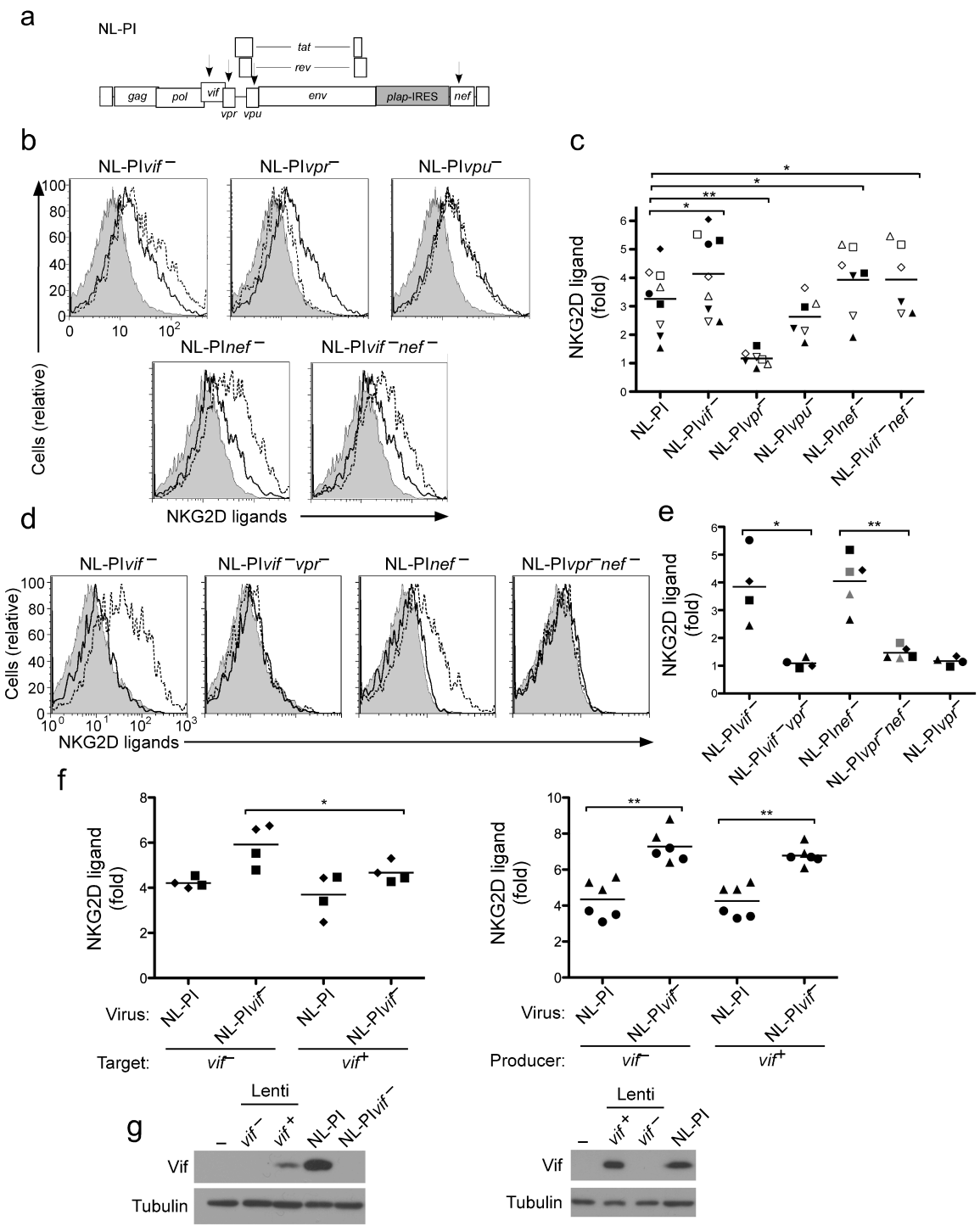

Figure 2.1: NKG2D ligand expression in HIV infected cells (**a**) HIV genome of NL-PI, which has been previously described[12, 40]. Locations of mutations in individual ORFs are indicated. **(b-f)** Flow cytometric analysis of NKG2D ligand expression by $CD3^+$ T cells. Cells infected with NL–PI (solid black line), NL–PI

accessory protein mutants (dashed black line) and mock-treated control (shaded gray histogram) are shown in part **(b)** whereas cells infected with NL–PI*vpr*– (solid black line), the indicated NL–PI accessory protein mutant (dashed black line) and mock-treated controls (shaded gray histogram) are presented in part **(d)**. **(c and e)** Summary plots of NKG2D ligand expression on infected versus mock-infected T cells. The fold difference in NKG2D ligand expression relative to mock-treated controls is shown for independent donors, each represented by a different character. Black bars represent the mean fold change in ligand expression for each virus. Statistical analyses were calculated by paired *t* test. * $p<0.05$, ** $p<0.01$. **(f)** Summary plots of NKG2D ligand expression on NL-PI or NL-PI*vif*– infected primary T cells with or without Vif rescue. Left panel, primary T cells co-transduced with NL-PI or NL-PI*vif* – and *vif*-expressing or control lentiviruses. Right panel, viruses used to infect primary T cells were prepared from *vif*-expressing or control 293T cells. Two independent donors assayed in triplicate are shown. * $p<0.05$, ** $p<0.01$. **(g)** Immunoblot analysis of whole cell lysates from primary T cell targets (left panel) and 293T producer cells (right panel). Data are representative of two independent donors. [2]

[2] The data in this figure was produced by Jason Norman and Michael Mashiba.

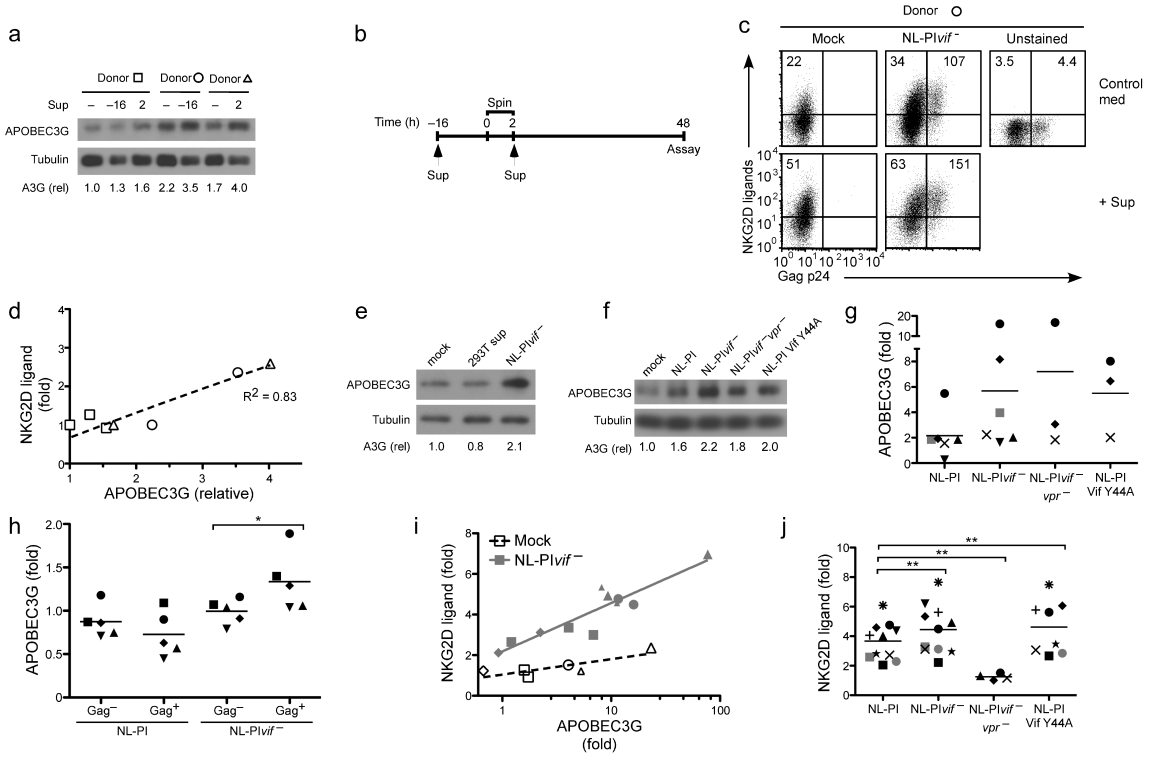

Figure 2.2: Upregulation of A3G correlates with NKG2D ligand upregulation. **(a)** Immunoblot analysis of T cells treated with media alone (-) or conditioned supernatant (Sup) (-16hr or 2hr) according to the experimental timeline in part **(b)**. **(c)** Flow cytometric analysis of $CD3^+$ T cells treated with conditioned supernatant (Sup) or control media (Control med) and infected with the indicated virus. The mean fluorescence intensity is indicated for the total Gag^- and Gag^+ populations. **(d)** Plot of relative A3G expression from part **(a)** versus NKG2D ligand normalized to control media treated cells. The linear best-fit line is indicated. R^2=0.83 **(e and f)** Immunoblot analysis of T cells infected with the indicated virus, control media (mock) or 293T cell supernatant. Data are representative of three independent donors. **(g and h)** Plot of relative A3G expression determined by **(g)** Immunoblot or **(h)** flow cytometric analysis of permeabilized cells infected with the indicated virus. Data obtained from each donor is indicated by unique symbols. *p<0.05 Donors in part **(g)** and **(h)** are independent. The mean fold change is indicated by the black bars. **(i)** Plot of A3G expression versus NKG2D ligand expression of mock-infected (white symbols) or NL-PI*vif*⁻ infected (gray symbols) cells with each independent donor indicated by a different symbol. R^2 for mock-infected =0.66, NL-PI*vif*⁻ R^2=0.84. **(j)** Summary of NKG2D ligand expression by cells infected with wild type or mutant NL-PI. Black bars indicate mean change in ligand expression. Each symbol represents a unique donor independent of those shown in **Fig. 1**. **p<0.02. [3]

[3] The data in this figure was produced by Jason Norman.

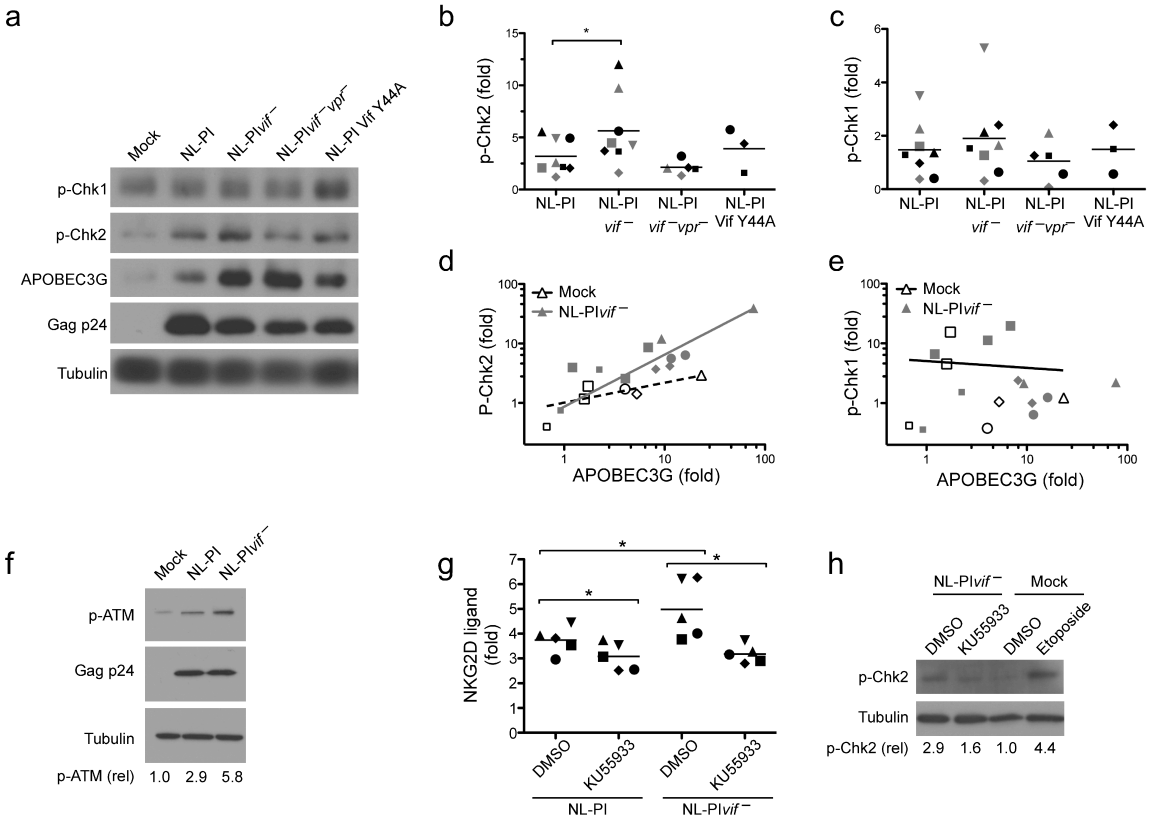

Figure 2.3: Activation of the DNA damage response in HIV infected primary T cells. (a) Representative immunoblot analysis of primary T cells infected with the indicated virus. **(b and c)** Summary plots of immunoblot analyses. **(b)** Chk2 phosphorylation at Thr_{68}; **(c)** Chk1 phosphorylation at $Ser_{345.}$ Each independent donor is represented by a unique symbol. The black bar inidcates fold change relative to mock. **(d and e)** Relative A3G expression versus **(d)** Chk2 phosphorylation at Thr_{68} or **(e)** Chk1 phosphorylation at Ser_{345}. Each independent donor is represented by a unique symbol. Open symbols; mock infected plus conditioned supernatant. Gray symbols; infected plus or minus conditioned supernatant. For **(d)**, mock R^2=0.75, NL- PI*vif*$^-$ R^2=0.92, p<0.02. For (**e**), R^2=0.02. **(f)** Western blot analysis of primary T cells infected with the indicated virus measuring ATM phosphorylation at Ser_{1981}. **(g)** Summary plot of NKG2D ligand expression on primary T cells infected with the indicated viruses and treated with the ATM inhibitor KU55933 (10mS, Calbiochem) or solvent control. Data from five independent donors is shown. The black bars indicate the mean NKG2D ligand upregulation. Statistical analyses were performed by paired *t* test. * p<0.05 **(h)** Western blot analysis of primary T cells infected with NL-PI*vif*$^-$ and treated with KU55933 (10ma), solvent control or Etoposide (10m,, Sigma). Band intensities

were quantified using Adobe Photoshop software, adjusted for background and normalized to a. ulin levels in the same lane. P-Chk2 expression relative to mock infected, solvent control is shown. Data are representative of three independent donors.[4]

[4] The data in this figure was produced by Jason Norman and Michael Mashiba.

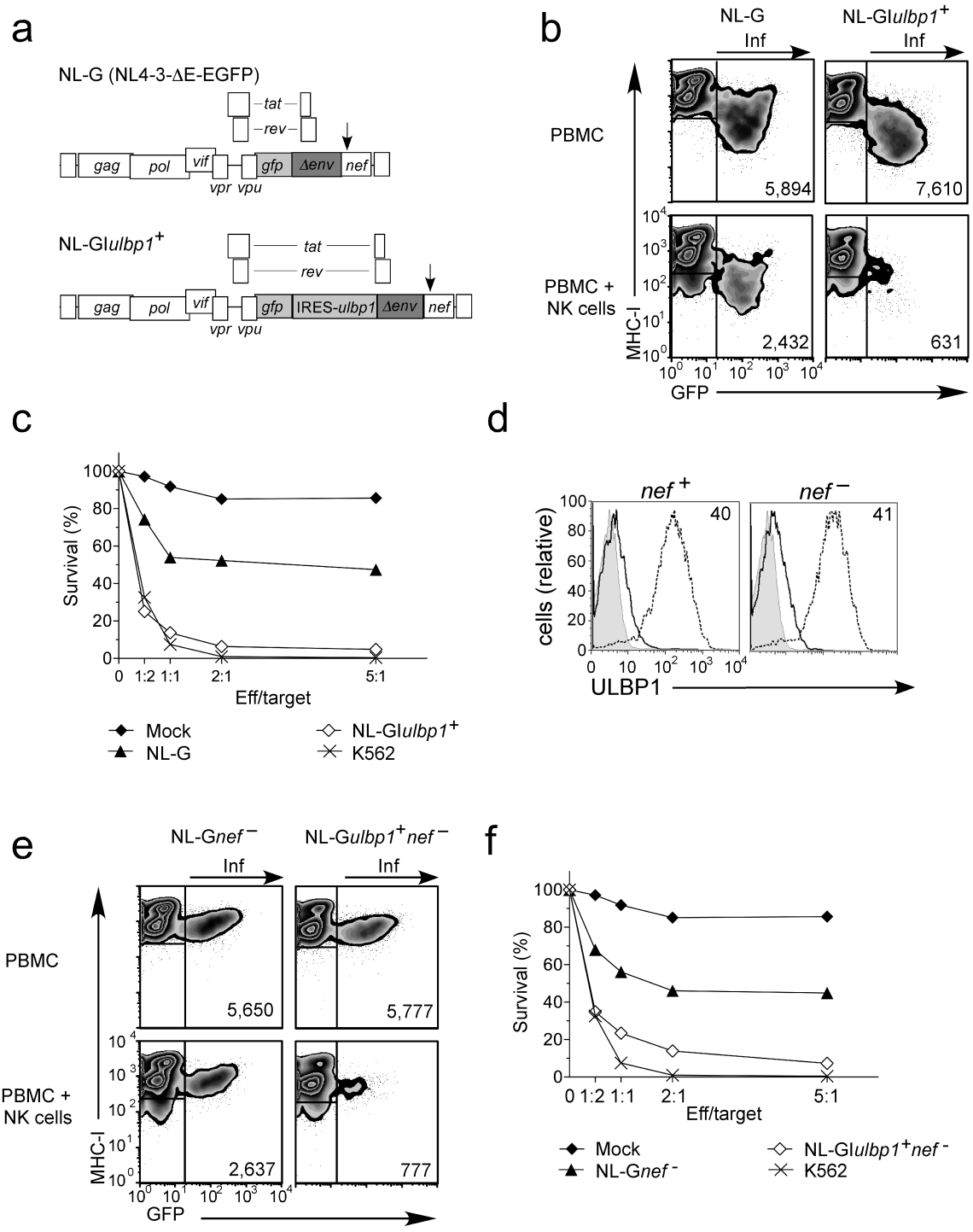

Figure 2.4: HIV–infected T cells are resistant to NK cell recognition unless an NKG2D ligand is overexpressed. (a) HIV genomes. NL4–3–DE–EGFP has been described[41]. Arrow indicates mutation in *nef*- construct. **(b)** Flow cytometric NK cell assay of primary T cells infected with the indicated virus 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. Infected cells (Inf) are indicated. The total number of GFP$^+$ cells normalized to counting beads is indicated. **(c)** Quantification of NK cell assay from part **(b)**. The data are representative of three independent donors. The effector to target cell ratio (Eff/target) is indicated. **(d)** Flow cytometric analysis of ULBP1 expression in PHA-activated primary T cells transduced with NL–GI*ulbp1*$^+$, +/– *nef* (dotted black line) or NL–G +/– *nef* viruses (solid black line). ULBP1 expression of the transduced, GFP positive cells is shown. The solid gray histogram is the isotype control. The fold change in ULBP1 expression relative to control virus is indicated. **(e)** Flow cytometric NK cell assay of PHA activated primary T cells infected with the indicated viruses and co-incubated with autologous NK cells. 7–AAD$^-$CD56$^-$CD16$^-$ GFP$^+$ cells are shown and the number (normalized to counting beads) is indicated. **(f)** Quantification of NK cell assay from part e. The data are representative of two independent donors.[5]

[5] The data in this figure was produced by Jason Norman.

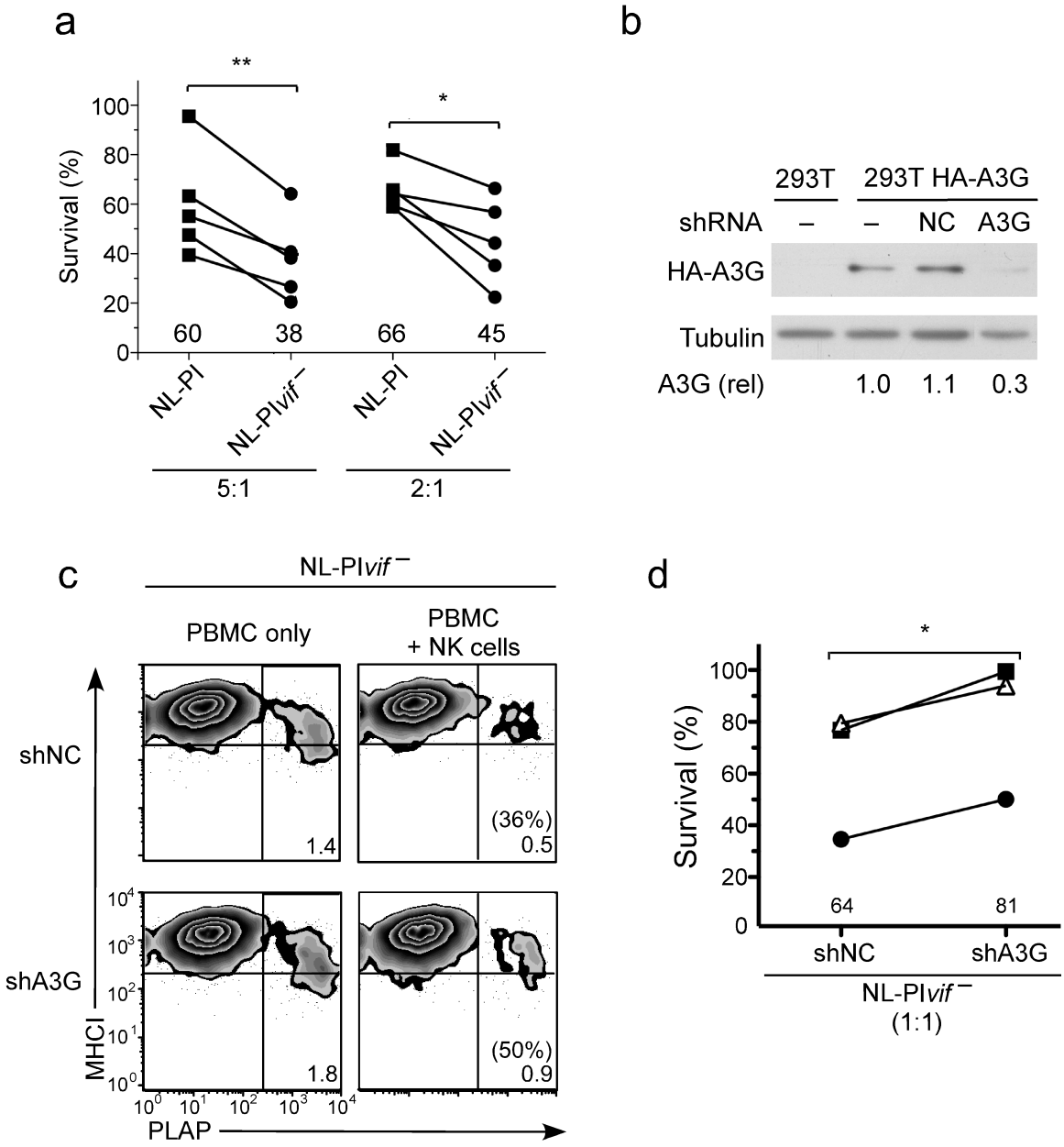

Figure 2.5: The effect of Vif on NK cell lysis. (a) Summary of flow cytometric NK cell killing assays from five independent donors. The percentage of infected cells surviving autologous NK cell treatment is shown. Mean T cell survival among the independent donors is indicated. * $p<0.05$, ** $p<0.01$. **(b)** Immunoblot analysis of 293T whole cell lysates transfected with the indicated shRNAs or untransfected (-). Band intensities were quantified using Adobe Photoshop software and normalized to tubulin expression. A3G expression relative to the untransfected control is shown. Data are representative of three experiments. **(c)** Flow cytometric NK cell assay of

PHA activated primary T cells infected with the indicated virus plus a GFP-expressing lentivirus encoding A3G–targeting or negative control shRNAs and co–incubated with IL–2 stimulated autologous NK cells at a 1:1 effector to target ratio in a 4 hour cytotoxicity assay. Plots show MHC–I and PLAP expression of GFP^+, 7–AAD^- $CD56^-$ $CD16^-$, live target cells. The percentage of infected cells with low MHC-I expression remaining after NK cell incubation is indicated. The percent cell survival with NK cells compared to without is shown in parentheses. **(d)** Summary of NK cell killing assay for three independent donors, indicated by different symbols. The mean cell survival at the indicated effector to target cell ratio (1:1) is indicated. In one of three experiments conditioned supernatant was required to observe an effect of A3G knockdown. Statistical analysis was calculated by paired *t* test. * $p<0.05$.[6]

[6] The data in this figure was produced by Jason Norman.

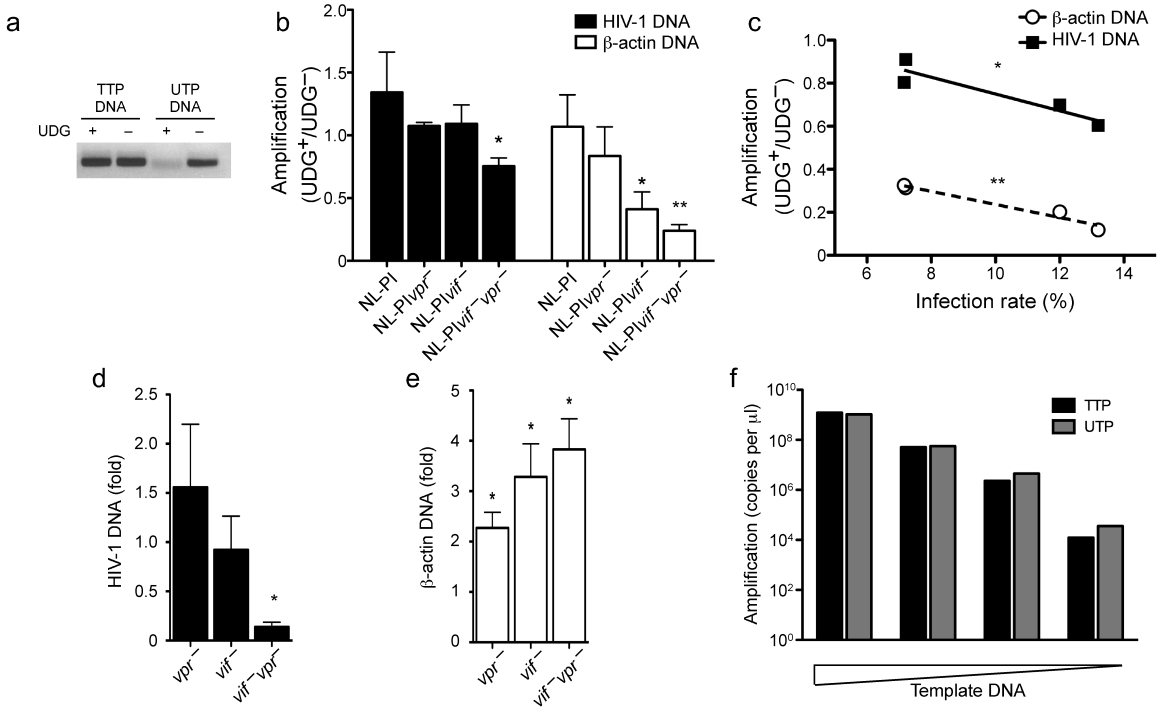

Figure 2.6: Vif and Vpr limit uridine incorporation in primary T cells. (a) Agarose gel of PCR products using NL-PI DNA containing dTTP or dUTP treated with recombinant UDG (+) or heat inactivated UDG (-) for 1 hour as template. **(b)** qPCR amplification results using total genomic DNA from primary T cells infected with NL-PI or the indicated virus mutant as template. DNAs were treated with UDG (+) or heat-inactivated UDG (-) prior to amplification. The graph shows the mean fold change in amplification with UDG+ treatment from four independent donors. Statistical significance was determined by comparing to the theoretical mean of 1.0, which would represent no effect of UDG treatment. * p<0.05, **p<0.002 **(c)** Graph of qPCR results from part **(b)** versus infection rate of NL-PI*vif*⁻*vpr*⁻ infected primary T cells. HIV-1 DNA; R^2=0.86, p=0.07. b0.07. orrelR^2=0.96, p=0.02. **(d and e)** qPCR amplification results of **(d)** total HIV DNA and **(e)** be)tal HIV DNA and results of t donors. Statistical significance was iruses. In part **(d)** the mean was normalized for infection rate and bthe mean was normalized for infection rate and tical significance was irus(d) * p<0.002, (e) *p<0.05 **(f)** qPCR amplification results of serially diluted NL-PI DNA templates containing dUTP or dTTP.[7]

[7] The data in this figure was produced by Jason Norman, Lucy McNamara and Adewunmi Onafuwa-Nuga.

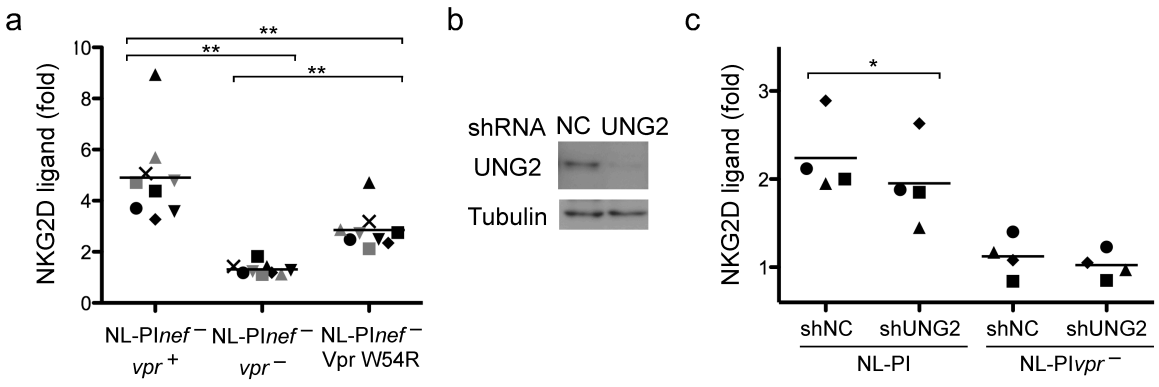

Figure 2.7: UNG2 binding by Vpr induces NKG2D ligand expression. (a and c) Summary plots of NKG2D ligand expression on activated primary T cells infected with the indicated viruses. The fold increase in NKG2D ligand expression relative to mock infected cells is shown. The mean expression for **(a)** eight and **(c)** four independent donors, indicated by different symbols, is represented by the black bars. Statistical analyses were performed by paired t test. * $p<0.05$, ** $p<0.01$. **(b)** Western blot analysis of CEM-SS T cells transduced with control or shUNG2-expressing lentivirus.[8]

[8] The data in this figure was produced by Jason Norman and Wenwen Shen.

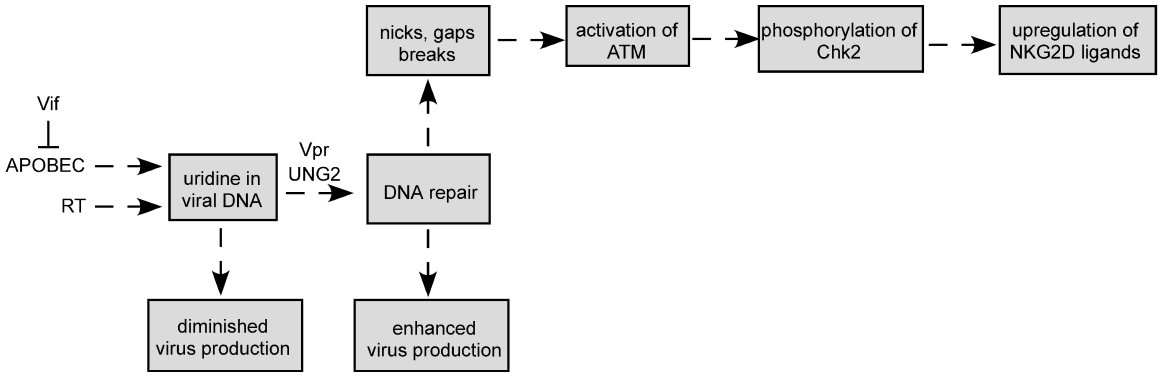

Figure 2.8: Proposed model for A3G-induced DNA damage response activation and NKG2D ligand expression. Arrows indicate activation and horizontal lines indicate inhibition. RT (reverse transcription) can also lead to incorporation of uridine.

Methods

Cell culture and viral infections.

We obtained pre-existing leukopaks lacking subject identifiers from The New York Blood Center. The use of these specimens was reviewed by the University of Michigan IRB and found not to be human subjects research. We isolated PBMCs by Ficoll-Paque density separation. We prepared $CD56^+$ NK cells from adherence-depleted PBMCs using commercially available kits (EasySep, StemCell Technologies). After isolation we maintained $CD56^+$ cells in RPMI 1640 with 10% human AB serum (Fisher scientific), penicillin, streptomycin, L-glutamine and 500 IU/ml rhIL-2[42]. PBMCs were prepared for HIV infection as previously described[14] except that they were also CD56-depleted (EasySep, StemCell Technologies). Virus production and infection of PBMCs have been previously described[12,43]

Viral constructs.

HIV NL-PI and *nef*$^-$, *vpr*$^-$, *vpu*$^-$, *vpr*$^-$*nef*$^-$ accessory protein mutants have been reported previously[12, 40]. *Vif* expression was disrupted by filling in the NdeI site at position 83 of *vif*. *Nef* expression was disrupted by filling in the XhoI site at position 101 of *nef*. We used a standard PCR-based mutagenesis strategy to generate NL-PI*vif*Y_{44}A and NL-PI*vpr*W_{54}R (see primers in **Table S1**).

FG12 shA3G and shUNG2, short hairpin RNA expression constructs targeting APOBEC3G and UNG2, were cloned using the primers in **Table S1** into the lentiviral

vector FG12[44] as described previously[43]. The negative control vector (FG12 shNC) was previously described[43].

To construct the Vif FG9 lentiviral expression vector, NL4-3 Vif was amplified by PCR (see primers in **Table S1)** and cloned into the BamHI and NotI sites of FG9[45].

To construct pNL-GI*ulbp1*$^{+}$, *ulbp* was amplified from cDNA (MegaMan Human Trascriptome Library, Stratagene; **Table S1**) and a PCR-based strategy was used to create the IRES*ulbp1* cassette from the amplicon (**see primers in Table S1**). The cassette was cloned into the NheI and BglII sites in the *env* open reading frame of pNL-G (pNL4-3-deltaE-EGFP)[41].

Flow cytometry.

Cell surface proteins (aside from NKG2D ligands), were stained in flow cytometry buffer plus human serum (PBS with 2% FBS, 1% human type AB serum, 1% HEPES and 1% NaN_3) using the antibodies listed in **Table S2** and fluorescently conjugated isotype-specific secondary antibodies (Invitrogen or Caltag), then fixed in 2% paraformaldehyde. For NKG2D ligand analysis, cells were stained with rhNKG2Dfc (R&D Systems) and anti-CD3 antibody (**Table S2**) in flow cytometry buffer without human serum. Intracellular Gag stains were performed as previously described[46]. For A3G staining, cells were fixed and permeabilized in 90% methanol, blocked in PBS-1% BSA-10% goat serum prior to staining with APOBEC3G antibody (Abcam) or normal rabbit serum control (Santa-Cruz) in PBS-1% BSA. Cells were

subsequently stained for Gag and FITC or GAR-488 conjugated anti-rabbit secondary antibodies (Biosource and Invitrogen) diluted in PBS-1% BSA for 30 min RT.

NK cell cytotoxicity.

Target cells (K562 cells and 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 incubated overnight at 37°C. Alternatively, target cells were infected with FG12 shRNA or NL-G viruses expressing GFP. Labeled target cells were co-incubated with purified, autologous NK cells at increasing effector:target ratios for 4 hours at 37°C and then 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 identified by light scatter parameters, 7-AAD exclusion, and $CD56^-/CD16^-/CFSE^+$(or GFP) staining. A constant number of fluorescent counting beads (Countbright beads, Invitrogen) was added to each tube and counted during cytometric acquisition. The normalized number of live cells was calculated as the number of cells times the fraction of beads counted. Percent target cell survival was determined as the percent normalized target cells remaining after NK cell incubation.

Immunoblot Analysis.

48 hours post HIV infection, PBMC lysates were isolated as previously described[47]. Normalized whole cell lysates were analyzed by immunoblot using the antibodies in **Table S2**. APOBEC3G[48] and Vif[49] antibodies were previously

described. Membranes were incubated with HRP-conjugated secondary antibodies (Invitrogen) and developed. Background-subtracted median band intensities were determined using Adobe Photoshop and normalized to tubulin for quantification.

Quantitative PCR analysis

Infected cells were harvested 48 hours post-infection. For DNA extraction, cells were resuspended in PBS and lysed with MagNa Pure Lysis/Binding buffer (Roche). Lysed cells were loaded on the MagNa Pure Compact and total DNA was extracted. Samples were treated with 10U UDG (NEB) and incubated at 37°C for 1h followed by 10 minutes at 95°C to inactivate the UDG. For samples with heat-inactivated UDG, DNA was added after UDG heat-inactivation.

For qPCR analysis, the TaqMan ProbeMaster kit (Roche) and the primers in **Table S3** were used. HIV-1 DNA was first subjected to 12 cycles of linear amplification using the 2nd-LTR-F-univ primer[46] to amplify the negative strand. Probe-2 was previously described[50].

Control TTP and UTP-containing templates were generated by PCR amplifying NL-PI using the 2nd-LTR-F and Gag-R-3 primers. For UTP control templates, dUTP instead of dTTP was used. Gel purified TTP and UTP-containing control templates were analyzed post UDG treatment by gel electrophoresis of re-amplified samples

Statistical analysis

Statistical significance in the flow cytometry and western blot data were assessed by paired *t* test. Regression analysis was performed using GraphPad Prism software.

For qPCR data, amplification efficiency (E) was calculated as E = 10^(-1/slope of target standard curve) – 1. Average Cq for three replicates of each sample was converted to Cq at 100% efficiency (Cq_{100}) = $Cq*Log_2(1+E)$ to permit comparison between different genes. ΔCt values for each sample were calculated as Cq_{100}(untreated) –Cq_{100}(UDG treated) and converted to quantity ratios (2^ΔCt). One sample t-tests were used to compare ΔCts and quantity ratios to the expected values if UDG treatment has no effect: 0 and 1, respectively. Total HIV-1 DNA was assessed by computing ΔCt values for non-UDG treated samples relative to b-actin and Ct values comparing each mutant virus to NL-PI. Ct values were converted to quantity ratios and normalized to infection rate. One sample t-tests were used to compare the normalized quantity ratios to the expected value of 1 if HIV-1 DNA amplification does not vary with Vif or Vpr mutation.

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Chapter 3

Vpr utilizes DCAF1-DDB1-CUL4 E3 ubiquitin ligase to overcome a macrophage-specific restriction of Env expression and virion production

Summary

The HIV accessory protein Vpr enhances infection of macrophages through an unknown mechanism. Recent studies have revealed that Vpr-dependent interactions with the DCAF1-DDB1-CUL4 E3 ubiquitin ligase and cell cycle arrest are linked to pathways that limit activation of innate immunity. Here we describe a restriction mechanism that is active in non-permissive, HIV-1 infected primary macrophages lacking Vpr, but not in permissive cell lines. In the absence of Vpr or upon silencing of DCAF1, HIV Env protein was targeted for degradation via a lysosomal degradation pathway. Loss of Env expression was associated with dramatically reduced virion production and spread in macrophages. HIVs harboring Vpr mutants that were defective at DCAF1 binding and G2/M cell cycle arrest were similarly defective at virion production and Env expression. Our work provides insights into a macrophage-specific restriction pathway targeting HIV Env that is counteracted by Vpr and its cellular cofactor DCAF1.

Introduction

To establish a persistent infection, lentiviruses encode accessory proteins that are not required for replication in some cell lines, but are necessary for infection *in vivo* (Malim and Emerman, 2008). Many of these accessory factors have been shown to counteract host restriction factors that can limit HIV infection(Collins and Collins, 2014). Interestingly, transformed cell and primary cell systems vary in the extent to which they express restriction factors targeted by these accessory proteins. For example, primary monocytic cells harbor a post-entry block to HIV-1 infection that can be overcome by the simian immunodeficiency virus (SIV) accessory protein Vpx (Berger et al., 2011; Sharova et al., 2008). Vpx binds a substrate adaptor of a cellular ubiquitin ligase complex [damaged DNA binding protein 1-cullin 4-associated factor 1 (DCAF1)] to promote ubiquitination and proteasomal degradation of cellular restriction factors SAMHD1(Laguette et al., 2011) and apolipoprotein B-editing complex 3A (APOBEC3A)(Berger et al., 2011). In the absence of Vpx, these restriction factors prevent productive infection of immature monocytic cells.

Despite its importance for infection of immature monocytic cells, no *vpx* gene has been found in any HIV-1 molecular clones and as such, HIV-1 is not able to infect immature monocytic cells that express high levels of SAMHD1 and APOBEC3A. However, Vpr-expressing HIV-1 is able to efficiently infect monocyte derived macrophages (MDM) that have lower levels of SAMHD1 and APOBEC3A (Ayinde et al., 2010; Chen et al., 2004; Connor et al., 1995; Heinzinger et al., 1994). Like Vpx, Vpr utilizes DCAF1 and the Rbx1/Cullin4A E3 ubiquitin ligase complex, however, the cellular targets of Vpr have only recently been identified. Elegant studies performed in transformed cell line systems demonstrated that Vpr binding to DCAF1 activates the structure specific endonuclease (SSE) regulator SLX4

complex. Activation of SLX4 leads to evasion of innate immune sensing of viral infection, possibly by enhanced processing of HIV-1 DNA replication intermediates (Laguette et al., 2014). However, the cell lines used for these studies are permissive to HIV infection in the absence of Vpr. Thus, the exact way in which escape from innate immune recognition facilitates HIV-1 infection of restricted cell types has not yet been determined.

To elucidate the crucial role of Vpr in HIV infection of nonpermissive cell types, we used two distinct HIV-1 molecular clones to characterize the molecular mechanism by which Vpr counteracts the restriction to HIV-1 infection in primary macrophages. In contrast to what is observed with Vpx-dependent SIV infection of immature monocytes, we found no restriction to the first round of infection of MDM by HIV-1. However, we noted a striking effect of Vpr on the amount of virions produced by infected MDM and we noted higher infection rates in subsequent rounds, particularly at low multiplicity of infection (MOI). Surprisingly, enhanced virion production in primary MDM depended on an intact *env* gene and HIV infected primary MDM lacking Vpr had dramatically reduced amounts of HIV Env protein due to increased lysosomal degradation. Based on studies using Vpr mutants and DCAF1 silencing, DCAF1 was required for Vpr to counteract the macrophage restriction of Env expression and virion production. Thus, innate immune evasion promoted by Vpr impacts HIV spread in macrophages by preventing the induction of a novel macrophage specific intrinsic antiviral pathway that targets HIV Env and virion production.

Results

Vpr overcomes a restriction to the spread of HIV-1 in macrophage cultures that is most apparent at low MOI.

To explore the mechanism through which Vpr enhances HIV-1 infection of primary MDM, we constructed a Vpr-null mutant of the 89.6 molecular clone (89.6*vpr*$^-$), which was isolated from the blood of an HIV infected person with AIDS (Collman et al., 1992). As expected based on prior published studies, virion production in 293T cells transfected with p89.6 or p89.6*vpr*$^-$ proviral DNA plasmids was equivalent over a range of DNA inputs (**Figure 3.1A**). Additionally, equal mass amounts of 89.6 and 89.6*vpr*$^-$ virus stocks were equally infectious in CEMx174 cells (**Figure 3.1B**) as described previously (Balliet et al., 1994). Finally, HIV 89.6 and 89.6*vpr*$^-$ were similarly infectious in primary CD4$^+$ T cells (**Figure 3.1C**, left panel).

In contrast, we noted striking differences in virion production by MDM infected with the same viral stocks of wild type and mutant HIVs, particularly at low inoculum (**Figure 3.1D**). The impact of Vpr on virus production was most pronounced when the virus was allowed to spread through the culture for eighteen days (up to 20-fold differences, **Figure 3.1C**, right three panels). Thus, 89.6*vpr*$^-$ is defective in infection of primary human MDM but behaves like wild type virus in permissive cells such as CEMx174 and 293T cells. These results are similar to those reported by others and confirm that 89.6*vpr*$^-$ behaves as expected (Balliet et al., 1994; Chen et al., 2004; Connor et al., 1995; Eckstein et al., 2001; Heinzinger et al., 1994; Kogan and Rappaport, 2011; Vodicka et al., 1998).

Vpr does not significantly enhance initial infection of primary human macrophages.

To determine whether the effect of Vpr on the level of virus measured in the supernatant of infected MDM resulted from a higher number of initially infected cells or from enhanced spread, we validated an intracellular HIV Gag staining protocol to ensure that we could measure true infection events rather than cell surface binding or endocytosis of viral particles by MDM (**Figure 3.2A**). At five days post infection (dpi) intracellular Gag staining and flow cytometry revealed a distinct population of cells that expressed HIV Gag in a manner that was completely inhibited by HIV reverse transcription and integration inhibitors, azidothymidine (AZT) and raltegravir, respectively (**Figure 3.2A**). Thus, we concluded that this assay detected *de novo* infection of MDM that was dependent on reverse transcription and integration.

At the earliest time points in which we could detect intracellular Gag (two dpi) MDM infected with an equal viral inoculum were equally infected plus or minus Vpr (**Figure 3.2B**, left panel). However by four dpi, we observed a higher frequency of infected MDM with Vpr-containing viruses (2.8-fold, $p < 0.05$, **Figure 3.2B**, right panel). These results were confirmed using a PCR assay that detects HIV DNA (**Figures 3.2C** and **3.3A**); Vpr did not stimulate the amount of cell associated provirus detected at two dpi but did increase the amount of amplifiable *gag* DNA by three to four-fold at four dpi (**Figures 3.2C** and **3.3A**). In MDM, reverse transcription and integration typically require two to three days for completion of the first round of infection (Spivak et al., 2011). Thus, at four to five dpi, the first round of replication has been completed and the second round of infection has begun. Therefore, to distinguish initial infection from spread, we inhibited subsequent rounds of infection by the addition of raltegravir to a subset of MDM at two dpi. We then

harvested all the cells on day four. In the absence of raltegravir, we again observed a 2.6-fold increase in the frequency of infected cells with Vpr-containing viruses (**Figure 3.2D**). However, in a side-by-side experiment using cells from the same donor, the addition of raltegravir abrogated this difference (**Figure 3.2D**). Thus, under the conditions of our assay, Vpr did not affect the initial infection of MDM and primarily acted by stimulating spread of virus to new target cells.

Vpr overcomes a macrophage-specific restriction of virion production

To better understand how Vpr promotes spreading infection of MDM cultures, we utilized the fact that the addition of raltegravir two dpi equalized infection rates by preventing spread (**Figure 3.2D**). The amount of raltegravir added was sufficient to fully inhibit any new HIV infections (**Figure 3.2A**). Under these conditions, we noted a five-fold increase in virion production by MDM infected with a Vpr-containing virus compared to HIV-infected MDM lacking Vpr, which was statistically significant across multiple donors (**Figure 3.4A**). Of note, even in the absence of raltegravir, the differences in virion production were much more striking than differences in infected cell number (**Figure 3.4B**). The apparent discrepancy between Vpr's effects on infected cell frequency (**Figure 3.2B**, right panel) and infected cell number (**Figure 3.4B**) is due to the fact that there are often fewer MDM in cultures treated with Vpr-containing viruses (**Figure 3.3A**).

We also observed dramatic effects of Vpr on virion production in MDM cultures in which HIV-1 was allowed to spread to saturation over 20 days, equalizing the infection rates based on flow cytometry (**Figures 3.4C** and **D**) and Gag DNA (**Figures 3.4E** and **3.3B**). Under these conditions of equal infection, Vpr increased virion production by five-

fold (p<0.01, **Figure 3.4F**). Altogether, our data demonstrate that Vpr overcomes an MDM-specific restriction of virion production.

HIV-1 Env is necessary for Vpr to maximally enhance virion production by MDM

Because Env and Gag are the major structural components of virions, we examined a potential role for Env in virion production by measuring virus produced by primary human MDM infected with HIV-1 89.6 Env-null and Env/Vpr-null mutants (89.6*env*$^-$ and 89.6*env*$^-$*vpr*$^-$). Env-null mutants were pseudotyped with 89.6 Env *in trans* to generate infectious virions. Because the Env-null mutants are unable to spread beyond one round of replication, MDM infected with equal mass amounts of wildtype 89.6, 89.6*vpr*$^-$, 89.6*env*$^-$ and 89.6*env*$^-$*vpr*$^-$ were all treated with raltegravir at two dpi to prevent the spread of infection by all of the viruses equally. Contrary to what was expected from experiments performed with transformed cell lines (**Figure 1A**), we observed a five-fold reduction in Vpr-dependent stimulation of virion production by MDM infected with viruses lacking *env* (**Figure 3.4A**). Thus, the ability of Vpr to maximize virion production depended upon Env and was specific to macrophages.

Vpr stimulates virion production by overcoming a macrophage specific restriction of Env production.

To determine whether the stimulatory effect of Vpr on virion production resulted from effects on Env protein levels, we performed western blot analysis of HIV-1 infected primary human MDM. As expected, there was no defect in Env expression in 293T transformed cells lines transfected with proviral DNA plus or minus Vpr (**Figure 3.5A**, left panel).

However, the amount of HIV-1 Env protein detected in MDM was Vpr-dependent (**Figure 3.5A**, center and right panels). Vpr consistently overcame an apparent restriction to the expression of the processed forms of Env (gp120 and gp41) (**Figures 3.5A**, center and right panels and **B**). The gp160 precursor form of Env was also affected but to a lesser extent (**Figure 3.5A**, center panel). In contrast, the levels of Gag precursor pr55 were similar between wildtype 89.6 and 89.6*vpr*- at 20 dpi (**Figures 3.5A**, center and right panels and **B**).

At earlier time points, the analysis was more complex because of differences in infection rates between the viruses. Therefore, to accurately compare the level of Env per infected cell at such time points, we examined serial dilutions of whole cell lysate and compared Env levels only for dilutions in which Gag expression was matched. Under these conditions, we again observed that Vpr had selective effects on Env expression even at 5dpi (**Figure 3.5C**). While we observed differences in the expression of both the precursor (gp160) and the processed (gp120) form of Env, differences in expression of the processed forms were more dramatic over time (**Figure 3.5C**).

Similar results were obtained with the wild type HIV molecular clone AD8 and a well-characterized AD8 Vpr-null mutant (Rey et al., 1998; Theodore et al., 1996). We observed no defect in Env expression by the mutant virus in transfected 293T cells (**Figure 3.5D**). However, MDM infected with AD8*vpr*- displayed restricted Env expression relative to Gag pr55. This was especially notable for Env gp41 where we observed an average of seven-fold more protein in MDM infected with wild type virus (**Figures 3.5E** and **F**). Again, we observed a more variable effect of Vpr on Env gp160 precursor in part because gp120 can be difficult to cleanly resolve from gp160 (**Figure 3.5E**). Thus, the ability of Vpr to

counteract an MDM-specific restriction of Env protein expression and virion production is conserved amongst HIV variants isolated from different HIV-1-infected people.

Using a similar analysis, we also observed that Vpr stimulated Env incorporation into virions by an average of two to three-fold (**Figures 3.6A-C**). However, the magnitude of the effect was not sufficient to affect infectivity under the conditions of our assays (data not shown). Thus, sufficient Env is incorporated into the residual virions to maintain their infectivity in the absence of Vpr.

Vpr-counteracts a post-translational restriction that targets Env for lysosomal degradation.

To determine the mechanism by which MDM restrict Env expression, we performed a pulse-chase analysis of Env protein using infected MDM at ten dpi. Over the time course of our assay, Vpr did not affect the quantity of the precursor form of Env (gp160) synthesized within the one hour pulse (n = 8, **Figure 3.7A** and data not shown). However Vpr increased the half-life of the processed form (gp120) from 3.3h to 7.7h ($p < 0.0001$, **Figures 3.7A** and **B**). In contrast, there was no significant effect of Vpr on the half-life of HIV Gag pr55 (**Figure 3.7A** and data not shown). Based on quantitation of β-actin and HIV-1 *gag* DNA the numbers of infected cells added to the assay were similar (**Figures 3.7C** and **5D**). In sum these results indicate that Vpr counteracts a restriction of Env protein expression that decreases the half-life of Env.

To determine which degradative pathways affected Env protein expression in MDM infected with 89.6*vpr*- we treated cells pulse-labeled for 1h and chased with non-labeled media for 8h with ammonium chloride, an inhibitor of lysosomal degradation or MG132, an inhibitor of proteasomal degradation. Ammonium chloride but not MG132 partially

rescued Env gp120 expression ($p < 0.002$, Figures **3.7E** and **F**). In contrast, ammonium chloride treatment did not significantly affect Gag pr55 levels (**Figure 3.7G**). This demonstrates that Vpr counteracts a cellular restriction of Env expression that promotes lysosomal degradation of Env.

Vpr causes accumulation of PLK-1 and turnover of MUS81, DCAF1 and UNG2 in MDM and primary cell targets of HIV-1.

Recent studies have shown that inducible Vpr expression in a HeLa cell line activates the structure specific endonuclease regulator SLX4 complex (Laguette et al., 2014). In this cell line system, induction of Vpr increases polo-like kinase-1 (PLK-1) levels and stimulates the ubiquitination and turnover of MUS81-EME1 endonucleases (Laguette et al., 2014) in a manner that requires the DCAF1-DDB1-CUL4 E3 ubiquitin ligase complex. We show here that this pathway is intact in primary human MDM and $CD4^+$ lymphocytes (**Figure 3.8A** and **B**). Compared to infected cells lacking Vpr, wild type HIV infection led to the accumulation of PLK-1 and increased the turnover of MUS81 and DCAF1 in primary cells (**Figure 3.8A**). In primary $CD4^+$ T cells, we additionally noted decreased amounts of the Vpr-interacting protein uracil-DNA glycosylase 2 (UNG2) as previously reported (Priet et al., 2003; Wen et al., 2012). In MDM, the levels of UNG2 were not assayable due to lower expression levels (data not shown). Interestingly, DCAF1 was diminished as early as five hours post-infection (hpi) in MDM, which demonstrates that Vpr packaged into virions is sufficient for initiation of this pathway (**Figure 3.8C**). Vpr-dependent downmodulation of DCAF1 at 5 hpi was completely reversed by the addition of the proteasome inhibitor MG132 (**Figure 3.8C**). These data confirm that the Vpr-DCAF1 pathway that activates SLX4

complex associated endonuclease activity and inhibits innate immune pathways is activated soon after entry of HIV-1 in primary human MDM.

DCAF1-dependent functional activities correlate with Vpr–dependent virion production and Env expression.

To examine whether the pathway activating the SLX4 complex and cell cycle arrest is also required for Vpr to counteract MDM restriction of Env and virion production, we examined a panel of Vpr mutants. Vpr Q65R and H71R display decreased DCAF1 binding and decreased downstream functions that require DCAF1 such as cell cycle arrest and activation of the SLX4 complex (Belzile et al., 2010; DeHart et al., 2007; Hrecka et al., 2007; Laguette et al., 2014). These mutations were directly inserted into the 89.6 molecular clone (89.6*vpr*^{Q65R} and 89.6*vpr*^{H71R}). Both mutant proteins were expressed in 293T cells and packaged into viral particles (**Figures 3.9A** and **B**). However, Vpr^{H71R} was not expressed and packaged as efficiently as wild type Vpr. As expected, wild type p89.6 increased the number of 293T cells arrested in G2 phase three-fold compared to cells transfected with p89.6*vpr*$^{-}$ (**Figure 3.10**). Cells transfected with Vpr mutants p89.6*vpr*^{Q65R} and p89.6*vpr*^{H71R} were both relatively defective for cell cycle arrest with p89.6*vpr*^{Q65R} arresting a greater number of cells than p89.6*vpr*^{H71R} (**Figure 3.10**). As expected, in the permissive 293T cell line, these mutants did not affect Env protein expression (**Figure 3.9A**).

In primary human MDM that were infected with the mutant constructs, we observed a correlation between each mutant's activity in the cell cycle arrest assay and their capacity to enhance virion production in MDM (R^2 = 0.98, **Figure 3.9C**). The cell cycle arrest

phenotype also correlated with Vpr-dependent stabilization of Env gp120 and gp41 levels (R^2 = 0.92 and 0.87 respectively, **Figures 3.9D-F**). We further observed correlations between Vpr's effects on Env and virion production (R^2 = 0.98 and 0.94, **Figures 3.9G** and **H**). These correlations suggest that these Vpr–dependent activities are the result of the same pathway.

To more directly demonstrate a causal relationship between the DCAF1-dependent pathway and the ability of Vpr to counteract the macrophage-specific restriction of virion production and Env expression, we silenced DCAF1 expression in primary human MDM. To accomplish this, we used an shRNA-expressing lentiviral construct that had been optimized to maximize silencing while limiting antiviral responses in MDM (Pertel et al., 2011). Using this system we reproducibly achieved efficient silencing of DCAF1 expression (**Figure 3.11A**). Remarkably, we observed that DCAF1 silencing dramatically reduced virion production (**Figure 3.11B**) and Env expression (**Figure 3.11C**) relative to Gag levels in wild type HIV-1 infected MDM. Indeed, without DCAF1 expression, virion production and Env expression were similar between wild type and Vpr mutant viruses (**Figures 3.11B** and **C**). Thus, these studies indicate that Vpr requires the DCAF1-DDB1-CUL4 E3 ubiquitin ligase pathway to overcome the restriction of virion production and Env expression in HIV-1 infected MDM.

Discussion:

Vpr is an HIV-1 accessory protein and virulence factor associated with high viral loads and progression to the acquired immunodeficiency syndrome (AIDS) (Lang et al., 1993; Somasundaran et al., 2002). *In vitro*, Vpr is needed for efficient infection of nonpermissive primary cells such as macrophages (Le Rouzic and Benichou, 2005). Recent studies in permissive transformed cell lines revealed that Vpr utilizes its cellular cofactor DCAF1 to activate the SSE regulator SLX4 complex and to avoid detection of HIV-1 by the innate immune system (Laguette et al., 2014). This study implied but did not demonstrate that triggering of the innate response in the absence of Vpr activates downstream effector pathways that would restrict infection in nonpermissive cells such as primary human macrophages. Here we have demonstrated that this Vpr- and DCAF1-DDB1-CUL4 E3 ubiquitin ligase-mediated mechanism counteracts a novel, downstream macrophage-specific pathway that restricts Env protein expression and virion production. These results provide an explanation for why *vpr* is so well conserved among lentiviruses(Lim et al., 2012), yet is not needed for propagation in many transformed cell lines that lack this restriction pathway (de Silva et al., 2012; Le Rouzic and Benichou, 2005; Yamashita and Emerman, 2005).

In agreement with other studies, we observed that Vpr enhances viral infection and spread in macrophages(Balliet et al., 1994; Chen et al., 2004; Connor et al., 1995; de Silva et al., 2012; Heinzinger et al., 1994). However, using a system in which we could clearly separate initial numbers of infected cells from viral spread, we found that Vpr enhanced virion production per infected cell. Remarkably, we found that the effect of Vpr on virus production was mediated through increased expression of Env, a necessary component of

HIV-1 virions that binds to the CD4 receptor and coreceptors (Freed and Martin, 1995a) to facilitate viral fusion to target cells as well as cellular fusion and syncytia formation.

The conclusion that Vpr stimulates virion production by counteracting a macrophage-specific restriction of Env expression is based on a series of observations. Firstly, in our studies, the capacity of Vpr to stimulate virion production was always linked to its capacity to stimulate Env (but not Gag) expression. Vpr stimulated both virion production and Env expression in macrophages but not 293T cells. In addition, both phenotypes were observed with wild type 89.6 and AD8 Vpr proteins but not Vpr-null mutants from each molecular clone. The magnitude of Vpr activity for both phenotypes correlated significantly in viruses containing 89.6 Vpr, Vpr^{Q65R} and Vpr^{H71R}. Moreover, DCAF1 silencing abrogated both phenotypes. Finally, null mutations in *env* reduced the ability of Vpr to stimulate virion production. In sum, these observations indicate that while virions can be made in the absence of Env, Env is necessary for maximal virus production in human primary macrophages. Therefore, the capacity of Vpr to overcome a macrophage-specific restriction in Env expression enables Vpr to facilitate virion production.

Vpr dependent phenotypes depend on its interaction with the Cul4A-DDB1-DCAF1 ubiquitin ligase (Lim and Emerman, 2011; Romani and Cohen, 2012; Srivastava et al., 2008), which activates SLX4-endonuclease complex stimulating cell cycle arrest, induces formation of FANCD2 foci indicative of DNA replication stress and promotes Vpr nuclear envelope localization (Belzile et al., 2010; DeHart et al., 2007; Hrecka et al., 2007; Laguette et al., 2014). Based on these observations, it is possible that activation of the SLX4 endonuclease complex suppresses accumulation of unprocessed HIV-1 DNA intermediates that could be sensed by the cell and induce the expression of *IFNα* and *IFNβ* that has been

observed in cells lacking Vpr (Cardozo and Pagano, 2007; Harman et al., 2011; Laguette et al., 2014).

Our results provide the first evidence that the Vpr-DCAF1-SLX4 pathway is linked to a virological endpoint in a restricted cell type. The observation that Vpr affects a late process in the viral lifecycle was initially surprising, because the efficient incorporation of Vpr into virions suggests it acts on a step in the viral lifecycle preceding integration(Lu et al., 1995). Indeed, the targeting of DCAF1 for proteasomal degradation by Vpr as early as five hours post infection suggests that Vpr packaged into virions is sufficient to initiate DCAF1-dependent pathways. Interestingly, the proteasome inhibitor, MG132, blocked the effect of Vpr on DCAF1 immediately post infection, but did not activate the macrophage restriction of Env expression when added after infection was established. Given that Vpr prevents activation of the innate immune response (Laguette et al., 2014), the effect of Vpr on late stages of infection likely results from its ability to counteract intrinsic antiviral pathways that become upregulated upon viral sensing (**Figure 3.12**). Thus, macrophage restriction of Env expression in the absence of Vpr is likely a downstream consequence of viral sensing and innate immune activation (**Figure 3.12**). Whether viral sensing in the absence of Vpr results in the upregulation of a cellular factor that binds and disrupts Env trafficking remains to be investigated.

It is interesting to note that MUS81, which is utilized by Vpr to prevent activation of the innate immune response, is also turned over by the proteasome (Laguette et al., 2014). In addition, we have noted that DCAF1 and UNG2 are similarly turned over via a Vpr-dependent pathway in primary cells. The effect of Vpr on UNG2 levels has been described previously in other cell types (Priet et al., 2003; Wen et al., 2012) but the role of UNG2

remains unclear. UNG2 has been linked to reduced accumulation of uracilated DNA intermediates in HIV infected primary $CD4^+$ T cells expressing Vpr (Norman et al., 2011). Whether UNG2 and the SLX4 complex work cooperatively to clear HIV intermediates and prevent immune activation in HIV infected primary cell systems is an interesting hypothesis that remains to be investigated.

In summary, we have determined that Vpr increases spread of HIV-1 in MDM by counteracting an MDM-specific restriction of Env expression that leads to lysosomal degradation of Env and reduced virion production. Notably, this pathway relies upon the expression of the Vpr cofactor DCAF1 and provides the first mechanism that ties a Vpr-dependent DCAF1 pathway to a viral infection phenotype in a restricted cell type. Macrophages represent an important conduit for HIV-1 infection of $CD4^+$ T cells(Sharova et al., 2005) and are infected during the acute phase of HIV-1 infection(Hladik et al., 2007). Thus, these studies provide important insights into how HIV evades the innate immune pathways that would otherwise recognize and restrict viral infection in primary cells that are the targets of HIV-1 *in vivo*.

Materials and Methods

Antibodies and cell lines

Antibodies to CAp24 [KC57 conjugated to phycoerythrin (PE) or fluorescein isocyanate (FITC)] were obtained from Beckman Coulter. Antibodies to the following proteins were used for immunoblot analysis; PLK-1 (sc-17783, Santa Cruz), MUS81 (ab14387, Abcam), DCAF1 (11612-1-AP Proteintech), UNG2 (EPR4371, Epitomics), Tubulin (T5168, Sigma), Gag, gp120, gp41, and Vpr [AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog Numbers 4250 and 288 were from Dr. Michael Phelan (Hatch et al., 1992). Catalog number 11557 was from Dr. Michael Zwick (Zwick et al., 2001). Catalog number 3951 was from Dr. Jeffrey Kopp.] HIV-Ig serum used for radioimmunoassay was obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH catalog 3957 from NABI and NHLBI) and has been described previously(Freed and Martin, 1995b).

CEMx174 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: 174xCEM from Dr. Peter Cresswell.

Viral constructs

p89.6 and pNL4-3 were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalog numbers 3552 and 114 from Dr. Ronald G. Collman and Dr. Malcolm Martin, respectively (Adachi et al., 1986; Collman et al., 1992). pNL-PI*vpr*$^{-}$ has been described previously(Norman et al., 2011). SIV3^{+}, psPAX2, pAPM-1221 (shNC) and pDCAF-APM.1-3 (shDCAF1) were obtained from Dr. Jeremy Luban (Pertel et al., 2011). pAD8 (HIV-1$_{AD8}$) and pAD8*vpr*$^{-}$ (HIV-1$_{AD8}$VprX) were obtained from Vicente Planelles, PhD. (Zimmerman et al., 2006).

p89.6*vpr*$^-$ was generated by filling in the Afl II site at position 5634 of p89.6 with Klenow polymerase and religating. p89.6*env*$^-$ was created by removing a BsaBI-StuI fragment from p89.6, as described(Carter et al., 2010). p89.6*env*$^-$ *vpr*$^-$ was made by ligating the EcoRI-ApaI fragment from p89.6*vpr*$^-$ into p89.6*env*$^-$. p89.6vpr^{Q65R} and p89.6vpr^{H71R} were constructed by substituting synthetic mutant oligonucleotides into the EcoRI and SalI sites in p89.6. pNL4-3*vpr*$^-$ was generated by ligating the SpeI-SalI fragment from pNL-PI*vpr*$^-$ into pNL4-3.

Virus stocks were obtained by transfection of 293T cells with provirus expression plasmids using polyethylenimine, as described (McNamara et al., 2012). Viral supernatants were collected at 48h and centrifuged at 1500 rpm to remove cell debris. Virus was stored at -80°C and quantified by CAp24 ELISA, as described (Salmon and Trono, 2007).

Cell culture and viral infection

Leukocytes isolated from anonymous donors by apheresis were obtained from New York Blood Center Component Laboratory (Long Island City, NY). Peripheral blood mononuclear cells (PBMC) were purified by Ficoll density gradient separation, as described(Norman et al., 2011). CEMx174 cells were cultured in R10, and infected with HIV-1 by spinoculation as described above(Salter et al., 1985).

CD14$^+$ monocytes were isolated by positive selection with an EasySep magnetic sorting kit (StemCell Technologies). Monocyte-derived macrophages (MDM) were obtained by culturing monocytes in R10 (RPMI-1640 with 10% fetal bovine serum (Gibco, Invitrogen), penicillin (10 Units/ml), streptomycin (10 µg/ml), L-glutamine (292 µg/ml), M-CSF (50 ng/ml, R&D systems) and GM-CSF (50 ng/ml R&D systems)) for 7-9 days, as

described (Lahouassa et al., 2012). MDM were incubated with HIV-1 for 4h, washed with phosphate buffered saline (PBS) and cultured in fresh medium, as described (Peeters et al., 2002).

$CD4^+$ lymphocytes were prepared as follows; Adherence-depleted PBMC were depleted of $CD56^+$ cells (Easy Sep, StemCell Technologies) and $CD8^+$ cells (Dyna beads, Sigma) by negative selection. $CD4^+$ lymphocytes were stimulated in R10 and phytohaemagglutinin (PHA, 5μg/ml). IL-2 (500 IU/ml) was added to the culture 24h following PHA stimulation. $CD4^+$ T cells were infected with HIV-1 by spinoculation (2500rpm at 25°C for 2-3h) 48-72h following PHA stimulation, as described (Norman et al., 2011). Infected cells were maintained in R10 and IL-2 until analyzed.

shRNA knockdown of DCAF1 was performed as previously described (Pertel et al., 2011). Briefly, we spinoculated primary monocytes with VSV-G-pseudotyped $SIV3^+$ for 2 hours with 10 μg/ml polybrene to allow Vpx-dependent downmodulation of SAMHD1. Cells were then incubated overnight in RPMI+10% certified endotoxin-low FBS (Invitrogen) with M-CSF (50 ng/ml, R&D systems) and GM-CSF (50 ng/ml R&D systems) plus 20μg VSV-G-pseudotyped lentivirus containing an shRNA cassette targeting luciferase (shNC) or DCAF1(shDCAF1). Following an overnight incubation, the cells were cultured for 3 days in fresh medium before addition of 10μg/ml puromycin for 3 additional days prior to infection.

Flow cytometry staining

Intracellular staining for Gag CAp24 expression was performed as described previously (Carter et al., 2010). For cell cycle analysis, cells were permeabilized with 100%

ethanol and treated with 50μg/ml propidium iodide and 100μg/ml RNase type I-A in PBS as described (Zimmerman et al., 2006). Cell cycle distribution was modeled using FlowJo software (Cytek).

ELISA

Supernatant containing viral particles was lysed in Triton-X lysis buffer (0.05% Tween-20, 0.5% Triton-X-100, 0.5% Casein in PBS). Anti-p24 antibody (clone 183-H12-5C) was bound to NUNC Maxisorp plates. Lysed samples were captured for 1-2h and incubated with biotinylated anti-p24 antibody (clone 31-90-25). 31-90-25 was biotinylated with the EZ-Link Micro Sulfo-NHS-Biotinylation Kit (Pierce). Samples were detected using streptavidin-HRP (Fitzgerald) and 3,3',5,5'-Tetramethylbenzidine substrate, as described(Salmon and Trono, 2007). Virion production was normalized for infected cell number by dividing the CAp24 measured by ELISA in supernatant by the number of Gag+ cells acquired by flow cytometry within a fixed interval of time.

QPCR

DNA was isolated from 4 to 5 x 10^5 MDM using the DNeasy Blood & Tissue Kit (Qiagen). HIV-1 DNA and *ATCB* DNA were quantified by quantitative PCR (QPCR) using specific primers as described previously (Clouse et al., 1989; McNamara et al., 2013; Norman et al., 2011).

Western blotting

MDM whole cell lysate preparation. MDM infected with HIV-1 were washed with PBS before being lysed in Blue Loading Buffer (Cell Signaling). WCL were sonicated with a Misonix sonicator (Qsonica, LLC.) and clarified by centrifugation at 13000rpm.

Viral lysate preparation. Supernatant from infected cells was passed through a 0.45μm filter and virions were pelleted by ultracentrifugation at 25,000rpm, as described(Ono et al., 2007; Waheed et al., 2009). The virus-containing pellet was lysed in Blue Loading Buffer and clarified by centrifugation at 13000rpm.

Lysates were analyzed by immunoblot and protein levels were quantified using Adobe Photoshop as described(Norman et al., 2011).

Radioimmunoprecipitation Assay

Metabolic labeling of HIV-1 infected MDM was performed as described(Ono and Freed, 1999). MDM infected with 1μg CAp24 for ten days were labeled with 125μCi [^{35}S]Met/[^{35}S]Cys (Perkin Elmer) for 1h. Following metabolic labeling, cells were lysed immediately or incubated in non-labeled medium for an additional seven or fifteen hours. Where indicated, cells were treated with 20μM NH_4Cl, 2.5μM MG132, or an equal volume of water from 30min prior to labeling to just prior to lysis. Cells were lysed in Triton Lysis Buffer (300mM NaCl, 50mM Tris pH 7.5, 0.5% Triton X-100, 10mM Iodoacetamide, 1x protease inhibitor tablet (Roche)). HIV-1 proteins were precipitated with serum from HIV$^+$ individuals obtained from the NIH reagent repository as described(Freed et al., 1995; Freed and Martin, 1995b). Immunoprecipitated lysate was separated by SDS-PAGE and metabolically labeled protein was detected with a storage phosphor screen and a Typhoon System (Amersham Biosciences). Protein levels were quantified with ImageQuant TL 7.0

software as ^{35}S signal over an area of background (GE Healthcare). To determine the half-life of Env given that gp41 was not efficiently immunoprecipitated, the fraction of remaining gp120 at 8h or 16h was calculated as the ratio of measured ^{35}S in the gp120 band to calculated ^{35}S signal from gp120 at time zero (TO). The calculated ^{35}S signal from gp120 at T0 was obtained by multiplying by the fraction of cysteine and methionine residues predicted to be present within gp120 relative to gp160 (32/42) by the gp160 signal at T0 that was processed at each chase time. The processed gp160 signal at T0 was calculated by subtracting the unprocessed gp160 ^{35}S signal at each chase time from the actual ^{35}S signal measured in gp160 at T0.

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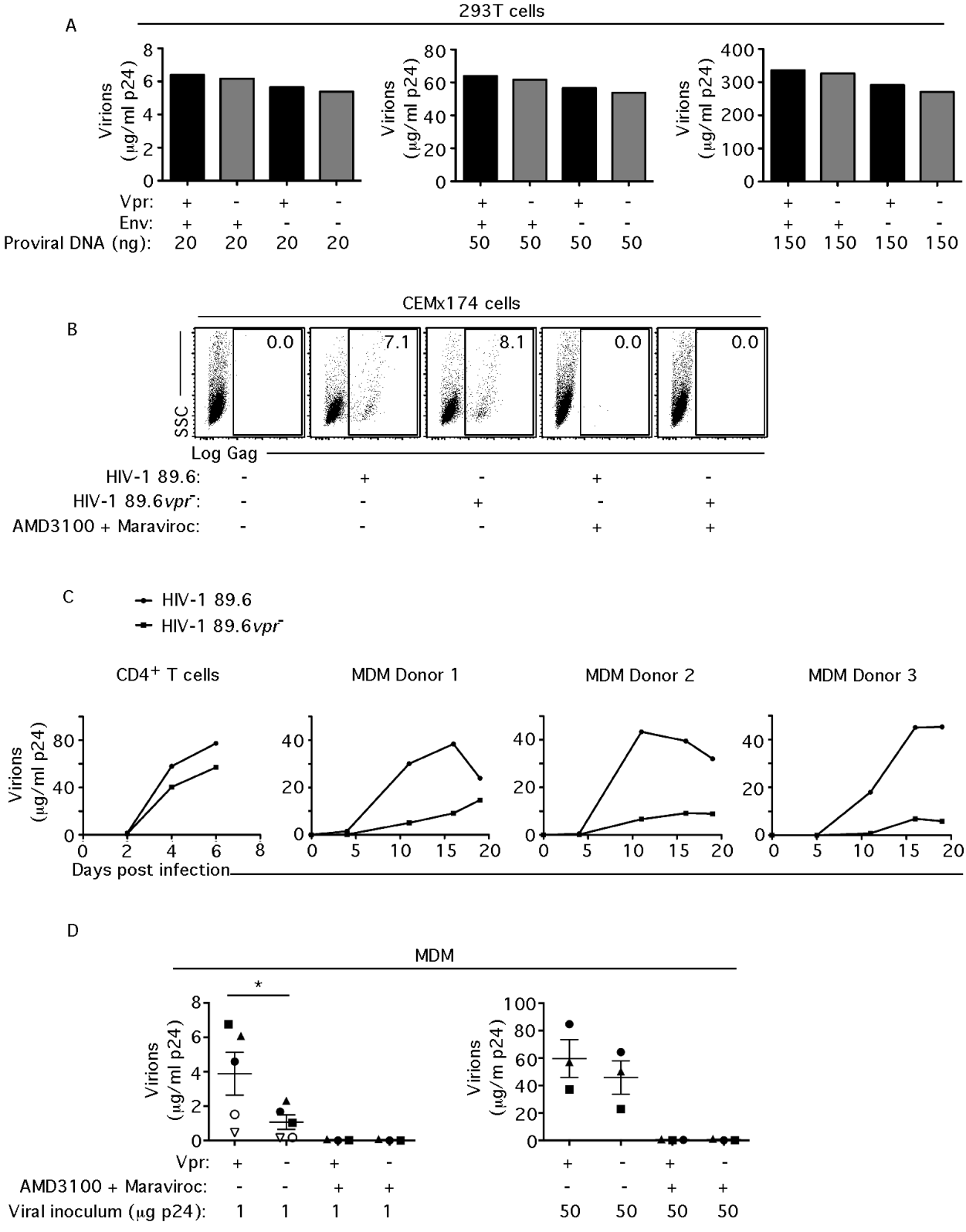
A
293T cells
Virions (µg/ml p24)
Vpr:
Env:
Proviral DNA (ng):
B
CEMx174 cells
0.0
7.1
8.1
0.0
0.0
SSC
Log Gag
HIV-1 89.6:
HIV-1 89.6vpr-:
AMD3100 + Maraviroc:
C
HIV-1 89.6
HIV-1 89.6vpr-
CD4+ T cells
MDM Donor 1
MDM Donor 2
MDM Donor 3
Virions (µg/ml p24)
Days post infection
D
MDM
*
Virions (µg/ml p24)
Vpr:
AMD3100 + Maraviroc:
Viral inoculum (µg p24):

Figure 3.1. Vpr increases p24 levels in the supernatant during a spreading infection in MDM. **(A)** Virion production by 293T cells transfected with proviral containing DNA plasmids expressing the indicated HIV-1 proteins. Virion production was quantified using a CAp24 ELISA of the supernatant 2 days after transfection **(B)** Flow cytometric analysis of HIV-1 Gag p24 expression in CEMx174 cells 2 dpi with 10μg of wildtype 89.6 or 89.6*vpr*-. **(C)** Virion production by primary human $CD4^+$ T cells or MDM infected with 1μg of the indicated virus for 6d or 18d, respectively. Error bars represent the standard error of the mean. * $p < 0.05$ (two-tailed paired *t*-test). **(D)** Summary plot of virion production by MDM infected for 5d with the indicated mass amounts of wildtype 89.6 or 89.6*vpr*-. Where indicated, Cells were treated with 10μg/ml AMD3100 and 20μM Maraviroc from -1h to 5d. [1]

[1] This figure was compiled by Michael Mashiba.

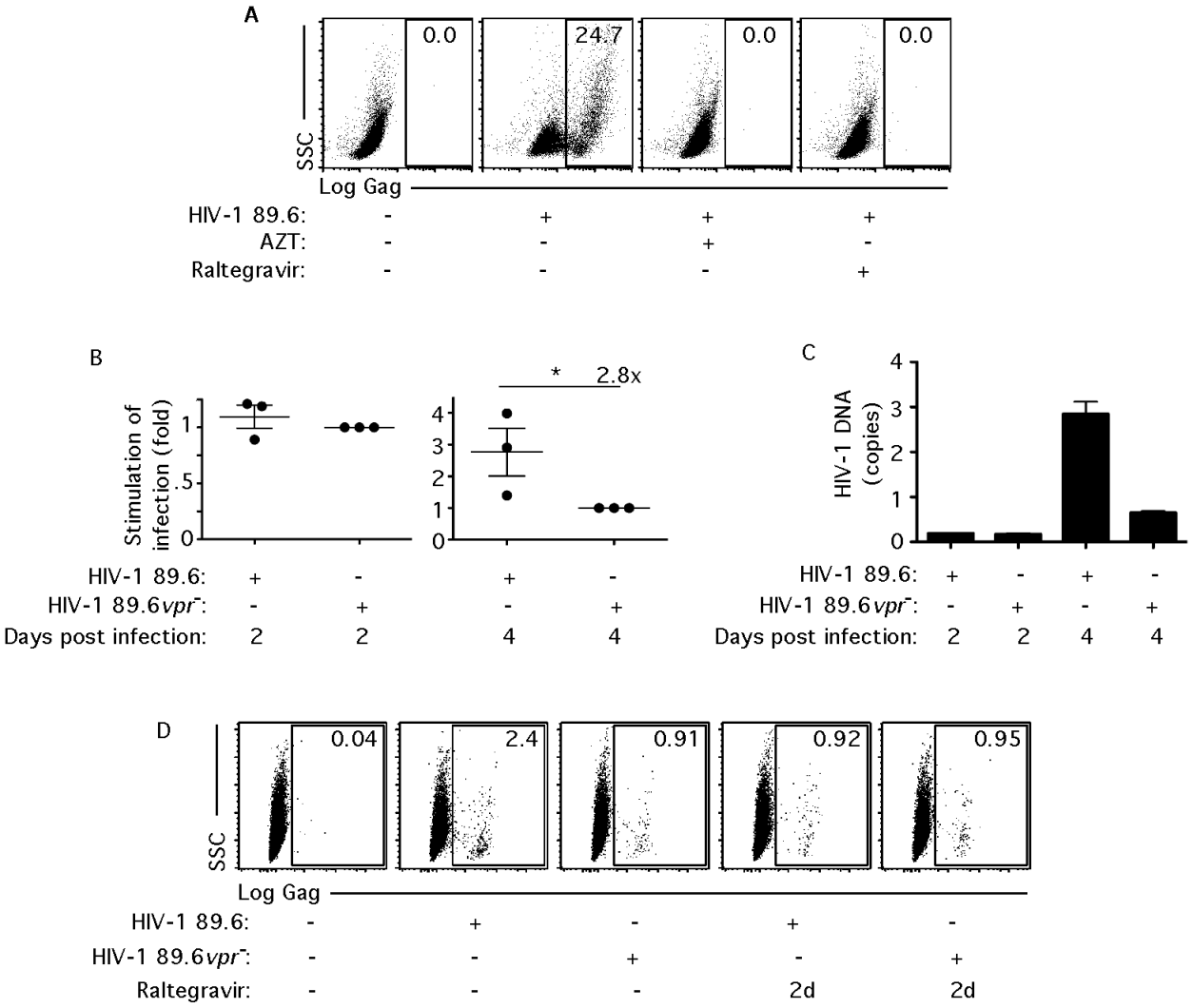

Figure 3.2. Vpr does not increase the first round of infection in primary human MDM cultures. **(A)** Flow cytometric analysis detecting bona fide infection of MDM by intracellular HIV Gag p24 stain. Cells were infected for 5d with 50μg of HIV-1 89.6 and treated with 20μM azidothymidine (AZT) or 2μM raltegravir where indicated. **(B)** Summary graph showing the fold difference in infection frequency observed in MDM treated with 1μg of wild type or Vpr mutant virus. By definition, the mutant virus normalizes to 1.0. **(C)** QPCR analysis of *gag* DNA in MDM infected with the indicated viruses. Levels of *gag* DNA were normalized for β–actin (*ATCB)* DNA levels to account for differences in cell number (Figure S2). Each bar represents the average of three replicates from the same donor. Error bars represent the standard error of the mean. * $p < 0.05$ (two-tailed paired *t*-test). **(D)** Flow cytometric analysis of intracellular HIV-1 Gag p24 expression in MDM infected with 1μg of the indicated viruses and analyzed at 4dpi. Cells were treated with 2μM raltegravir at 2dpi where indicated. [2]

[2] This figure was compiled by Michael Mashiba.

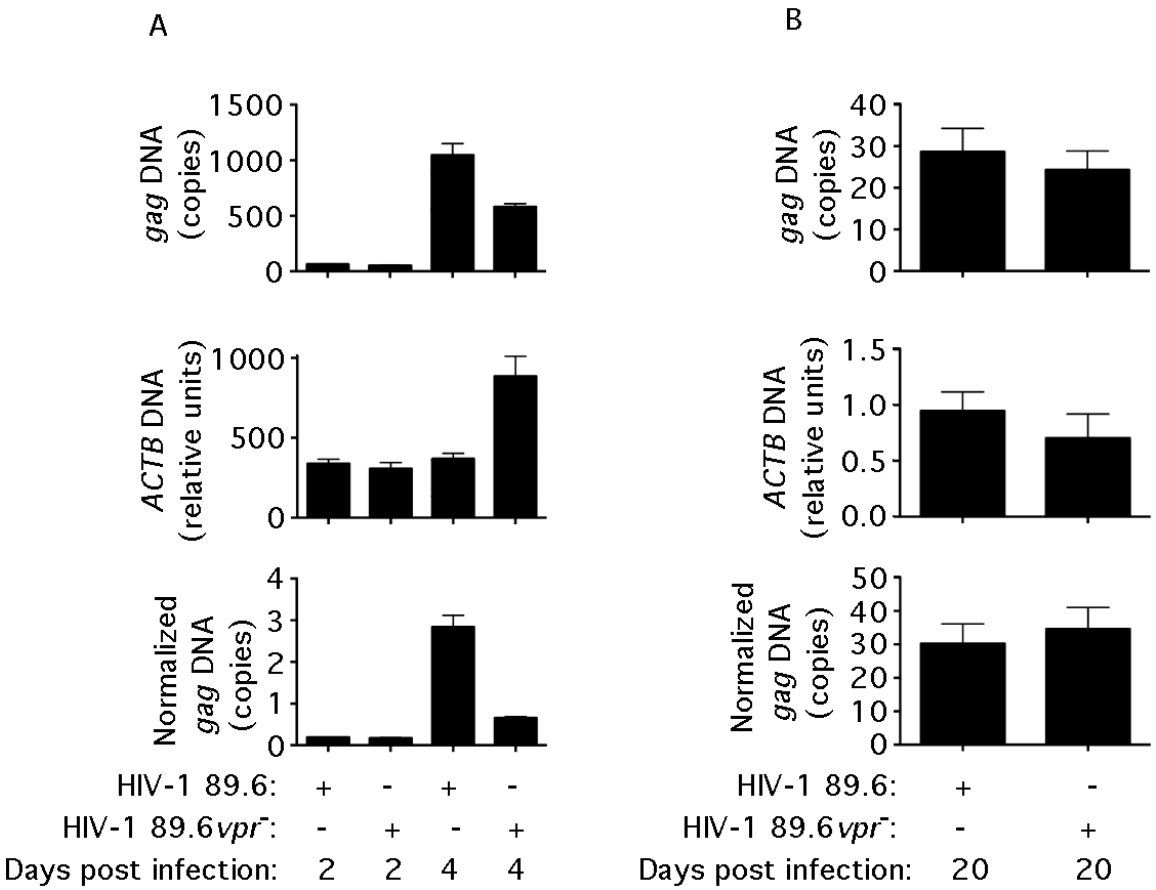

Figure 3.3. Calculation of normalized *gag* DNA in HIV-infected MDM. **(A and B)** QPCR analysis of the indicated genes in MDM infected with 1μg of wild type or Vpr mutant virus. Normalized *gag* DNA (bottom graphs) was adjusted for total cell number by dividing measured *gag* DNA (top graphs) by total *ACTB* DNA (middle graphs). Normalized *gag* DNA in bottom graphs in A and B are shown in figures 1C and 2E, respectively. Each bar represents the mean of three replicates from one experiment from a single donor. Error bars represent the standard error of the mean.[3]

[3] This figure was compiled by Michael Mashiba.

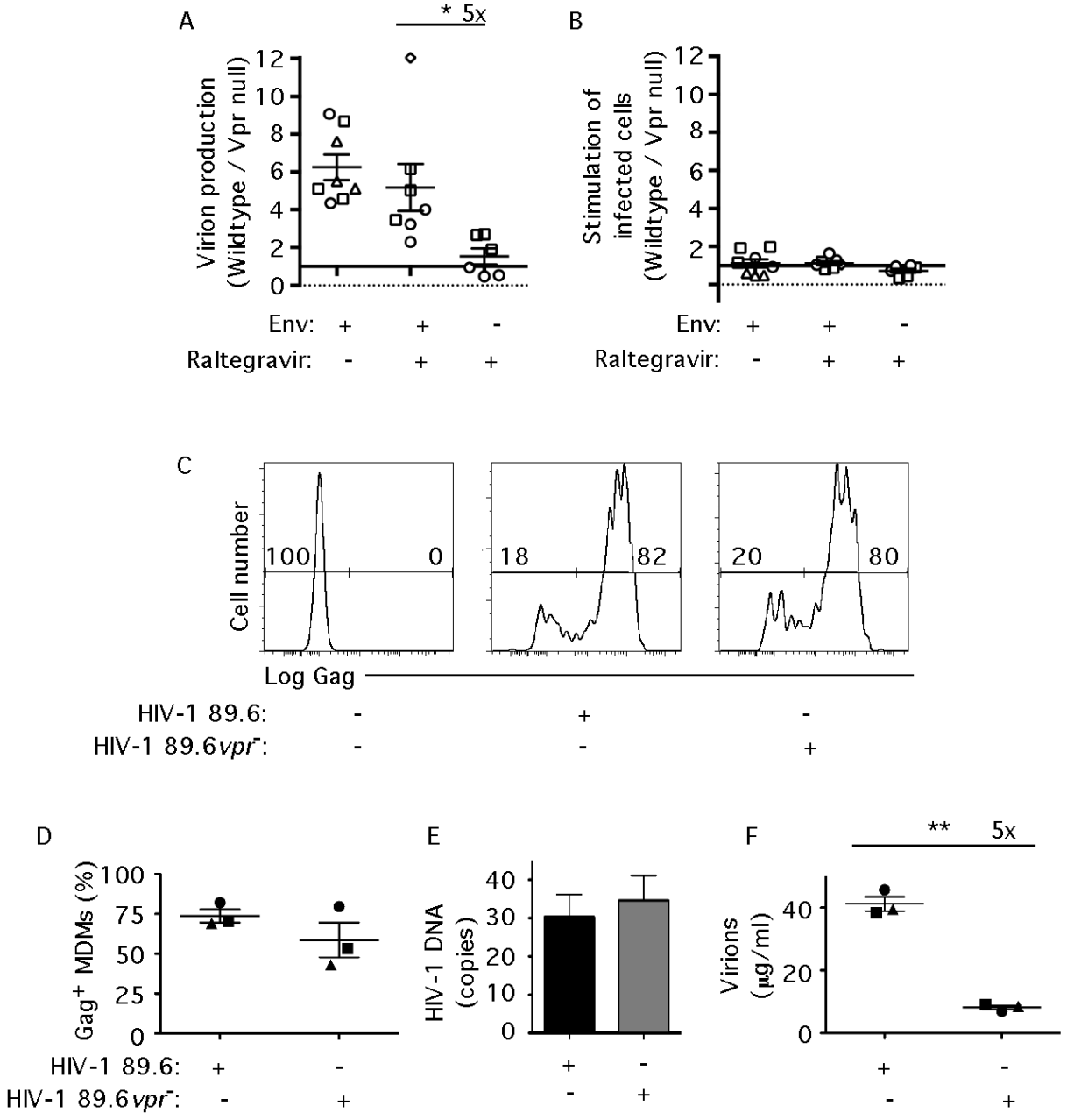

Figure 3.4. Vpr increases virion production in HIV infected primary MDM. **(A)** Summary graph of the effect of Vpr on virion production, calculated as the fold-change in virion production by MDM infected by wildtype 89.6 relative to 89.6*vpr*⁻. Virion production was normalized for the absolute number of Gag⁺ cells acquired by flow cytometry within a fixed interval of time. **(B)** Summary graph of the effect of Vpr on the number of infected cells, calculated as the fold change in the number of MDM infected by wildtype 89.6 relative to 89.6*vpr*⁻. Each shape in A and B represents a replicate from one experiment from a single

donor. Error bars represent the standard error of the mean. **(C)** Flow cytometric analysis of HIV-1 Gag p24 expression in MDM infected for 20d with 1μg of the indicated viruses. **(D)** Compilation of flow cytometry experiments staining for intracellular HIV-1 Gag p24 in MDM infected as in A. **(E)** QPCR analysis of *gag* levels in MDM from a duplicate well from the experiment in A. Levels of *gag* DNA were normalized for β–actin (*ATCB)* DNA levels to account for differences in cell number. **(F)** Virion production based on HIV-1 Gag p24 levels in the supernatant of MDM from three donors for which infection rates were shown in D. Error bars represent the standard error of the mean. * $p < 0.05$, ** $p < 0.01$ (two-tailed paired *t*-test). In D and F each symbol represents a different donor ($n = 3$). [4]

[4] This figure was compiled by Michael Mashiba.

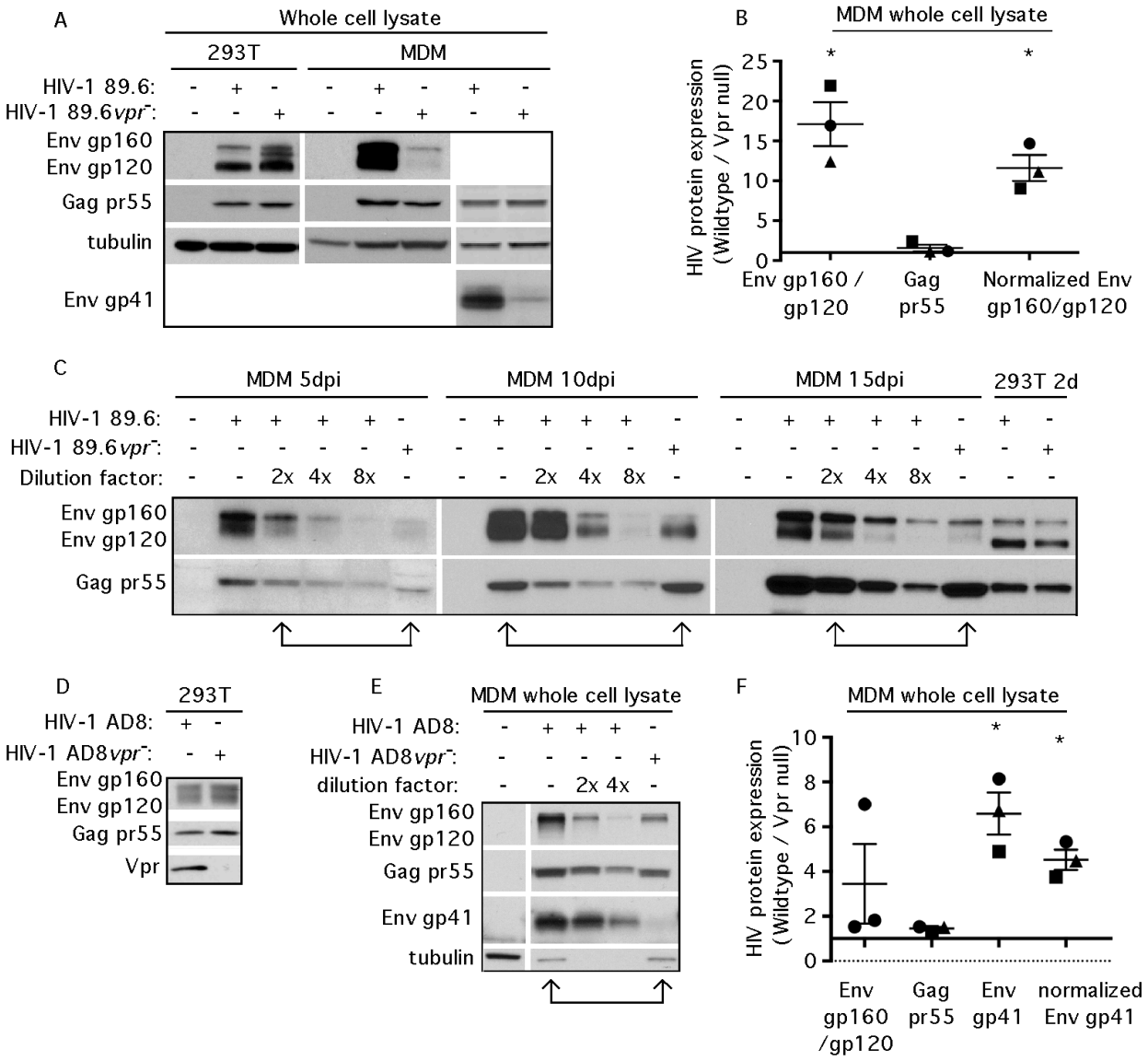

Figure 3.5. HIV 89.6 Vpr counteracts a macrophage restriction of Env expression. **(A)** Immunoblot analysis of HIV protein expression in whole-cell lysates (WCLs) from MDM infected for 20d with 1μg of the indicated viruses and from 293T cells transfected for 2d with the indicated proviral DNA plasmids. **(B)** Summary graph quantifying the fold increase in expression of the indicated HIV-1 proteins in MDM infected with wildtype 89.6 relative to 89.6*vpr*- as in A. Where indicated, gp160/gp120 was normalized for pr55 expression and each symbol represents a different donor from a separate experiment (n = 3). Error bars represent the standard error of the mean. * $p < 0.03$ (two-tailed paired t-test). **(C)** Immunoblot analysis of viral protein levels in WCL from MDM infected for the times indicated with 1μg of the indicated viruses and from 293T cells transfected for 2d

with the indicated proviral DNA plasmids. WCL from MDM infected with wildtype 89.6 was serially diluted as indicated. Arrows denote samples containing comparable levels of Gag pr55 in the presence or absence of *vpr*. **(D)** Immunoblot analysis of viral protein expression in 293T cells transfected for 2d with pAD8 or pAD8*vpr*$^-$ proviral DNA plasmids. **(E)** Immunoblot analysis of viral protein levels in whole cell lysate (WCL) from MDM infected for 6d with 1μg of wildtype AD8 or AD8*vpr*$^-$. Lysates from MDM infected by wildtype AD8 were serially diluted in loading buffer as indicated. **(F)** Summary graph of the effect of Vpr on expression of the indicated HIV-1 proteins, calculated as fold change in protein levels in MDM infected for 6d with 1μg of wildtype AD8 relative to AD8*vpr*$^-$. Where indicated, gp41 was normalized for pr55 expression. Each shape represents a different donor from a separate experiment (n = 3). Error bars represent the standard error of the mean. * p < 0.05 (two-tailed paired t-test).[5]

[5] This figure was compiled by Michael Mashiba. The western blot in Figure 3.5D was produced by David Collins.

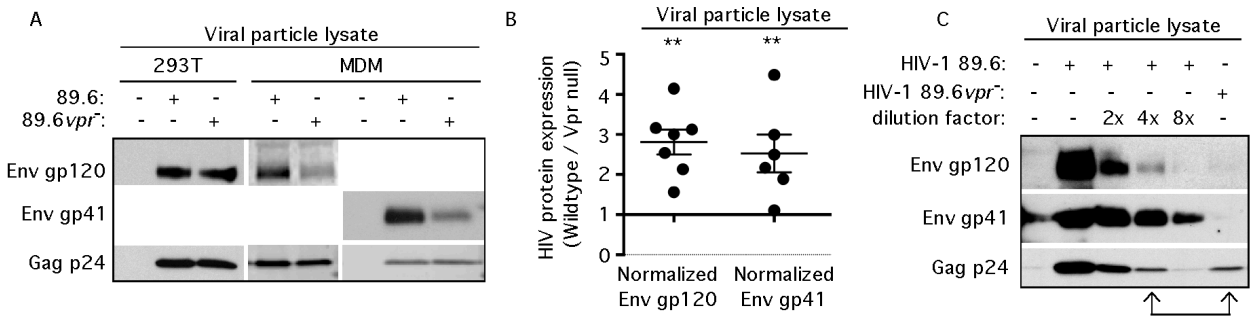

Figure 3.6. Vpr increases Env incorporation into virions. **(A)** Immunoblot analysis of HIV protein levels in viral lysates (VL) collected at 10dpi from cells infected with 1 μg wild type or mutant viruses. **(B)** Summary graph quantifying the fold increase in expression of the indicated HIV protein in VL from experiments performed as in A. Normalized gp160/gp120 and gp41 were adjusted for HIV Gag CAp24 content and each dot represents a different donor from a separate experiment (n = 7 (gp120) and 6 (gp41)). Error bars represent the standard error of the mean. ** $p < 0.002$ (two-tailed paired t-test). **(C)** Immunoblot analysis of HIV protein levels in VL collected from MDM infected as in A and serially diluted as indicated. Arrows denote samples containing comparable levels of Gag CAp24 in the presence or absence of *vpr*. [6]

[6] This figure was compiled by Michael Mashiba.

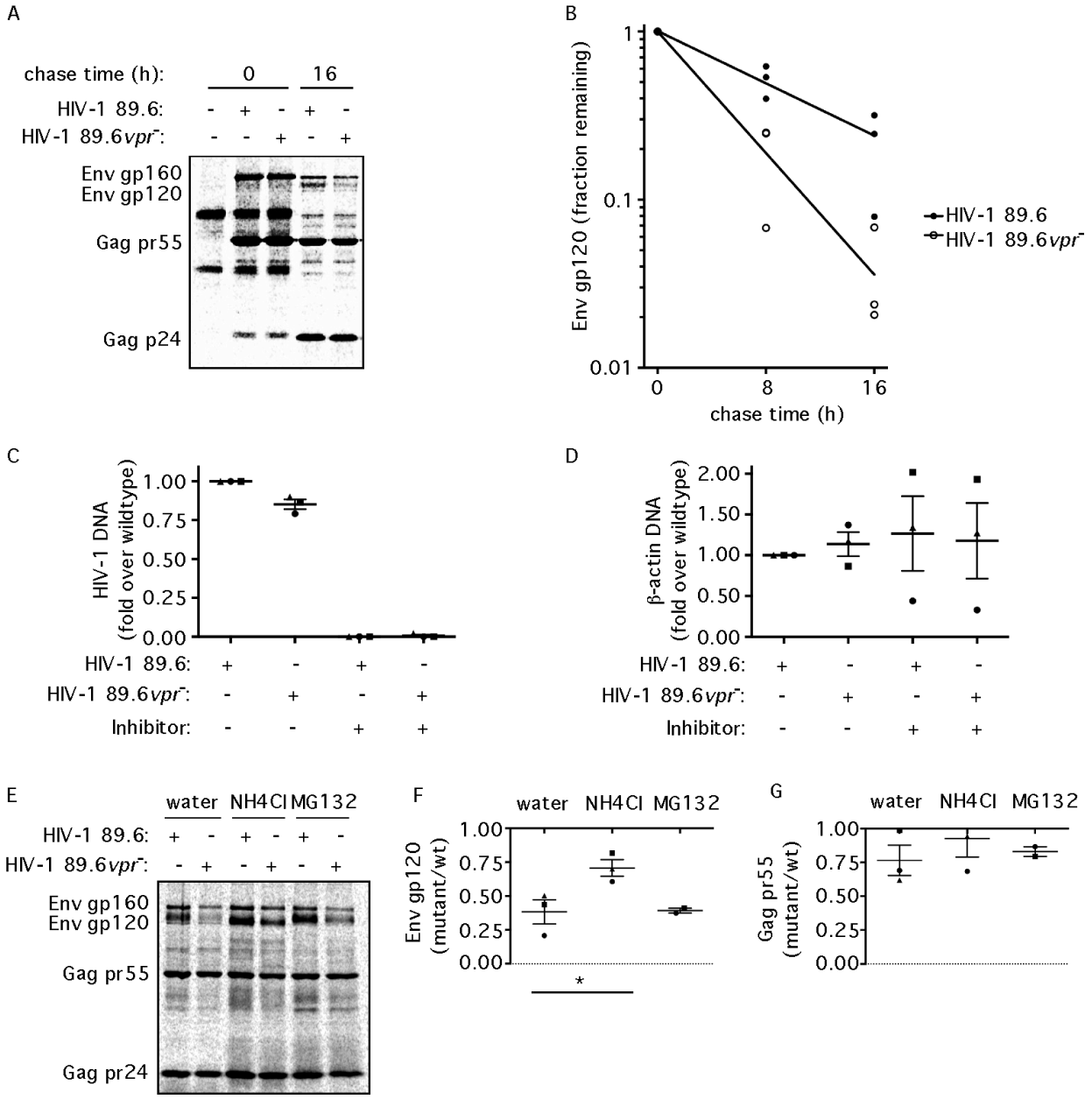

Figure 3.7. Vpr counteracts a restriction pathway that targets HIV Env for lysosomal degradation in primary human MDM. **(A)** Radioimmunoprecipitation assay of HIV proteins from primary human MDM infected with 1μg of wildtype 89.6, cultured for 10d prior and metabolically labeled with [^{35}S]Met/Cys for 1h. The labeled cells were chased for the indicated time period, immunoprecipitated and subjected to SDS-PAGE. **(B)** Summary of quantified data from the experiment(s) performed as in A. The fraction of gp120 remaining at 8 or 16h relative to T0 was calculated as described in Materials and Methods. Each dot represents a different donor from a separate experiment (n = 3). Best-fit curves were

obtained by non-linear regression analysis. (C and D) qPCR analysis of *gag* **(C)** and β-actin **(D)** DNA levels in cells analyzed in B. Cells were treated with 10μg/ml AMD3100 and 20μM maraviroc or 2μM raltegravir during infection where indicated. Each shape represents a different donor from a separate experiment (*n* = 3). **(E)** Radioimmunoprecipitation assay of HIV proteins from primary human MDM infected and radiolabeled as described for **(A)**. As indicated, labeled cells were chased for 8h with or without inhibitors of lysosomal and proteasomal degradation (20μM NH_4Cl and 2.5μM MG132 respectively). **(F and G)** Quantification of Env gp120 **(F)** and Gag pr55 **(G)** levels in cells treated as in E. Env expression was calculated as the fold change in protein measured in WCL from MDM infected by 89.6*vpr*⁻ relative to wildtype 89.6. Each shape represents a different donor from a separate experiment (*n* = 3). Error bars represent the standard error of the mean. * $p < 0.05$ (two-tailed paired *t*-test).[7]

[7] This figure was compiled by Michael Mashiba.

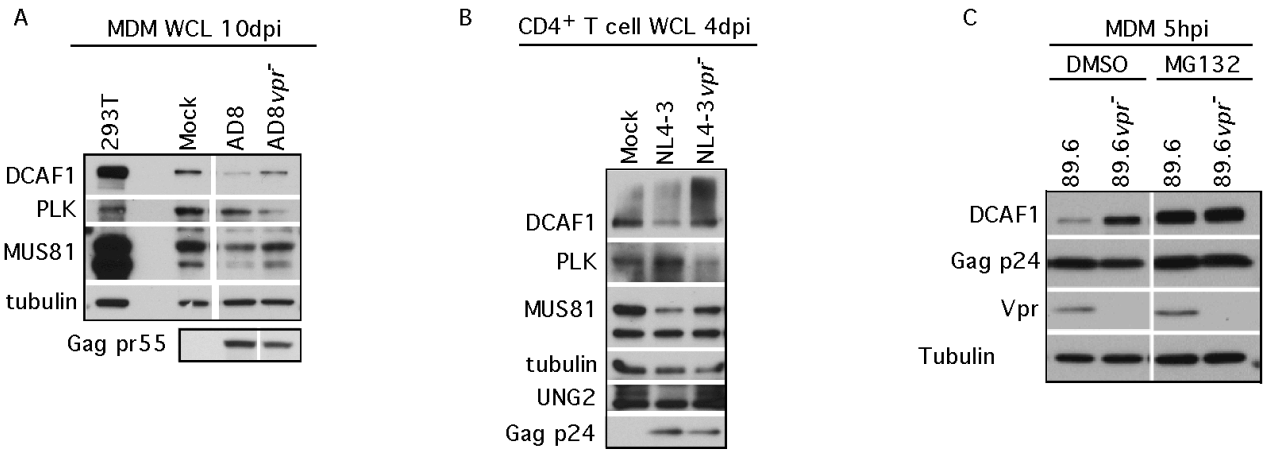

Figure 3.8. Vpr causes accumulation of PLK and turnover of MUS81, DCAF1 and UNG2 in MDM and primary cell targets of HIV-1. **(A-C)** Immunoblot analysis of whole cell lysates (WCL) from cells infected with the indicated virus. Where indicated, cells were treated with 2.5μM MG132 or DMSO from -2 to 5hpi. [8]

[8] This figure was compiled by Michael Mashiba.

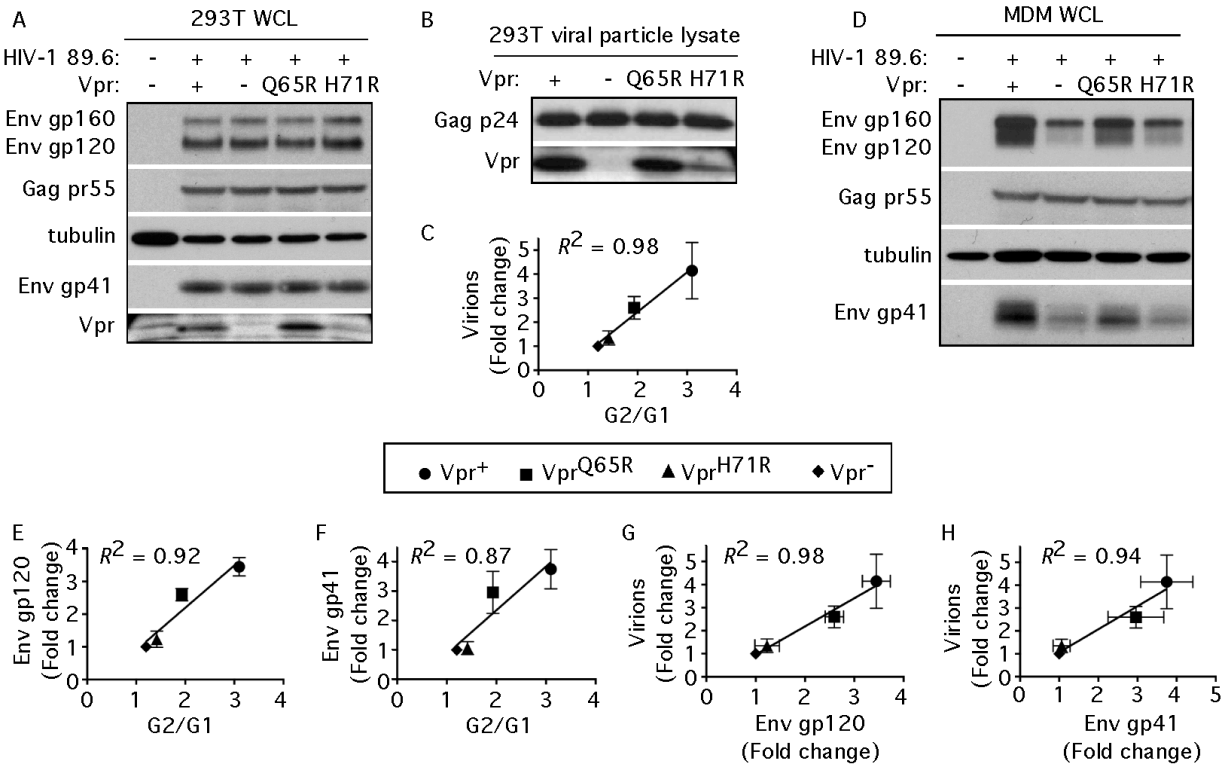

Figure 3.9. DCAF1-dependent functional activities correlate with Vpr–dependent virion production and Env expression. **(A, B and D)** Immunoblot analysis of whole cell lysates (WCL, **A and D**) or viral particle lysate **(B)** from the indicated cells transfected with the indicated proviral plasmid **(A and B)** or infected with the indicated virus **(D)**. **(C, E-H)** Graphical analysis demonstrating correlations between each of the Vpr activities measured for wild type and mutant Vpr constructs. G2/G1 indicates cell cycle arrest activity in transfected 293 T cells. Virion production was assessed at 10dpi with 1μg of HIV-1 (n = 6) and is expressed as the fold difference between wild type and mutant viruses. Env fold change is the ratio of the amount of Env produced in MDM infected with wild type virus divided by the amount of Env produced by MDM infected with mutant virus as measured by western blot analysis (n = 4). Error bars represent the standard error of the mean. Best-fit curves and R^2 values were obtained by linear regression analysis.[9]

[9] This figure was complied by Michael Mashiba. 89.6*vpr*^{Q65R} and 89.6*vpr*^{H71R} mutants were cloned by David Collins.

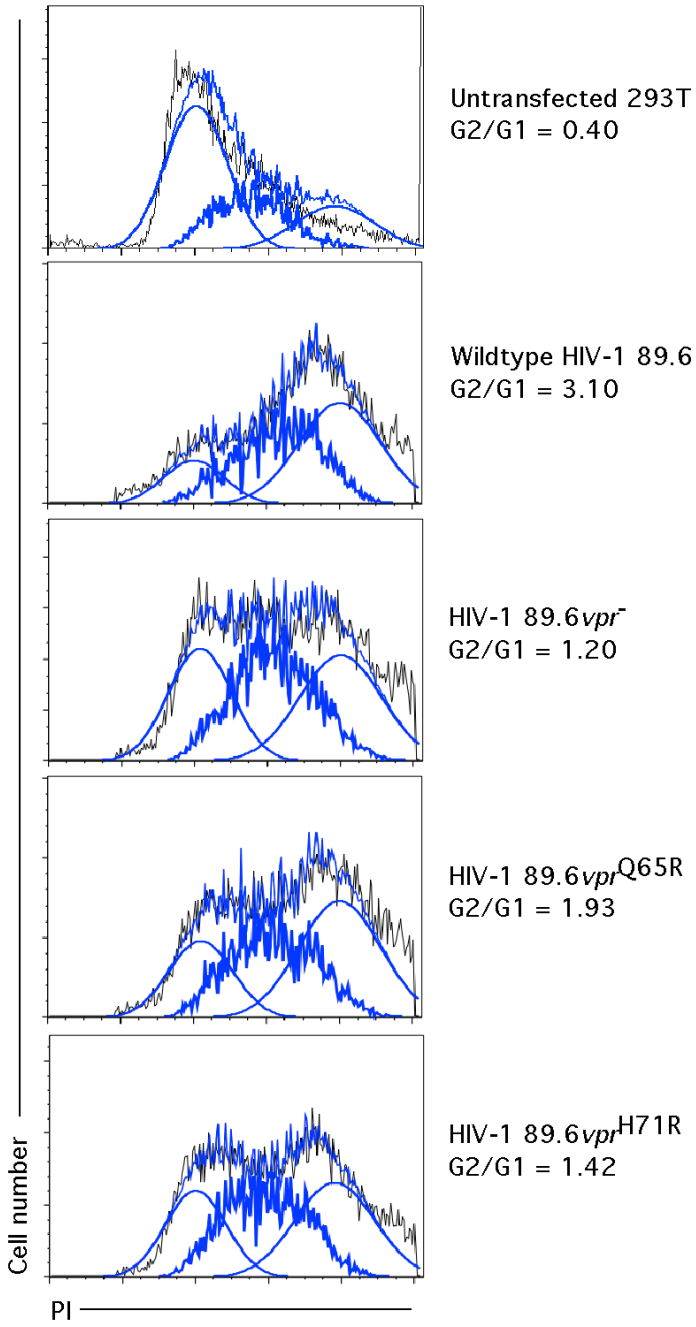

Figure 3.10. Cell cycle analysis of 293T cells expressing Vpr mutants. Flow cytometric DNA content analysis of Gag$^+$ 293T cells transfected for 2d with the indicated HIV-1 proviral DNA plasmids. Black histograms represent raw data and blue histograms represent G1, S, G2 and summated model populations predicted by the Watson algorithm.[10]

[10] This figure was compiled by Michael Mashiba. 89.6*vpr*^{Q65R} and 89.6*vpr*^{H71R} mutants were cloned by David Collins.

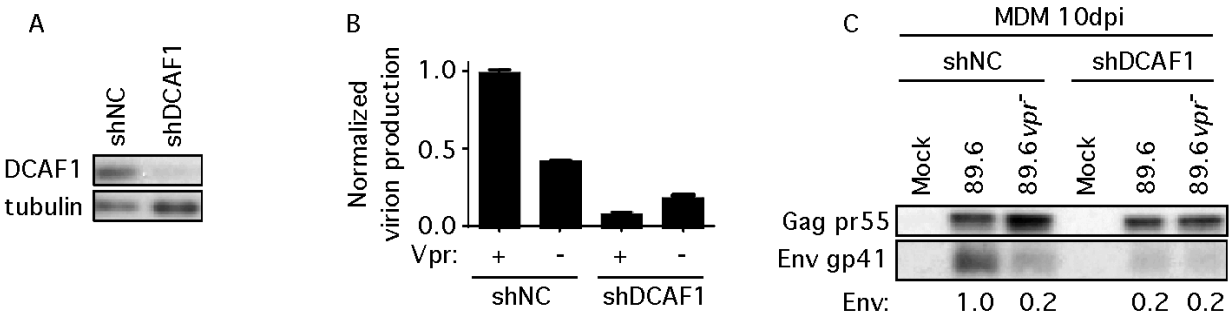

Figure 3.11. DCAF1 is required for Vpr to counteract the macrophage-specific restriction to virion production and Env expression. **(A)** Immunoblot analysis of whole cell lysates prepared from MDM transduced with a lentivirus expressing an shRNA targeting DCAF1 (shDCAF1) or luciferase (shNC) (Pertel et al., 2011). **(B)** Normalized virion production by cells treated as described for A and then infected with 1μg of wildtype 89.6 or 89.6*vpr*⁻ for 10 days. **(C)** Immunoblot analysis of WCL from MDM treated as described in B. Data are representative of two experiments performed using cells from independent donors. Error bars represent the standard error of the mean. [11]

[11] This figure was compiled by David Collins.

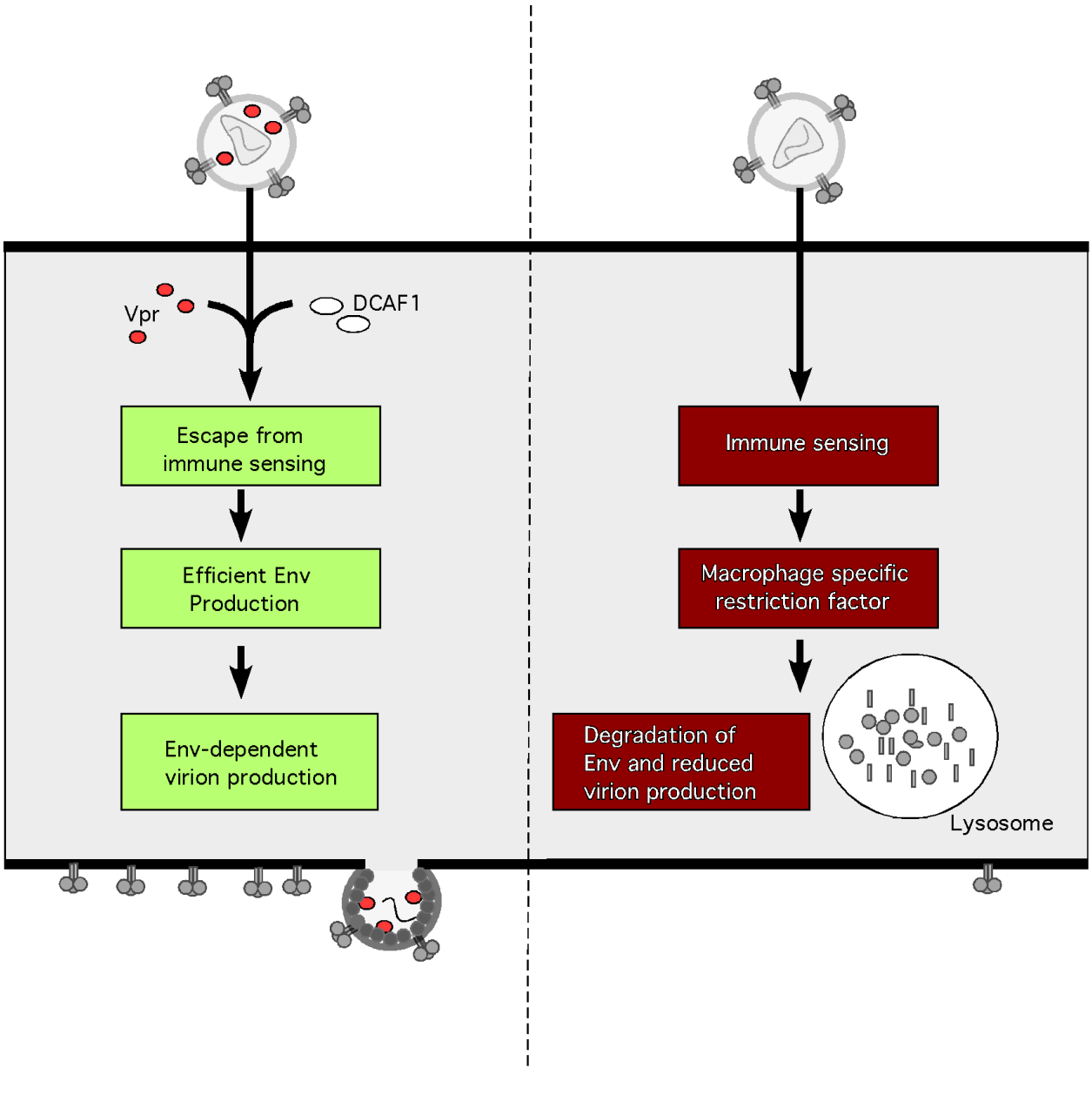

Figure 3.12. Proposed model for Env-dependent virion production by Vpr.[12]

[12] This figure was created by Kathleen Collins.

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Chapter 4:

Characterization of a novel primary macrophage cell culture system and description of a possible role for C-type lectins

Introduction

Vpr is a conserved lentiviral accessory protein that has been reported to recruit the structure-specific endonuclease (SSE) regulator SLX4 complex to escape immune recognition of HIV, and to cause cell cycle arrest (Laguette et al., 2014). However, neither SSE activation nor cell cycle arrest have been linked to HIV infectivity in existing cell lines. The development of relevant models in cell culture has often presented a barrier to understanding mechanisms of lentiviral escape from host innate immunity. For example, the observation that the lentiviral accessory protein Vpx provided a replication advantage in myeloid cells (Sharova et al., 2008) led to the identification of myeloid restriction factors counteracted by Vpx, including SAMHD1 (Berger et al., 2011; Laguette et al., 2011). Vpr has been reported to enhance infection of myeloid cells by utilizing the same DCAF-DDB1-Cul4A E3 ubiquitin ligase complex as Vpx, but the effect of Vpr on macrophage infectivity *in vitro* has appeared to be less dramatic than that of Vpx (Ayinde et al., 2010; Eckstein et al.,

2001). The target of the Vpr-associated E3 ubiquitin ligase is unknown, but Vpr has been proposed to downregulate a macrophage-specific restriction factor that is active *in vivo* under conditions not yet replicated in cell culture(Malim and Emerman, 2008).

In order for HIV to infect a cell, the HIV envelope (Env) glycoprotein protein initiates entry by binding the host CD4 receptor and co-receptors CCR5 or CXCR4. Several factors are known to inhibit entry *in vivo* that are not commonly present in cell culture. First, HIV-infected people acquire antibodies that neutralize Env, and these antibodies co-evolve with Env (Liao et al., 2013). Secondly, mannose-binding lectin (MBL) is a host serum lectin that activates complement, opsonization and neutralization of HIV by binding mannose residues on HIV Env through its carbohydrate recognition domain (CRD) (Ezekowitz et al., 1989; Ji et al., 2005). More recently, it was discovered that the antiretroviral drugs AMD3100 and Maraviroc also block HIV infection by inhibiting the interaction between Env and CXCR4 and CCR5, respectively (Emmelkamp and Rockstroh, 2007; Hendrix et al., 2000). Therefore, HIV encounters obstacles to entry *in vivo* that are not commonly factors *in vitro*.

In addition to CD4 and coreceptors, Env binds to several other host proteins on the surface of the cell. HIV is known to utilize host C-type lectin receptors (CLRs), such as human mannose receptor (hMR) and dectin 1, DC-specific ICAM3-grabbing non-integrin (DC-SIGN) to mediate trans-infection of $CD4^+$ T cells by dendritic cells(Geijtenbeek et al., 2000; Nguyen and Hildreth, 2003). However, CLRs serve several important functions in innate immunity as well. CLRs share a Ca^{2+}-dependent carbohydrate recognition domain that binds non-self terminal mannose residues on pathogenic glycoproteins, including HIV-1 Env (Geijtenbeek and Gringhuis, 2009; Lennartz et al., 1989; Wileman et al., 1986). Thus, DC-SIGN and hMR, which are expressed specifically on macrophages and dendritic cells, are

pattern recognition receptors (PRRs) that mediate phagocytosis of pathogens such as *Mycobacterium tuberculosis* and *Candida albicans*(Ezekowitz et al., 1991). CLRs are also required for the initiation of signaling pathways in response to pathogenic stimuli, leading to activation of transcription factors, such as nuclear factor-κB (NF-κB) (Geijtenbeek and Gringhuis, 2009; Gringhuis et al., 2007). Thus, in addition to being subverted by HIV for trans-infection, CLRs represent an important mechanism of innate immunity in macrophages. However, it is not known if hMR or DC-SIGN inhibit HIV infection through pathways that have been characterized for other pathogens.

Here, we report that HIV Vpr downregulates hMR and DC-SIGN, and provide preliminary evidence suggesting that hMR inhibits HIV infection of monocyte-derived macrophages (MDM). The identification of hMR as an antiviral factor downmodulated by Vpr arose from analysis of a novel cell culture system, and should be investigated further. Treatment of MDM with entry inhibitors modeling *in vivo* barriers to entry amplified the effect of Vpr on infectivity from two fold to greater than 100 fold. By reproducing *in vivo* obstacles to HIV infection in cell culture, this study illuminates several novel pathways utilized by Vpr to enhance infection of macrophages.

Results

Vpr overcomes a restriction in MDM treated with compounds blocking HIV-1 entry

To identify cell culture conditions in which Vpr enhances infection, we compared replication fitness of the wildtype dual-tropic molecular clone HIV-1 89.6 to that of the Vpr-null mutant 89.6*vpr*$^-$. HIV-1 89.6*vpr*$^-$ was as infectious as wildtype 89.6 in permissive CEMx174 cells at a range of multiplicities of infection (MOIs) (**Figure 4.1A**), as reported

previously (Balliet et al., 1994). Therefore, CAp24 levels in our virus stocks correlated with infectivity, and mutation of *vpr* did not alter infectivity in permissive cells.

Because Vpr has been proposed to enhance HIV-infection at an early stage of replication in MDM (Heinzinger et al., 1994; Laguette et al., 2014; Malim and Emerman, 2008), we asked whether reagents that decrease the efficiency of HIV entry might amplify the requirement for Vpr in MDM. AMD3100 and Maraviroc are antiretroviral drugs that inhibit entry by blocking the interaction between HIV-1 Env and the coreceptors CXCR4 and CCR5, respectively (Emmelkamp and Rockstroh, 2007; Hendrix et al., 2000), however their efficacy in MDM is not known. As expected, these compounds blocked high MOI HIV infection of CEMx174 cells and $CD4^+$ T lymphocytes efficiently in the presence or absence of *vpr* {**Figure 4.1B**, (Carter et al., 2011) and data not shown}. Also as predicted, infection of MDM at the same high MOI in the absence of entry inhibitors did not reveal an effect of *vpr* on infection frequency, which ranged from 40-80% at five days post infection (dpi) (**Figures 4.2A, top row, and 4.2B**). Surprisingly, however, AMD3100 and Maraviroc treatment of MDM inhibited 89.6*vpr*$^-$ infectivity over 100-fold more than wildtype 89.6 ($p < 0.005$, **Figures 4.2A, second row and 4.2B**). Furthermore, infection of MDM treated with AMD3100 and Maraviroc at higher multiplicities of infection (MOI) enhanced infection by wildtype 89.6, but not 89.6*vpr*$^-$ (**Figure 4.3**). These results demonstrated that *vpr* overcame a restriction to HIV-infection in AMD3100 and Maraviroc-treated MDM.

To confirm that this *vpr*-dependent HIV infection was genuine, we examined whether it could be blocked by the reverse transcriptase inhibitor azidothymidine (AZT). AZT treatment completely blocked Gag expression when added at the time of infection (**Figure 4.2A, third row**), demonstrating that the *vpr*-dependent infection we observed

required reverse transcription, and was not caused by viral particles from the inoculum binding to the cell surface. AZT treatment of AMD3100 and Maraviroc-treated MDM at 24 or 48 hours post infection (hpi) also reduced the frequency of Gag-expressing cells measured at five days by three- to five-fold (**Figure 4.2A, fourth and fifth rows**). Therefore, we observed that *vpr* enhanced infection of MDM treated with AMD3100 and Maraviroc as early as one dpi and possibly as late as two dpi to the time of cell harvest at five dpi.

We next wished to determine the mechanism through which AMD3100 and Maraviroc treatment created a requirement for *vpr* in MDM. Because AMD3100 and Maraviroc inhibit entry of HIV-1, we asked whether other compounds known to inhibit entry reproduced a *vpr* requirement for infection. Anti-Env and anti-CD4 antibodies are known to inhibit entry by blocking the interaction between HIV Env and CD4(Gorny et al., 1989; Oravecz and Norcross, 1993). Indeed, treatment of MDM with anti-Env or anti-CD4 antibodies potently inhibited infection by HIV-1 89.6*vpr*- ten to twenty fold more than wildtype 89.6 (**Figure 4.4A**). Of note, the percentage of MDM infected by wildtype 89.6 in the presence of neutralizing antibodies was similar to that in the presence of AMD3100 and Maraviroc (3 to 4 percent, **Figure 4.4A**). In sum, we concluded that reagents known to interfere with HIV-1 entry through the CD4 receptor and CCR5 and CXCR4 coreceptors induced a requirement for *vpr* in MDM. This finding is interesting because Env-neutralizing molecules are known to be present in high quantities in human serum(Ezekowitz et al., 1989), opening the possibility that Vpr has evolved to counteract these *in vivo* barriers to infection.

Having discovered that reagents hindering entry elicited a *vpr* requirement in MDM, we explored whether drugs acting on later replication events revealed a similar phenotype. However, neither the reverse transcriptase inhibitor AZT nor the integrase inhibitor Raltegravir induced a *vpr* requirement for infection of MDM at maximally effective concentrations (**Figure 4.4B**, top row) or at sub-optimal concentrations (**Figure 4.4B**, second and third rows). Thus, we found that compounds inhibiting reverse transcription or integration did not evoke a *vpr* phenotype in MDM.

Vpr mutants defective at DCAF1 binding are unable to enhance HIV infection of MDM treated with entry inhibitors

Vpr is known to interact with a DCAF1-DDB1-Cul4A E3 ubiquitin ligase complex that has been proposed to enhance HIV replication by degrading an unknown cellular restriction factor(Ahn et al., 2010; Belzile et al., 2010; Laguette et al., 2014; Wen et al., 2012). However, identification of this restriction factor has been challenging because Vpr does not dramatically enhance replication in existing cell culture conditions. To examine whether DCAF1 is required to enhance *vpr*-dependent infection under our conditions, we employed the Vpr mutants Q65R and H71R that bind DCAF1 less efficiently than wildtype Vpr {**Chapter 3, Figures 3.6 and 3.10** (Belzile et al., 2010; DeHart et al., 2007; Laguette et al., 2014)}. Both Vpr mutants were expressed in 293T cells and packaged into virions, but Vpr H71R was expressed at a lower level than wildtype (**Chapter 3, Figure 3.6**). As expected, these Vpr point mutations did not affect infectivity of HIV-1 89.6 at a high MOI (**Figure 4.5, top row**). However, AMD3100 and Maraviroc treatment diminished infection by HIV-1 89.6*vpr*^{Q65R} and 89.6*vpr*^{H71R} to the level of the *vpr*-null virus, whereas wildtype HIV-1 89.6 infected 0.7 percent of MDM (**Figure 4.5, second row**). Therefore, we

concluded that *vpr*-dependent infection of MDM treated with entry inhibitors required the interaction of Vpr with the substrate adaptor DCAF1.

Mannose receptor and DC-SIGN are host antiviral factors counteracted by Vpr

Although CD4 is the major host receptor utilized by HIV for entry, minority receptors such as hMR have been reported to mediate entry into astrocytes in the central nervous system(Trujillo et al., 2007). To determine if hMR permitted HIV entry into macrophages treated with AMD3100 and Maraviroc, we treated MDM with an antibody previously demonstrated to inhibit the interaction between hMR and HIV Env (Liu et al., 2004). As expected, treatment with hMR blocking antibody did not have a significant effect on the frequency of MDM infected at a high MOI in the absence of inhibitors of CD4-mediated entry (**Figure 4.6, top row**). Unexpectedly, in MDM treated with AMD3100 and Maraviroc, anti-hMR antibody increased the frequency of cells infected by wildtype HIV-1 89.6 from one to two percent (**Figure 4.6, second row**). Importantly, hMR blocking antibody did not rescue infection by HIV-1 89.6*vpr*$^-$ in the presence of AMD3100 and Maraviroc (**Figure 4.6, second row**). These preliminary data suggest that hMR counteracts HIV infection of MDM when entry is inhibited pharmacologically, although infection remained Vpr-dependent.

Because hMR demonstrated antiviral activity in MDM under our assay conditions, we hypothesized that Vpr counteracts hMR. To determine if Vpr regulates hMR levels, we examined hMR expression in MDM infected by wildtype HIV-1 89.6 or 89.6*vpr*$^-$ at seven and ten days post infection, time points at which high numbers of cells were infected. Indeed, infection of MDM by wildtype HIV-1 89.6 reduced hMR levels sixty percent by seven dpi,

and ninety percent by ten dpi, but 89.6*vpr*⁻ had a more subtle effect on hMR (**Figure 4.7A**). Downregulation of hMR by *vpr* ranged from no effect to five-fold in experiments performed on cells from different donors ($n = 7$, $p < 0.05$, **Figures 4.7A-D**). Interestingly, these effects of *vpr* on hMR expression appeared to be more dramatic than the effect of *vpr* on the frequency of infected cells, suggesting that infection was not required for hMR downmodulation (**Figure 4.7A**). Of note, the effect of *vpr* on hMR expression correlated with its effects on HIV-1 Env and DCAF1 observed previously (**Figure 4.7A** and **Chapter 3, Figures 3.5 and 3.8**). Altogether, these results demonstrate that Vpr counteracts hMR by downregulating hMR expression.

The finding that *vpr* lowers the amount of hMR detected by western blot is surprising given that HIV is not known to affect total hMR levels. It has previously been shown that the HIV accessory protein Nef removes hMR from the surface of MDM, but this surface downmodulation does not affect the total level of hMR(Vigerust et al., 2005). Nevertheless, to determine if Nef is required for Vpr-mediated downregulation of total hMR levels, we examined hMR levels in MDM infected with HIV-1 89.6 mutants lacking *nef*, *vpr*, or both genes (89.6*nef*⁻). Mutation of *vpr* had a much more dramatic effect on HIV-mediated hMR downregulation than mutation of *nef*, despite similar infection frequencies and Gag pr55 expression between MDM infected by 89.6*vpr*⁻ or 89.6*nef*⁻ (**Figure 4.7D**, second and third lanes). Also, the presence of *vpr* correlated with hMR downregulation even in the absence of *nef* (**Figure 4.7D** third and fourth lanes). Thus, we concluded that hMR downregulation by *vpr* did not depend on *nef*.

We next wished to determine if the downregulatory effect of *vpr* on hMR is specific to that molecule or if *vpr* regulates other CLRs known to interact with HIV Env. DC-SIGN is

such a molecule expressed on the surface of dendritic cells (DC) that mediates HIV trans-infection of $CD4^+$ T cells by DC (Geijtenbeek and Gringhuis, 2009; Geijtenbeek et al., 2000). MDM from four donors were examined for DC-SIGN expression, but only cells from two donors expressed detectable levels of DC-SIGN (data not shown). In MDM from these donors, however, infection by HIV-1 89.6 downmodulated DC-SIGN 70 to 80 percent compared with 89.6*vpr*$^-$ or mock infected cells (**Figures 4.7C and D**). In one donor, we were surprised to observe 70% DC-SIGN downregulation in the total cell population considering that only 3% of cells were infected by HIV-1 89.6 (**Figure 4.7C**). These results opened the possibility that infection was not required for *vpr* to downmodulate DC-SIGN. In sum, while MDM stimulated under our conditions did not consistently express DC-SIGN, this molecule was downmodulated by *vpr* when it was expressed.

Finally, given that infection of MDM in the presence of entry inhibitors requires the interaction between Vpr and DCAF1, we asked whether DCAF1 is involved in the basal regulation of hMR and DC-SIGN. To examine the effect of DCAF1 on hMR and DC-SIGN, we silenced DCAF1 in MDM using an shRNA-expressing lentivirus(Pertel et al., 2011). In the absence of DCAF1, we observed that levels of hMR and DC-SIGN were two-fold and four-fold higher, respectively (**Figure 4.7D**). This result is consistent with a model in which DCAF1 regulates basal levels of hMR and DC-SIGN.

Discussion and future directions

The identification of primary cell culture conditions in which Vpr enhances replication has been a barrier to identification of host restriction factors counteracted by Vpr. Here, we report novel cell culture conditions under which Vpr enhances HIV

replication in primary MDM more dramatically than in any previously characterized system. The fact that anti-hMR antibody increases HIV infection two-fold suggests that hMR restricts HIV infection of macrophages. In subsequent experiments, we found that Vpr downregulates the CLRs hMR and DC-SIGN. Additional studies are needed to determine whether these effects of Vpr are linked (see discussion below and **Chapter 5**.)

The conservation of *vpr* among lentiviruses suggests it confers a significant replication advantage, but *vpr* deletion mutants have relatively mild phenotypes in existing cell culture models(Lim and Emerman, 2011; Lim et al., 2012), obstructing efforts to understand the molecular mechanism of *vpr*. Vpr has been reported to enhance the infection of MDM two to three fold {**Chapter 3, Figures 3.2A-D** and (Sherman et al., 2003)}. We discovered that treatment of MDM with reagents inhibiting HIV entry increased the effect of Vpr on infectivity to greater than 100 fold. The addition of entry inhibitors to MDM cultures may replicate diffusible barriers to *in vivo* infection that may intensify selection for lentiviruses expressing *vpr*. About twenty percent of HIV-1 infected individuals develop antibodies that neutralize envelopes from a broad library of HIV-1 isolates, and evidence suggests co-evolution of HIV Env and neutralizing antibodies *in vivo* (Liao et al., 2013). Additionally, host serum lectins such as MBL opsonize and neutralize HIV by binding HIV Env(Ezekowitz et al., 1989; Ji et al., 2005). Vpr may provide HIV with a mechanism of escape from neutralization by antibodies or lectins, and may explain why these endogenous immune mechanisms do not effectively control HIV in infected people (McCoy and Weiss, 2013). Therefore, the entry inhibitors used in our study represent a valuable tool to study Vpr by modeling conditions under which HIV-1 infects MDM *in vivo*.

Understanding the mechanism whereby entry inhibitors amplify the effect of Vpr on infection of MDM will require further investigation. Given that cell-to-cell spread has been reported to be resistant to neutralizing antibodies and certain antiretroviral drugs (Martin and Sattentau, 2009; Sigal et al., 2011), one possibility is that Vpr enhances cell-to-cell spread in our system. In addition, the observation that entry inhibitors did not influence the Vpr requirement for HIV infection of CEMx174 T cells or primary $CD4^+$ T cells open the possibility that macrophages express or upregulate restriction factors that T cells do not. MDM-specific pattern recognition receptors or mechanisms of viral entry may also amplify the effect of Vpr on infection when entry is inhibited pharmacologically. Inhibition of entry may stabilize an HIV replication intermediate at the plasma membrane, leading to virion endocytosis and detection by the innate immune system as has been proposed previously {**Figure 4.8** and (Laguette et al., 2014)}.

Another intriguing possibility is that CD4-dependent entry inhibitors limit infection to a minority population of cells expressing unique restrictions to lentiviral infection counteracted by *vpr*. Indeed, CD4-independent infection of minority populations of astrocytes has been reported previously (Liu et al., 2004). The observation that AMD3100 and Maraviroc, CD4-blocking antibody, and Env neutralizing antibodies permit infection of similar percentages of MDM by wildtype HIV-1 89.6 would be consistent with the existence of such a sub-population. The requirement for Vpr to bind DCAF1 to enhance infection of MDM treated with entry inhibitors suggests that the unidentified host target of the Vpr-DCAF1-DDB1-Cul4A E3 ubiquitin ligase restricts HIV under these conditions.

Here, we report that Vpr lowers the level of hMR in MDM infected by HIV (**Figure 4.8**). The finding that Vpr downregulates CLRs likely has significant implications for

macrophage innate immunity to HIV infection. hMR is an important pattern recognition receptor that mediates the phagocytosis and lysosomal degradation of glycosylated pathogens such as *Pneumocystis carinii, Mycobacterium tuberculosis* and *Candida albicans* by macrophages(Ezekowitz et al., 1991). It remains to be determined if hMR initiates clearance of HIV by macrophage phagocytosis. Furthermore, hMR and DC-SIGN may be required for macrophages to secrete cytokines in response to HIV infection (Geijtenbeek and Gringhuis, 2009). These receptors are required for efficient cytokine production in response to a variety of pathogens in cooperation with canonical pattern recognition receptors including Toll-like receptors 2 and 4 (TLR2 and TLR4) (Gazi and Martinez-Pomares, 2009; Geraldino et al., 2010; Zhang et al., 2004). Indeed, hMR has been reported to mediate noninfectious entry of HIV-1 into macrophages (Trujillo et al., 2007), raising the possibility that hMR-mediated endocytosis of HIV triggers an antiviral response that prevents infection (**Figure 4.8**). Therefore, downregulation of hMR and DC-SIGN by Vpr may disable macrophage innate immunity to HIV.

In addition, Vpr downmodulation of CLRs may be the mechanism through which Vpr enhances Env levels in HIV-infected MDM (**Chapters 3 and 5**). The interaction between the extracellular CRD of CLRs and mannose residues on Env has the potential to interfere with Env trafficking and stability. CLRs such as DC-SIGN target ligands to the lysosome for degradation(Engering et al., 2002), and have the potential to degrade Env through this mechanism as well. The specific expression of CLRs in macrophages but not in 293T cells or primary $CD4^+$ T cells correlates with the macrophage specificity of Vpr's enhancement of Env expression. The hypothesis that Vpr enhaces Env levels through CLRs is discussed in more detail in **Chapter 5**.

The low HIV infection rates sufficient to observe CLR downregulation by Vpr in whole cell lysate increase the likelihood that infection is not required for this activity. Given that Vpr is actively incorporated into virions through the p6 region of Gag(Bachand et al., 1999), one possibility is that virion fusion is sufficient to regulate hMR levels. Delivery of Vpr to regulate the innate immune system may be a prerequisite to HIV infection of MDM. An interesting study reported that HIV-1 Nef removes hMR from the surface of MDM (Vigerust et al., 2005). Unlike CD4, however, hMR is not degraded following surface downmodulation by Nef (Vigerust et al., 2005). Also, Nef is not packaged into virions, suggesting that the downmodulation of hMR that we observed may be Nef-independent. Therefore, this is the first report that HIV encodes a gene that downregulates total hMR levels.

It is still unknown if Vpr downregulates hMR through the DCAF1-DDB1-Cul4A E3 ubiquitin ligase directly, or prevents the upregulation of hMR by interfering with innate immune recognition of HIV {**Figure 4.8** and (Laguette et al., 2014)}. The observation that DCAF1 knockdown increases hMR and DC-SIGN levels suggests that the DCAF1-DDB1-Cul4A E3 ubiquitin ligase plays a role in the basal regulation of CLR levels. Similarly, basal turnover of UNG2, the most established Vpr target, is also dependent on DCAF1(Wen et al., 2012). Therefore, Vpr may target hMR and DC-SIGN for ubiquitin-mediated proteasomal degradation through the DCAF1-DDB1-Cul4A E3 ubiquitin ligase just like it downregulates UNG2. Alternatively, because DCAF1 regulates the type I interferon response(Laguette et al., 2014), one possibility is that DCAF1 regulates CLR expression through the interferon pathway. Further experiments will be required to uncover the molecular mechanism through which Vpr downregulates CLRs.

Some insight into this mechanism might arise from our preliminary observation that Vpr also downregulates DC-SIGN (Geijtenbeek et al., 2000). Although DC-SIGN and hMR have very different cytoplasmic tails, they contain dileucine (DC-SIGN, SDXXXLL) or similar (hMR, SDTKDLV) sequences that have been reported to interact with the clathrin adaptor protein 2 (AP-2) (Sancho and Reis e Sousa, 2012; Vigerust et al., 2005). Furthermore, the CT of DC-SIGN, hMR and other lectins expressed in MDM, such as MBL or Dectin-1 contain a conserved tyrosine residue within NPXY or LXXY sequences necessary for CLR-mediated phagocytosis (Sancho and Reis e Sousa, 2012; Vigerust et al., 2005). Further investigation will be required to determine if CLRs contain a common cytoplasmic tail motif targeted by Vpr. hMR and DC-SIGN also share CRDs in their extracellular regions(Sancho and Reis e Sousa, 2012). Given that Vpr also enhances HIV-1 Env levels (**Chapter 3**), and Env interacts with hMR and DC-SIGN(Geijtenbeek and Gringhuis, 2009), it is also possible that Vpr downregulates these CLRs through Env. Further experiments infecting MDM with Env-null viruses are needed to determine whether Env is necessary for Vpr to downregulate CLRs.

Finally, we present evidence that inhibition of HIV-1 89.6 infection by hMR can be rescued with hMR-blocking antibody. If hMR were to initiate phagocytosis of virions from the inoculum, one possibility is that hMR-mediated phagocytosis decreases the effective multiplicity of infection, which would be rescued by hMR-neutralizing antibody. Interestingly, hMR antibody did not rescue infection by HIV-1 89.6*vpr*$^-$, possibly suggesting that hMR inhibits HIV in intracellular compartments within MDM inaccessible to neutralizing antibody. In fact, eighty to ninety percent of mannose receptor protein has been reported to be intracellular (Vigerust et al., 2012; Wileman et al., 1984). Alternately,

it is possible that Vpr counteracts redundant CLRs such as DC-SIGN, MBL or Dectin-1 that inhibit infection by 89.6*vpr*⁻ in the presence of hMR-neutralizing antibody. Thus, while we have established hMR as a host protein having antiviral activity we may be only beginning to identify a number of host restriction factors counteracted by Vpr in our system.

In summary, we have developed novel cell culture conditions that model HIV infection of macrophages *in vivo*, leading to the preliminary characterization of hMR as a host antiviral factor downregulated by Vpr. Further work is needed to identify other host factors downregulated by Vpr, and to understand the mechanism through which entry inhibitors magnify the effect of Vpr on HIV infectivity. The molecular mechanism behind the conservation of *vpr* among lentiviruses has been a long-standing topic of interest in HIV research. This study will accelerate our potential to study *vpr* and may lead to a better understanding of innate immunity to lentiviral infection.

Materials and Methods

Antibodies and cell lines

Antibodies to CAp24 [KC57 conjugated to phycoerythrin (PE) or fluorescein isocyanate (FITC)] were obtained from Beckman Coulter. Antibodies to the following proteins were used for immunoblot analysis: DCAF1 (11612-1-AP Proteintech), Tubulin (T5168, Sigma), Gag CAp24, gp120, gp41, DC-SIGN and CD4 AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog Numbers 4250 and 288 were from Dr. Michael Phelan (Hatch et al., 1992). Catalog number 11557 was from Dr. Michael Zwick (Zwick et al., 2001). Catalog number 5443 was from Drs F. Baribaud, S. Pohlmann, J.A. Hoxie and R.W.

Doms. Catalog numbers 723 and 724 were from Dr. James Hildreth. Anti-hMR goat serum was obtained from Dr. Philip Stahl (Liu et al., 2004; Wileman et al., 1984).

CEMx174 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: 174xCEM from Dr. Peter Cresswell.

Cell culture and viral infection

Leukocytes isolated from anonymous donors by apheresis were obtained from New York Blood Center Component Laboratory (Long Island City, NY). Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll density gradient separation, as described(Norman et al., 2011).

$CD14^{+}$ monocytes were isolated by positive selection with an EasySep magnetic sorting kit (StemCell Technologies). Monocyte derived macrophages (MDM) were differentiated as described{**Chapter 3** and (Lahouassa et al., 2012)}. MDM were incubated with virus stocks and half of the media was replaced with fresh R10 every other day.

shRNA knockdown of DCAF1 was performed as previously described {**Chapter 3** and (Pertel et al., 2011)}.

Viral constructs and virus stocks

p89.6 was obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalog number 3552(Collman et al., 1992). p89.6*vpr*$^{-}$, p89.6*vpr*^{Q65R} and p89.6*vpr*^{H71R} have been described previously (**Chapter 3**). Virus stocks were produced by transfection of 293T cells with provirus expression plasmids using polyethylenimine, as described(McNamara et al., 2012). Viral supernatants were collected at 48h and

centrifuged at 1500 rpm to remove cell debris. Virus was stored at -80°C and quantified by p24 ELISA, as described {**Chapter 3** and (Salmon and Trono, 2007)}.

Flow cytometry staining and antibodies

Intracellular staining for Gag CAp24 expression was performed as described previously {**Chapter 3** and (Carter et al., 2010)}. Data was analyzed using FloJo software (Cytek).

Western blotting

MDM whole cell lysate was prepared and analyzed by immunoblot as described previously (**Chapter 3**). Lysates were analyzed by immunoblot using autoradiography and protein levels were quantified using Adobe Photoshop as described(Norman et al., 2011).

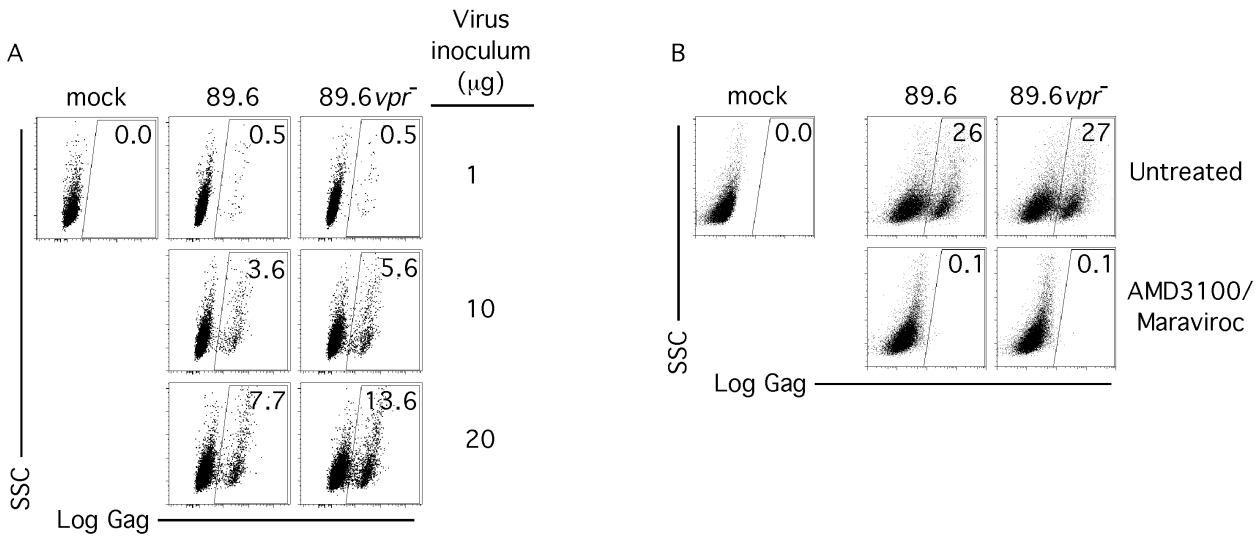

Figure 4.1. AMD3100 and Maraviroc inhibit HIV-infection of CEMx174 cells. **(A)** Flow cytometric analysis of CAp24 expression in CEMx174 cells infected for 2d with the indicated mass amounts of HIV-1. **(B)** Flow cytometric analysis of CEMx174 cells infected for 2d with 100μg of the indicated HIV-1 and treated with 10μg/ml AMD3100 and 20μM Maraviroc where indicated. [1]

[1] The data in this figure was produced by Michael Mashiba.

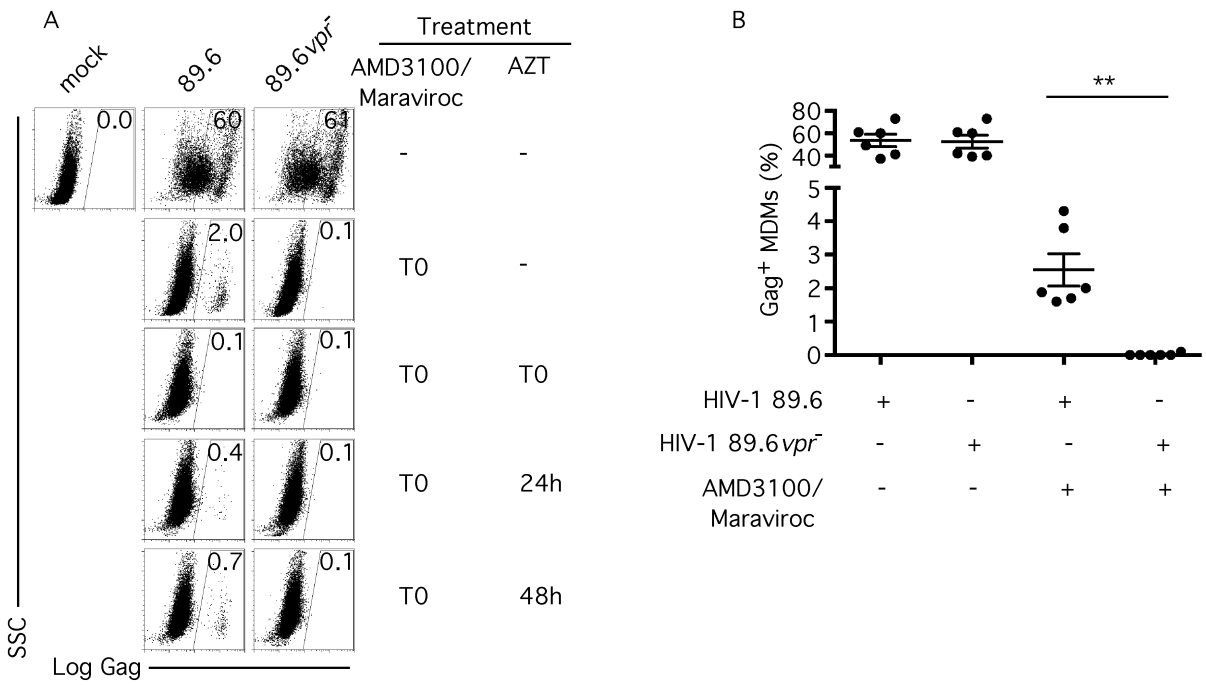

Figure 4.2. MDM pretreated with AMD3100 and Maraviroc exhibit a potent restriction to HIV-1 89.6 infection that is counteracted by Vpr. **(A)** Flow cytometric analysis of CAp24 expression in MDMs infected with 100μg of the indicated HIV-1 and treated with 10μg/ml AMD3100 and 20μM Maraviroc and/or 20μM azidothymidine (AZT) at the indicated times post infection. **(B)** Summary graph of experiments performed as in A. Each dot represents a separate experiment from a different donor. Error bars represent the standard error of the mean. ** $p < 0.005$.[2]

[2] The data in this figure was produced by Michael Mashiba.

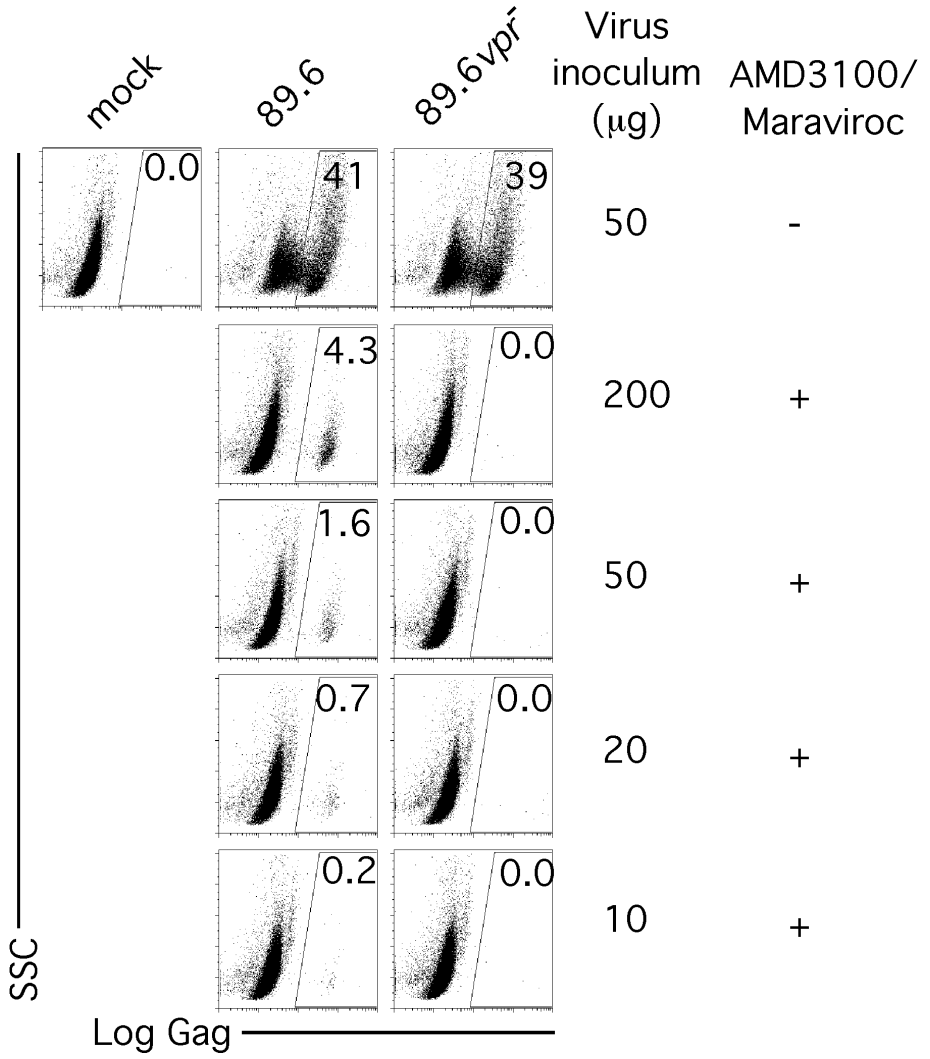

Figure 4.3. Vpr-mediated infection of MDM treated with AMD3100 and Maraviroc is dependent on the virus inoculum. Flow cytometric analysis of CAp24 expression 5dpi in MDM infected with the indicated quantity of HIV-1 89.6 or 89.6*vpr*- and treated with 10μg/ml AMD3100 and 20μM Maraviroc.[3]

[3] The data in this figure was produced by Michael Mashiba.

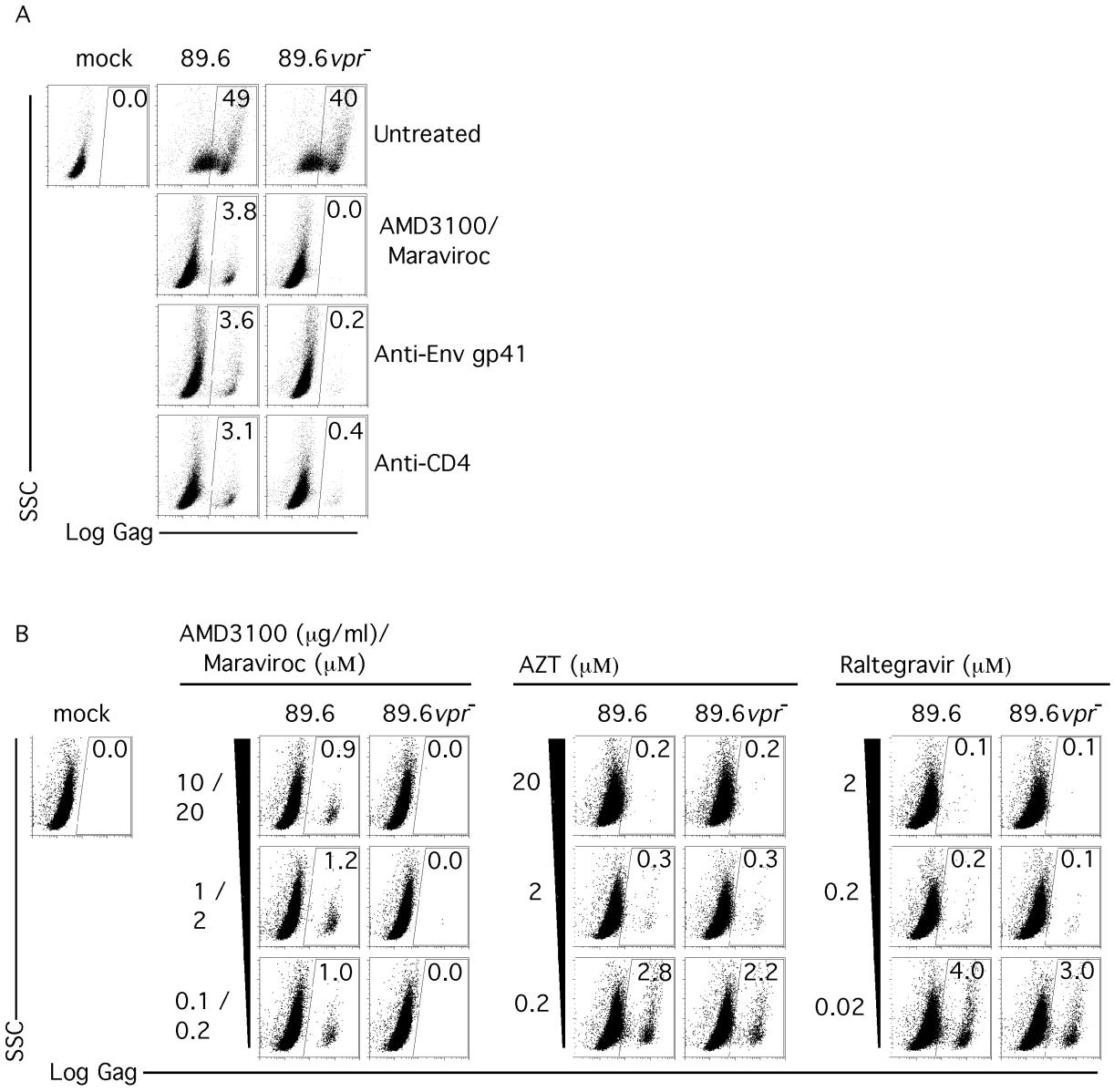

Figure 4.4. Vpr partially overcomes a restriction to HIV-infection in MDM treated with reagents blocking entry, but not reverse transcription or integration. **(A)** Flow cytometric analysis of CAp24 expression 5dpi in MDM infected with 200μg of the indicated HIV-1 and treated with 10μg/ml AMD3100 and 20μM Maraviroc, anti-gp41 antibody, or anti-CD4 antibody. **(B)** Flow cytometric analysis of CAp24 expression at 5dpi in MDM infected with 200μg of the indicated HIV-1 and treated with concentrations of AMD3100 and Maraviroc, Raltegravir or AZT indicated at the left of each panel. [4]

[4] The data in this figure was produced by Michael Mashiba.

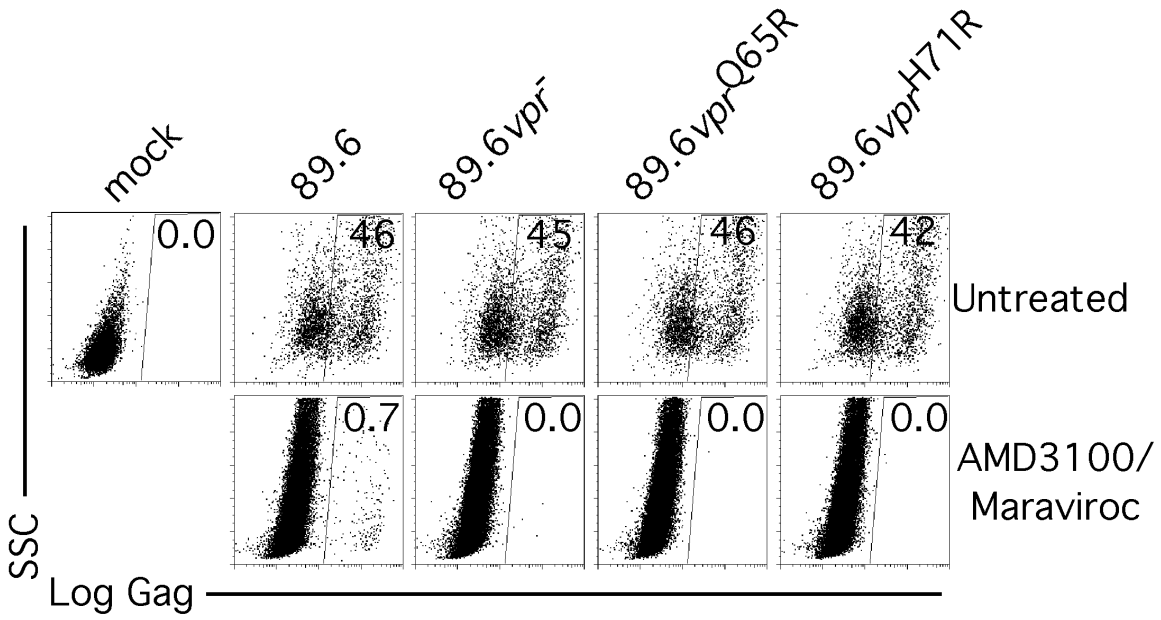

Figure 4.5. Mutants defective at DCAF1 binding are also unable to overcome a restriction to HIV-infection of MDM treated with AMD3100 and Maraviroc. Flow cytometric analysis of CAp24 expression in MDM infected with 50μg of wildtype HIV-1 89.6 or the indicated mutant viruses and treated with 10μg/ml AMD3100 and 20μM Maraviroc where indicated. [5]

[5] This experiment was performed by Michael Mashiba. David Collins cloned 89.6*vpr*^{Q65R} and 89.6*vpr*^{H71R}.

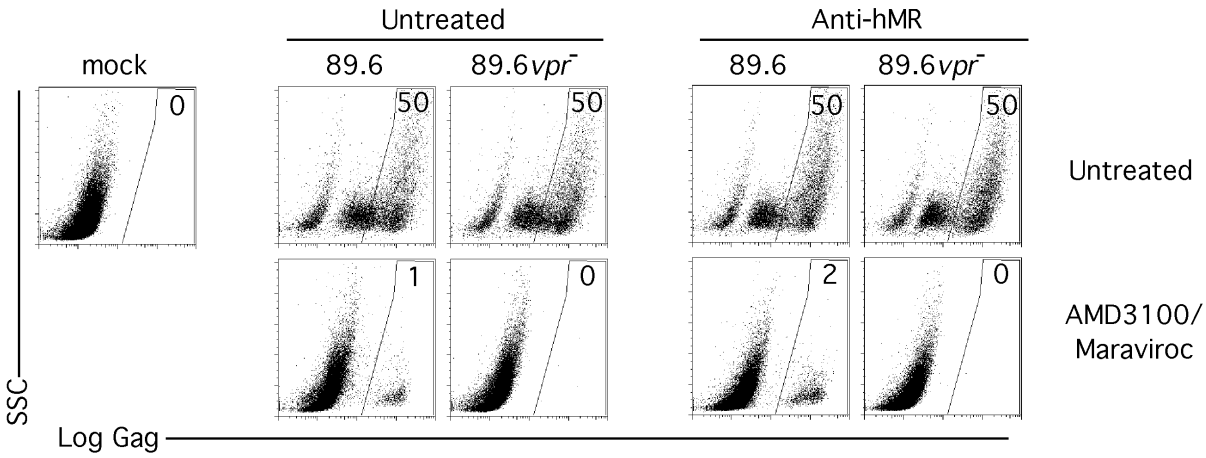

Figure 4.6. Mannose receptor blocking antibody enhances HIV infection of MDM treated with AMD3100 and Maraviroc. Flow cytometric analysis of CAp24 expression at 5dpi in MDM infected with 100μg of the indicated HIV-1 and treated with 10μg/ml AMD3100 and 20μM Maraviroc or anti-hMR neutralizing serum where indicated.[6]

[6] The data in this figure was produced by Michael Mashiba.

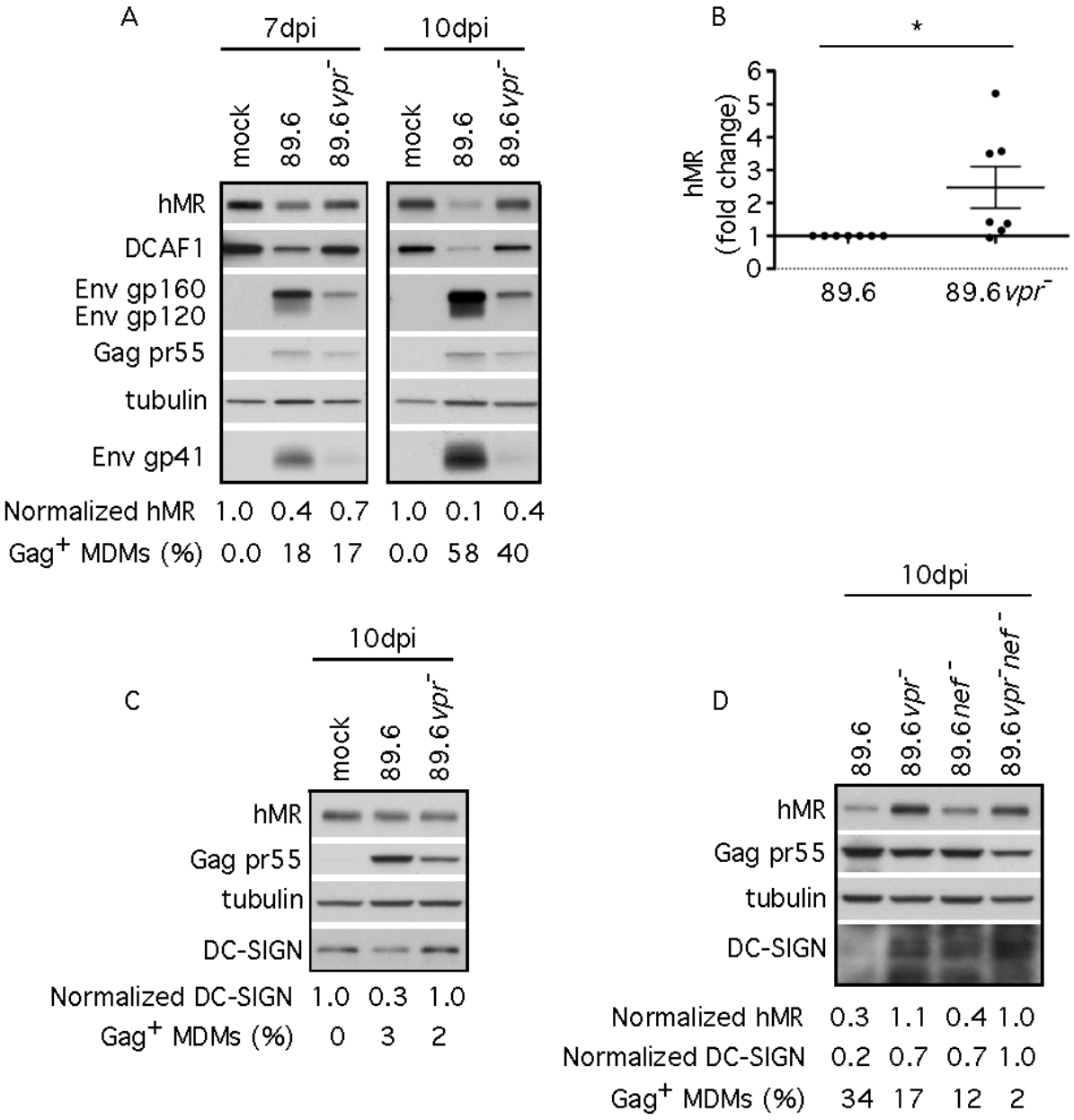
A
7dpi
10dpi
mock
89.6
89.6vpr-
hMR
DCAF1
Env gp160
Env gp120
Gag pr55
tubulin
Env gp41
Normalized hMR 1.0 0.4 0.7 1.0 0.1 0.4
Gag+ MDMs (%) 0.0 18 17 0.0 58 40
B
*
hMR (fold change)
0
1
2
3
4
5
6
89.6
89.6vpr-
C
10dpi
mock
89.6
89.6vpr-
hMR
Gag pr55
tubulin
DC-SIGN
Normalized DC-SIGN 1.0 0.3 1.0
Gag+ MDMs (%) 0 3 2
D
10dpi
89.6
89.6vpr-
89.6nef-
89.6vpr-nef-
hMR
Gag pr55
tubulin
DC-SIGN
Normalized hMR 0.3 1.1 0.4 1.0
Normalized DC-SIGN 0.2 0.7 0.7 1.0
Gag+ MDMs (%) 34 17 12 2
E
shNC
shDCAF1
hMR
DCAF1
tubulin
DC-SIGN
Normalized hMR 1.0 2.1
Normalized DC-SIGN 1.0 4.1

Figure 4.7. Vpr and DCAF1 downregulate mannose receptor and DC-SIGN in MDM. **(A)** Immunoblot analysis of MDM infected with 1μg of HIV-1 89.6 or HIV-1 89.6*vpr*-. Normalized hMR was quantified by densitometry and normalized for loading by tubulin content. Infection frequencies were determined by flow cytometric analysis of CAp24 expression. **(B)** Summary graph quantifying the fold increase in hMR expression in MDM infected for ten days with 1μg of 89.6*vpr*- relative to wildtype 89.6. Each dot represents a different donor from a separate experiment ($n = 7$). Error bars represent the standard error of the mean. * $p < 0.05$. (two-tailed *t*-test). **(C and D)** Immunoblot analysis of MDM from separate donors infected by the indicated viruses as in B. Normalized hMR and DC-SIGN were quantified as in A. **(E)** Immunoblot analysis of MDM 7d following transduction with a lentivirus expressing an shRNA targeting DCAF1 (shDCAF1) or negative control (shNC). Normalized hMR and DC-SIGN were determined as in A-C.[7]

[7] The data in this figure was produced by Michael Mashiba.

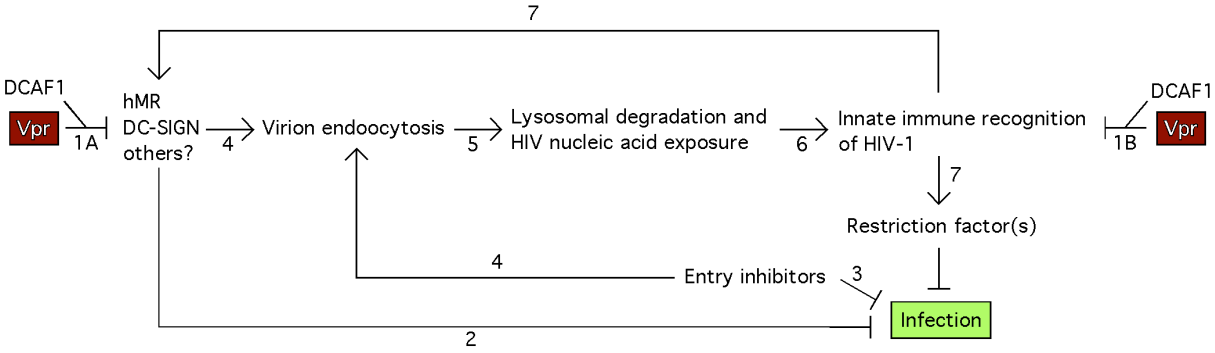

Figure 4.8. Proposed model for restrictions to HIV infection overcome by Vpr in primary MDM. Arrows indicate activation and horizontal lines indicate inhibition. (1A) Vpr utilizes the DCAF1-DDB1-Cul4A E3 ubiquitin ligase to downregulate CLRs on the surface of MDM such as hMR and DC-SIGN. (2) These MDM-specific lectins bind to glycosylated regions of HIV Env, inhibiting viral entry by preventing the interaction between Env and CD4, CXCR4 or CCR5. (3) Entry inhibitors such as AMD3100 and Maraviroc or Env-neutralizing antibodies also block the interaction between Env and CD4 or CXCR4 and CCR5. (4) Virions that cannot enter the cell through the infectious pathway are endocytosed, exposing viral nucleic acid (5) to detection by pattern recognition receptors (6), leading to an upregulation of host factors that restrict HIV infection, perhaps including hMR and DC-SIGN (7). (1B) Alternately, Vpr may suppress innate immune recognition of HIV-1, leading to an effect of Vpr on hMR and DC-SIGN indirectly through transcriptional downregulation. [8]

[8] The data in this figure was produced by Michael Mashiba.

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Chapter 5

Discussion

Lentiviral accessory protein phenotypes in relevant cell types present an opportunity to learn about innate immunity to HIV infection. Previously, the observation that myeloid cells maintain a post-entry block to lentiviral infection counteracted by Vpx led to the characterization of the host restriction factor SAMHD1 (Kaushik et al., 2009; Yu et al., 1991). This dissertation explores the molecular mechanisms behind Vpr, a homologous accessory protein that enhances HIV replication in macrophages. In Chapter 2, we found that Vpr and Vif cooperatively increase stability of HIV DNA. The mechanism we propose is that Vpr initiates repair of uracilated HIV DNA by recruiting the uracil DNA glycosylase UNG2, activating the DNA damage repair pathway and upregulating NKG2D ligands. In the Appendix, we begin to explore the hypothesis that Vpr protects uracilated HIV DNA from degradation or immunodetection through its interaction with UNG2. The Vpr-DCAF1-DDB1-E3 ubiquitin ligase was recently found to suppress the interferon response to HIV infection by recruiting the SLX4 complex to process HIV DNA in HeLa cells (Laguette et al., 2014). In Chapter 3, we explore the downstream implications of that pathway during HIV infection of MDM and identify host restrictions of HIV virion production and Env expression overcome by Vpr. Finally, in Chapter 4, we develop cell

culture conditions that amplify the effect of Vpr on MDM infection and begin to examine C-type lectin receptors as host antiviral factors counteracted by Vpr.

Experiments utilizing cell lines (**Figure 5.1A**), primary $CD4^+$ T cells (**Figure 5.1B**) and primary MDM (**Figure 5.1C**) have uncovered several cell-type-specific Vpr phenotypes that can or have the potential to be traced back to uracilation of genomic or HIV DNA. In this chapter, I will place the phenotypes observed in Chapters 2-4 into the context of current knowledge of accessory proteins and HIV-1 replication in different cell types. Experiments that may provide additional mechanistic detail for these phenotypes will also be described, as well as speculative mechanistic models. The following major conclusions were reached in this dissertation:

1) Uracilation of DNA leads to upregulation of NKG2D ligands
2) Vpr facilitates spread of HIV-1 and virion production in macrophages
3) Vpr increases Env levels in HIV-infected macrophages
4) Vpr-dependent virion production and Env expression require DCAF1
5) The ability of Vpr to enhance virion production depends on Env
6) Vpr overcomes a restriction in macrophages induced by entry inhibitors
7) Mannose receptor is a host restriction factor degraded by Vpr

Incorporation of uridine into HIV DNA leads to upregulation of NKG2D ligands

NK cells are a crucial component of the innate immune response to HIV (Gasser et al., 2005). NK cell recognition depends on a balance of inhibitory and activating surface molecules on HIV-infected cells, including activating ligands for the NK cell NKG2D

receptor (Lanier, 2008). In Chapter 2, we report that HIV upregulates NKG2D ligands on infected $CD4^+$ T cells. While several studies have shown that Vpr is necessary for this upregulation(Norman et al., 2011; Richard et al., 2010), it is assumed that that this phenotype is a byproduct of a Vpr activity that enhances HIV infection. Remarkably, we found that Vif suppresses this upregulation, and our investigation into the molecular mechanism through which Vpr and Vif influence NKG2D ligand expression has uncovered valuable insight into the uracilation of HIV DNA (**Figure 5.1B**).

It has long been known that Vif degrades APOBEC3G in virus-producing cells to prevent packaging of the antiviral factor into virions(Sheehy et al., 2002; Sheehy et al., 2003). In Chapter 2, we demonstrate that APOBEC3G degradation by Vif in the target cell decreases uracil incorporation into HIV DNA, suppressing NKG2D ligand expression by limiting activation of the DNA damage response pathway [(Croxford and Gasser, 2011; Gasser et al., 2005) and **Figure 5.1B**]. Therefore, this Vif activity initially characterized in the producer cell takes on new meaning for NK cell recognition of HIV-infected target cells.

While Vif limits DNA uracilation, we propose that Vpr may upregulate NKG2D ligands by initiating repair of uracilated DNA. Our hypothesis that Vpr recruits UNG2 to repair uracilated HIV DNA is based on several observations. First, we detected elevated DNA uracilation and lower amounts of HIV DNA in the absence of Vpr and Vif. Second, Vpr is known to associate with and recruit UNG2 into virions(Chen et al., 2004; Priet et al., 2003a). Also, we found that the W54R Vpr mutation affecting the interaction between Vpr and UNG2 attenuated NKG2D upregulation by HIV. And finally, we observed that UNG2 silencing attenuates Vpr-dependent NKG2D upregulation. Silencing of UNG2 presumably prevents the activation of the DNA damage repair pathway by nicks, gaps or breaks in HIV

DNA downstream of uracil excision (**Figures 5.1C**). Thus, Vif inhibits uracilation of HIV DNA, and Vpr enhances repair.

Our observation in primary $CD4^+$ T cells that Vpr and Vif limit uridine incorporation into host beta-actin DNA as well as HIV DNA suggests that HIV regulates uracilation of genomic DNA. Of note, a recent study in H9 cells also found an effect of Vpr on uracilation of genomic DNA, but reached the opposite conclusion that Vpr-mediated degradation of UNG2 cripples the ability of the cell to excise uracil from genomic DNA, resulting in increased uracilation of genomic DNA (Eldin et al., 2014). That study used a similar approach to the one used in Chapter 2 to digest uracilated DNA with recombinant uracil DNA glycosylase, but quantified DNA containing resulting apurinic sites by ELISA rather than quantitative PCR. Thus, cell type or assay-specific differences may account for apparent contradictions in Vpr's effect on genomic DNA uracilation. Future studies will be required to define conditions under which Vpr increases or decreases uracilation of genomic and HIV DNA, and to understand the full implications of uracilation for HIV infection.

Our hypothesis that Vpr recruits UNG2 to repair HIV DNA could be tested further by examining the effect of UNG2 silencing on the Vpr-induced DNA damage response, including phosphorylation of ATR, ATM and CHK2. Given that HIV DNA is highly uracilated even in cell lines (Yan et al., 2011), and Vpr has been demonstrated to cause ATR phosphorylation in HeLa cells (Zimmerman et al., 2006), such a silencing experiment could be performed in a cell line. In addition, Vpr-mediated cell cycle arrest has been proposed as a downstream consequence of SLX4 and ATR recruitment [**Figures 5.2A and B**, (Laguette et al., 2014)]. Observing the effect of UNG2 silencing on cell cycle arrest may

determine if cell cycle arrest results from aberrant DNA structures downstream of uracil repair, or from the direct recruitment of SLX4 by Vpr (**Figure 5.1B**).

In particular, it will be interesting to understand whether the pathway we describe in Chapter 2 leading from uracilation of HIV DNA to NKG2D ligand upregulation intersects with recently characterized pathways leading from HIV DNA sensing to interferon production(Laguette et al., 2014; Yan et al., 2010). In HeLa cells, it seems clear that Vpr activates SLX4 to process HIV DNA and avoid triggering the interferon response(Laguette et al., 2014). Also, non-specific activation of SLX4 has been proposed to cause cell cycle arrest [**Figures 5.2A and B,** (Laguette et al., 2014)]. Because DCAF1 is required for Vpr-mediated cell cycle arrest and suppression of interferon, it would be interesting to observe the effect of DCAF1 silencing on Vpr-mediated NKG2D ligand expression, activation of the DNA damage response pathway, and repair of uracilated HIV DNA. Mutants defective in DCAF1 binding examined in Chapter 3 could be used to explore the same question.

An interesting question that arises from the Laguette et al. study is why Vpr activates the SLX4 complex (SLX4com). One possibility is that cell cycle arrest is the goal, but two observations suggest that the SSE regulator initiates degradation of aberrant reverse transcription (RT) intermediates to avoid triggering an antiviral response (**Figures 5.2C and D**). First, Vpr mediates the association of SLX4 with HIV DNA, and second, silencing of SLX4 leads to accumulation of HIV DNA(Laguette et al., 2014). It is likely that certain RT intermediates resembling stalled replication forks can be substrates for SLX4com endonucleases such as MUS81-EME1, which nick genomic DNA near interstrand crosslinks [**Figures 5.2A and B**, (Cybulski and Howlett, 2011)]. One possible scenario is presented in **Figures 5.2C and D**, in which Vpr may recruit SLX4com to hairpin structures

resembling stalled replication forks in HIV (-) sense DNA to promote degradation of immunogenic HIV DNA. Widespread uracilation of HIV DNA (Yan et al., 2011) and uracil excision by UNG2 may lead to creation of abasic (AP) sites (**Figure 5.2C**). Reverse transcription stalls at AP sites(Cancio et al., 2004), which may not be repaired by host base excision repair (BER) in HIV single stranded DNA. Such RT intermediates may contain pathogen associated molecular patterns (PAMP) that upregulate type I interferon(Laguette et al., 2014). Therefore, Vpr-mediated recruitment of SLX4-associated endonucleases such as MUS81-EME1 may initiate degradation of these aberrant RT intermediates by host exonucleases such as TREX1 by exposing 3' ends of single-stranded HIV DNA (**Figure 5.2D**). Such a model would support the hypothesis that Vpr degrades UNG2 because the uracil DNA glycosylase promotes HIV DNA degradation and exposes the preintegration complex to immunodetection.

Another possibility is that unprocessed uracilated HIV DNA is the PAMP that triggers interferon production (**Figures 5.1B and 5.2B**). It would be interesting to observe the interferon response to HIV DNA after modulating uracil incorporation through treatment with canonical dNs or dU as in the Appendix. Also, because it has been shown that uricilated DNA transfected into HT29 cells is rapidly degraded and poorly expressed relative to non-uracilated DNA(Weil et al., 2013), it would be interesting to determine if transfection with uracilated DNA upregulates type I interferon more than non-uracilated DNA. Also, given that mutation of Vif leads to increased NKG2D ligand expression through increased APOBEC3G-mediated uracilation, it would be interesting to observe the effect of mutating Vif on interferon production. And finally, since UNG2 may initiate repair or recognition of uracilated HIV DNA, future studies might examine the effect of UNG2

silencing on interferon. These experiments would test the hypothesis that uracilated HIV DNA is the PAMP recognized by the host leading to the upregulation of interferon. Thus, we might tie effects of Vpr and Vif on uracilation of HIV DNA not only to NKG2D upregulation but also to interferon signaling.

Vpr enhances spread of HIV-1 in macrophages by increasing virion production

Vpr has been demonstrated to enhance HIV-1 replication in macrophages to a greater extent than in $CD4^+$ T cells (Heinzinger et al., 1994). Interestingly, in the primary macrophage model characterized in Chapter 3, 89.6 Vpr has a minimal effect on the first round of replication. Rather, Vpr increases viral particle production from infected cells, which enhances the infection rate at later time points.

The apparent lack of an effect of Vpr on the frequency of infected cells in the first round of replication is unexpected because the recruitment of Vpr into viral particles by Gag p6 (Kondo et al., 1995; Lavallee et al., 1994; Lu et al., 1995; Wang et al., 1994) suggests that Vpr performs an important early function. However, we do not rule out the possibility that Vpr may enhance the first round of replication in other cell culture models. Independent groups have continued to confirm that Vpr can enhance nuclear import of the preintegration complex in non-dividing macrophages under some conditions (Caly et al., 2008; Nitahara-Kasahara et al., 2007), although initial claims that Vpr is required for nuclear import in all non-dividing cells have been challenged (Yamashita and Emerman, 2005). Indeed, our observation in the Appendix that entry inhibitors amplify the effect of Vpr within the first 24h of infection suggests that we have also observed an effect of Vpr on the first round of replication, although the mechanism is yet to be discovered.

The experiments in Chapter 3 represent the first study combining flow cytometry and ELISA methods to demonstrate that Vpr enhances virion production per infected cell. Another study found that Vpr enhances virion production in single-round infection of dendritic cells (de Silva et al., 2012), but that study did not investigate whether Vpr affected virion production or the number of infected cells. Others have demonstrated in several reporter cell lines that Vpr increases HIV transcription by recruiting p300/CREB-binding protein (CBP) coactivators to the LTR (Kino et al., 2002; Subbramanian et al., 1998). However, in primary macrophages infected with HIV for 20 days, we found that 89.6 Vpr does not significantly affect the rate of Gag synthesis, suggesting that Vpr does not affect transcription of HIV mRNA in our assay. It is possible that differences in transcription factor expression between cell types account for published observations that Vpr enhances transcription from the LTR.

Finally, it would be interesting to understand how the ability of Vpr to enhance virion production varies among molecular clones. The observation that AD8 Vpr also enhances virion production (data not shown) suggests at least some conservation of this activity. NL4-3 Vpr, which differs from 89.6 Vpr at eight amino acids, has been used most commonly to demonstrate the effect of Vpr on nuclear import of the preintegration complex (Bukrinsky et al., 1992; Caly et al., 2008; Fletcher et al., 1996) and transcription from the LTR (Kino et al., 2002; Subbramanian et al., 1998). Indeed, Vpr genes from different lentiviruses are already known to have different properties. Most notably, HIV-1 Vpr genes cannot mediate degradation of SAMHD1, whereas SIV African green monkey (SIVagm) Vpr can (Lim et al., 2012). Further investigation is needed to understand

whether the selective pressures that account for Vpr conservation differ among lentiviruses, and whether Vpr represents a family of genes with overlapping activities.

Vpr increases Env levels in macrophages

Env is a necessary component of the HIV-1 particle because it mediates binding of virions to the CD4 receptor and coreceptors on permissive cells (Freed and Martin, 1995a). Env is also a cytotoxic factor that induces syncytia formation and apoptosis in infected cells (Ferri et al., 2000; Watkins et al., 1997). In Chapter 3, we found that Vpr enhances Env levels through a post-translational mechanism. While the molecular mechanism for this observation has not yet been characterized, we present several hypotheses below that arise from the data.

Future mechanistic studies might focus on whether the intracellular or extracellular regions of Env are targeted in the absence of Vpr. The cytoplasmic tail (CT) of gp41 is required for the incorporation of Env into viral particles in MDMs, but not in a number of cell lines (Murakami and Freed, 2000b). One possibility is that Vpr adds a posttranslational modification to the Env CT, preventing Env internalization from the plasma membrane and Env degradation. Indeed, in MDM infected by Vpr-null HIV, we recovered Env by treating cells with ammonium chloride. In future experiments, it would be interesting to examine the effect of Vpr on Env following deletion of the Env CT. However, these experiments may be technically challenging because Env CT mutants are unable to spread in primary MDM(Freed and Martin, 1995b, 1996; Murakami and Freed, 2000b) and we have only been able to observe effects of Vpr on Env during spreading infections.

Alternatively, Vpr may target the extracellular region of Env. We found that Vpr increases the steady-state level of intracellular Env in MDM, but not in 293T cells. One possible explanation for the observation that Vpr has a phenotype in macrophages may be that these cells process and traffic Env differently than other cell types. In MDM, the extracellular region of Env is glycosylated with N-acetyllactosamine repeats on complex chains, altering the interaction between glycosylated regions of Env and the carbohydrate recognition domains (CRD) of the C-type lectin receptor DC-SIGN (Lin et al., 2003). If C-type lectin receptors play a role in Env trafficking through their extracellular carbohydrate recognition domains, differences in Env glycosylation may be responsible for cell-type differences in susceptibility to trafficking by C-type lectin receptors. Additionally, in Chapter 4 we observed that Vpr downregulates the C-type lectin receptors DC-SIGN and hMR (**Figure 5.3A**). Thus, Vpr may facilitate Env trafficking by downregulating C-type lectin receptors, a process that may be most crucial in macrophages because of a unique Env glycosylation pattern (**Figures 5.1C and 5.3A and B**). C-type lectin receptors are also expressed specifically in macrophages and dendritic cells, raising the possibility that they are myeloid-specific restriction factors counteracted by Vpr. Indeed, it has been previously suggested that Vpr overcomes a restriction factor consitutively expressed in MDM (Malim and Emerman, 2008).

While MDM may express unique restriction factors constitutively, another possibility is that MDM upregulate unique restriction factors in response to HIV infection (**Figure 5.1C**). The observation that Vpr affects Env posttranslationally was unexpected because the efficient incorporation of Vpr into virions suggested a mechanisim preceding target cell expression of Vpr (Lu et al., 1995). The incorporation of Vpx into SIV particles,

for example, facilitates reverse transcription by downmodulating the triphosphohydrolase, SAMHD1 (Goldstone et al., 2011; Laguette et al., 2011; Lahouassa et al., 2012). One hypothesis is that Vpr's effect on an early process prevents the upregulation of a host restriction factor that acts late. We have demonstrated in Chapter 2, for example, that Vpr cooperates with Vif to limit uridine incorporation into HIV-1 DNA. Perhaps Vpr, by limiting the uridine detected by a host pattern recognition receptor, prevents the upregulation of a restriction factor that targets Env. Some evidence suggests that Vpr suppresses the upregulation of type I interferon in HeLa cells and primary dendritic cells treated with HIV-1 (Harman et al., 2011; Laguette et al., 2014). Therefore, Vpr may prevent the upregulation of an interferon-stimulated gene that targets Env (**Figures 5.1C and 5.3B**).

Additionally, several possible mechanisms exist in which Vpr may enhance Env levels independently of any restriction factors. For example, perhaps the direct interaction of Vpr with Gag affects the efficiency of Env incorporation and viral particle release. Vpr is packaged into viral particles by association with the p6 region of Gag (Lu et al., 1995). Immunoelectron microscopy indicates that Vpr localizes to the plasma membrane just beneath Env, and the proximity of p6 to the plasma membrane has led to speculation that p6 is a core-envelope linker (Gelderblom et al., 1987; Wang et al., 1994). In the absence of Gag, Env is rapidly internalized from the cell surface, presumably to reduce immunodetection of infected cells (Checkley et al., 2011; Egan et al., 1996). Thus, it would not be surprising if Vpr were to influence Env internalization and degradation through an interaction with p6. Vpr enhancement of Env levels may in fact result from mislocalization of Gag because Env is rapidly endocytosed in the absence of Gag (Egan et al., 1996).

It is interesting to note that the effect of Vpr on Env incorporation into viral particles is less dramatic than the effect of Vpr on intracellular Env. If Env were passively incorporated into viral particles, it would be expected that the Env incorporated onto particles would be proportional to intracellular Env. While Env is passively incorporated into viral particles in 293T cells and HeLa cells, it was shown that in primary MDMs, Env is actively recruited to sites of viral particle assembly through a mechanism requiring the cytoplasmic tail of gp41 (Checkley et al., 2011; Murakami and Freed, 2000b). Although MDMs infected with 89.6*vpr*$^{-}$ contain less Env, it is likely that budding viral particles acquire a sufficient quantity of Env by active recruitment to maintain infectivity in our assays. It is also possible that the three-fold effect of Vpr on Env incorporation into viral particles has a more dramatic effect on infectivity *in vivo*, under conditions in which Env can be neutralized by antibodies (Hausmann et al., 1987; Liao et al., 2013). Additionally, given that the non-covalent association between gp120 and gp41 is known to be labile (Checkley et al., 2011), an excess of Env could protect free virus circulating *in vivo* from a complete loss of Env over time.

Another possibility is that other accessory proteins promote Env incorporation onto virions in the absence of Vpr. An interesting study found that Nef also increases virion incorporation of Env (Schiavoni et al., 2004). Although the mechanism for this observation is not known, we hypothesize that Nef promotes Env trafficking by downmodulating cell surface proteins known to interact with the extracellular region of Env, including CD4(Chen et al., 1996) and hMR(Vigerust et al., 2005). Thus, Nef may complement lower total Env expression in the absence of Vpr by enhancing trafficking of Env to sites of virion assembly. Indeed, our preliminary data suggests that Env incorporation is undetectable on virions

collected from MDM infected by HIV lacking both *nef* and *vpr* (data not shown). Therefore, in future experiments, it would be interesting to determine if *vpr* and *nef*-null virus collected from primary MDM is non-infectious. Future investigation will be required to understand the full mechanism and physiologic significance of Vpr's effect on Env.

Env is required for Vpr to maximally enhance virus production

In Chapter 3, we reported that Env is required for Vpr to maximally enhance virion production. This finding is surprising because it has been demonstrated that Env is not required for the release of viral particles in multiple cell lines and in primary macrophages (Murakami and Freed, 2000b). In fact, Gag expression is sufficient for the production of virus-like particles (VLPs, (Freed, 1998). However, the effect of Env on viral particle assembly and release had not yet been studied in a quantitative assay. We have demonstrated by ELISA that Env has a quantitative effect on Vpr-mediated virion production.

One possibility is that Env directly facilitates viral particle release in macrophages. Env co-localizes with Gag at sites of viral particle assembly in a cell-type dependent manner (Murakami and Freed, 2000b). However, existing evidence does not support a direct role for Env in viral particle release because Env-null mutants are able to form virus-like particles (Murakami and Freed, 2000a). Chimeric virus containing an NL4-3 backbone and an *env* gene from AD8 (NL-AD8) does not demonstrate an Env dependency in a semi-quantitative virus release assay (Murakami and Freed, 2000b). One possibility is that Gag and Env from the same isolate co-evolve to interact most efficiently, and that AD8 Env is not compatible with NL4-3 Gag. Prior to this study, the effect of 89.6 Env on the release of

particles formed by 89.6 Gag had not been tested. Furthermore, we examined the role of Env in particle release at a relatively low multiplicity of infection (MOI). Gag assembly and budding may not depend on Env at high MOI used in previous studies. Therefore, the hypothesis that 89.6 Env directly facilitates viral particle release has not been examined experimentally until now.

While the role of Env in virion production is surprising, HIV has been known to encode proteins that facilitate virion release. HIV-1 Vpu counteracts tetherin (BST-2), a host restriction factor that inhibits the release of viral particles from the plasma membrane (Neil et al., 2008). HIV-2 Env localizes tetherin to a perinuclear compartment and also enhances virion release (Hauser et al., 2010). Although most SIVs counteract tetherin with Nef, the *env* gene of a *nef* deleted SIV acquired a compensatory mutation enhancing anti-tetherin activity in rhesus macaques (Serra-Moreno et al., 2011). In future studies, to determine if Vpr promotes virion release through tetherin, it would be interesting to visualize tetherin localization and expression in the presence or absence of 89.6 Vpr or 89.6 Env.

Like tetherin, C-type lectin receptors also interact with HIV Env and have the potential to restrict HIV release (**Figures 5.1C and 5.3D**). Also, as described in Chapter 4, Vpr downregulates the C-type lectin receptors hMR and DC-SIGN (**Figure 5.3A**). Interestingly, these C-type lectin receptors are considered pattern recognition receptors because they recognize non-self carbohydrate residues on pathogenic glycoproteins, leading to phagocytosis of pathogens such as *Mycobacterium tuberculosis* and *Candida albicans* (Ezekowitz et al., 1991). Therefore, C-type lectin receptors might mediate phagocytosis of virions by macrophages in addition to inhibiting release (**Figure 5.3E**). To

determine if Vpr enhances virion production by downmodulating C-type lectin receptors, it would be interesting to measure virus in the supernatant of infected MDM after neutralizing these lectins with D-mannose. Of note, it was shown that HIV Nef also downmodulates hMR, suggesting that HIV encodes redundant or complimentary activities against hMR (Vigerust et al., 2005). Further experiments are needed to test the hypothesis that Vpr enhances virion release by downmodulating surface receptors (**Figures 5.1C** and **5.3D**).

It is interesting to observe donor variability in the requirement of Env for Vpr to affect virus production per infected cell. Differences in expression of tetherin or C-type lectin receptors may account for this variablility. Alternatively, if an MDM surface receptor restricts viral particle release in macropahges, it is possible that Vpr and Env both affect the receptor through independent mechanisms. For example, Vpr may prevent the interferon-mediated transcriptional upregulation of the inhibitory receptor. Because Vpr also enhances Env levels, high Env levels may downmodulate the receptor from the plasma membrane. Such a model would explain the persistence of Vpr-mediated viral particle release in the absence of Env in some donors.

Another possibility is that Env enhances virion production by favoring syncytia formation. Env expression on the surface of infected cells induces the formation of multinucleated syncytia by interacting with CD4 on uninfected cells. In Chapter 3, we noted that fewer cells were observed in the presence of Vpr. One explanation for that observation might be that the increased Env observed in infected MDM leads to increased syncytia formation. Viruses that replicate in the nucleus such as herpes simplex virus benefit from syncytia formation by gaining additional replication sites while avoiding host

immunodetection of cell-free virus (Ogle et al., 2005; Sattentau, 2008). In future studies, it would be interesting to determine if Vpr enhances syncytia formation. To determine if cell fusion is sufficient to enhance virion production, heterokaryons between infected and uninfected cells might be induced with polyethylene glycol (PEG). Thus, cell fusion might enhance virion production by increasing cellular substrates for virion production that might be low in non-dividing MDM.

In conclusion, our mutant analysis clearly demonstrates *env* is an important factor in *vpr*-mediated viral particle release. Because Vpr's effect on viral particle production was not completely reversed in one donor, it is possible that in a subset of donors, Vpr acts on viral particle release independently of Env. The release of HIV-1 particles may be regulated by several host factors, including tetherin, CD4 and C-type lectin receptors (Chu et al., 2012; Geijtenbeek et al., 2000), any of which could be manipulated by Vpr. Therefore, future experiments will be required to understand the mechanism for this surprising result.

Future directions

Vpr enhancement of spread from macropahges to CD4$^+$ T cells

We have linked Vpr's effect on Env to virion production, but Env is required for cell-to-cell infection as well (Ono, 2010; Waki and Freed, 2010). Therefore, the possibility arises that Vpr facilitates the spread of HIV-1 between MDM or from MDM to CD4$^+$ T lymphocytes. An MDM-T cell co-culture system has not, to our knowledge, been used to study Vpr previously. Such experiments would provide novel insight into the effect of Vpr on the interaction between the two major targets of HIV-1.

Indeed, it is possible that the effect we observed in Chapter 3 of Vpr on spread within pure populations of MDM results from enhanced cell-to-cell spread instead of cell-free infection. A transwell assay (Carr et al., 1999) would determine if direct contact between cells is necessary for Vpr-mediated infectivity. Another possibility is that Vpr induces the secretion of a soluble factor that increases permissivity, as has been described previously for Nef (Swingler et al., 2003). These observations may help explain the association between *vpr* and disease progression *in vivo* (Lang et al., 1993; Somasundaran et al., 2002), despite the absence of phenotypes in many cell types *in vitro* (de Silva et al., 2012).

In future studies, it will be interesting to test the hypothesis that the observed *vpr*-dependent Env phenotype will lead to a *vpr*-dependent cell-to-cell spread phenotype. A recent study found that domains in matrix that are necessary for Env incorporation into viral particles are also necessary for cell-to-cell spread, suggesting that the localization of Env to sites of viral particle assembly correlates with an intact cell-to-cell spread mechanism (Monel et al., 2012). Emerging data suggests that cell-to-cell spread may be resistant to antiretroviral drugs and neutralizing antibodies because in this mode of infection, large aggregations of viral particles enter the target cell through an HIV-1 Env-mediated virological synapse (Abela et al., 2012; Sigal et al., 2011). If cell-to-cell infection is the predominant mode of infection in patients receiving antiretroviral drugs, Vpr may be a significant virulence factor *in vivo*. Indeed, we demonstrated in Chapter 4 that Vpr enhances infection of MDM most dramatically following treatment with drugs blocking HIV entry. Therefore, the effect of Vpr on Env may enhance cell-to-cell spread under conditions relevant to HIV infected people.

The effect of Vpr on UNG2 and DCAF1 levels in HIV-infected macrophages

Vpr is a pleiotropic protein that mediates the degradation of UNG2 via the DCAF1-DDB1-CUL4A E3 ubiquitin ligase. The association of Vpr with a host protein involved in DNA repair implies that Vpr has a function during stages of replication exposing HIV cDNA to uracilation. In this section, I discuss preliminary data presented in the Appendix, and future experiments that may establish a role for Vpr in DNA repair.

Macrophages employ unique antiviral mechanisms such as an elevated dUTP/dTTP ratio and high A3A and A3B expression compared to $CD4^+$ T cells (Kennedy et al., 2011; Peng et al., 2007). Because these conditions have been demonstrated to increase uracilation of HIV DNA, repair of deoxyuridine may be a prerequisite to HIV replication in this cell type. Emerging data suggests that UNG2 permits replication of HIV in macrophages by repairing uracilated HIV cDNA (Norman et al., 2011; Priet et al., 2005; Priet et al., 2003b). However, studies in several cell lines show that Vpr protects uracilated HIV DNA from degradation by downmodulating UNG2(Weil et al., 2013), and experiments presented in the Appendix confirm UNG2 degradation by Vpr. Future experiments may address the apparent contradiction between proviral and antiviral roles for Vpr.

Because UNG2 is necessary for virion infectivity in macrophages(Priet et al., 2005), one possibility is that UNG2 is protected from degradation in specific cell types such as macrophages. Preliminary experiments in the Appendix suggested that Vpr did not facilitate UNG2 degradation as efficiently in MDM as in 293T cells. While differences in Vpr expression levels may account for this discrepancy, one hypothesis is that Vpr does not completely degrade UNG2 in MDM because the host factor repairs uracilated HIV DNA.

Given that DCAF1 is required for proteasome-mediated degradation of UNG2 (Wen et al., 2012), one possibility is that UNG2 is protected from degradation in HIV-infected MDM because DCAF1 levels are low compared to other cell types. Indeed, we observed that DCAF1 is low in MDM compared to 293T cells, and that Vpr further mediates proteasomal degradation of DCAF1 in HIV-infected MDM. Therefore, the observation that UNG2 is not completely degraded in HIV-infected MDM is consistent with a model in which HIV requires UNG2 for replication in macrophages.

To test the hypothesis that low DCAF1 levels impair degradation of UNG2 by Vpr in MDM, it would be interesting to determine if overexpression of DCAF1 in MDM enhances the ability of Vpr to mediate UNG2 degradation. DCAF1 is also required in HeLa cells to suppress the basal type I interferon response(Laguette et al., 2014). Because DCAF1 is a Vpr cofactor and suppresses the interferon pathway, overexpression of DCAF1 may even increase the permissivity of MDM to HIV, or may amplify the effect of Vpr on infection of MDM.

Like myeloid cells, quiescent CD4+ T cells are also relatively resistant to infection by HIV for reasons that are not fully understood (Baldauf et al., 2012; Wu, 2012; Yan and Lieberman, 2012). Given our results, it would be interesting to determine whether DCAF1 expression is also low in quiescent $CD4^+$ T cells relative to activated cells, preventing Vpr-mediated UNG2 degradation in the majority of circulating T cells that encounter virus. Some evidence suggests that quiescent $CD4^+$ T cells exposed to HIV-1 undergo pyroptosis, an innate immune response to incomplete reverse transcripts causing a clinical decline in $CD4^+$ T cell numbers (Doitsh et al., 2010). UNG2 may play a significant role in the detection of incomplete HIV DNA containing dU and may be required for the induction of pyroptosis.

Therefore, Vpr may promote resting $CD4^+$ T cell death by facilitating UNG2-mediated immunodetection of HIV cDNA.

Finally, it is possible that Vpr-mediated DCAF1 degradation is a byproduct of Vpr's effect on its primary target. The expression of several substrate adaptors is tightly regulated by substrate expression (Cardozo and Pagano, 2004). UNG2 is a known substrate of the DCAF1-DDB1-CUL4A E3 ligase complex, but we found that UNG2 silencing had no effect on DCAF1 levels. However, UNG2 may not be the only target of this E3 complex. DCAF1 may be downmodulated in a negative feedback mechanism following the Vpr-mediated degradation of more than one substrate. Thus, downmodulation of DCAF1 may reflect efficient degradation of several putative targets in macrophages.

Conclusion

Vpr is universally conserved among lentiviruses, suggesting it confers a significant replication advantage *in vivo*. In cell lines, primary $CD4^+$ T cells and primary MDM, however, Vpr expression has variable consequences (**Figure 5.1A-C**). In this chapter, we have discussed demonstrated or hypothetical overlap between these cell culture models around a central mechanism in which Vpr may limit the immunodetection or degradation of uracilated HIV DNA. Such a model may begin to explain the wide-ranging historical observations connected to Vpr.

As the question marks in **Figure 5.1** suggest, significant work remains to trace the pleiotropic effects of Vpr back to uracilation of HIV DNA. In our current model, it is confusing and possibly incorrect that Vpr appears to affect HIV replication at three seemingly independent steps (**Figure 5.1**). Future studies may find, for example, that Vpr

activates the SLX4 complex by recruiting UNG2 to repair uracilated HIV DNA, leading to a downmoduation of C-type lectin receptors by inhibiting the interferon pathway. However, several accessory proteins have pleiotropic effects by targeting separate host factors. For example, Nef downmodulates MHC-I (Collins et al., 1998), CD4 (Chen et al., 1996), CD8 (Leonard et al., 2011) and hMR (Vigerust et al., 2005). Further study of the molecular mechanism through which Vpr enhances HIV infection may lead to novel therapies to enhance the immune response to HIV infection.

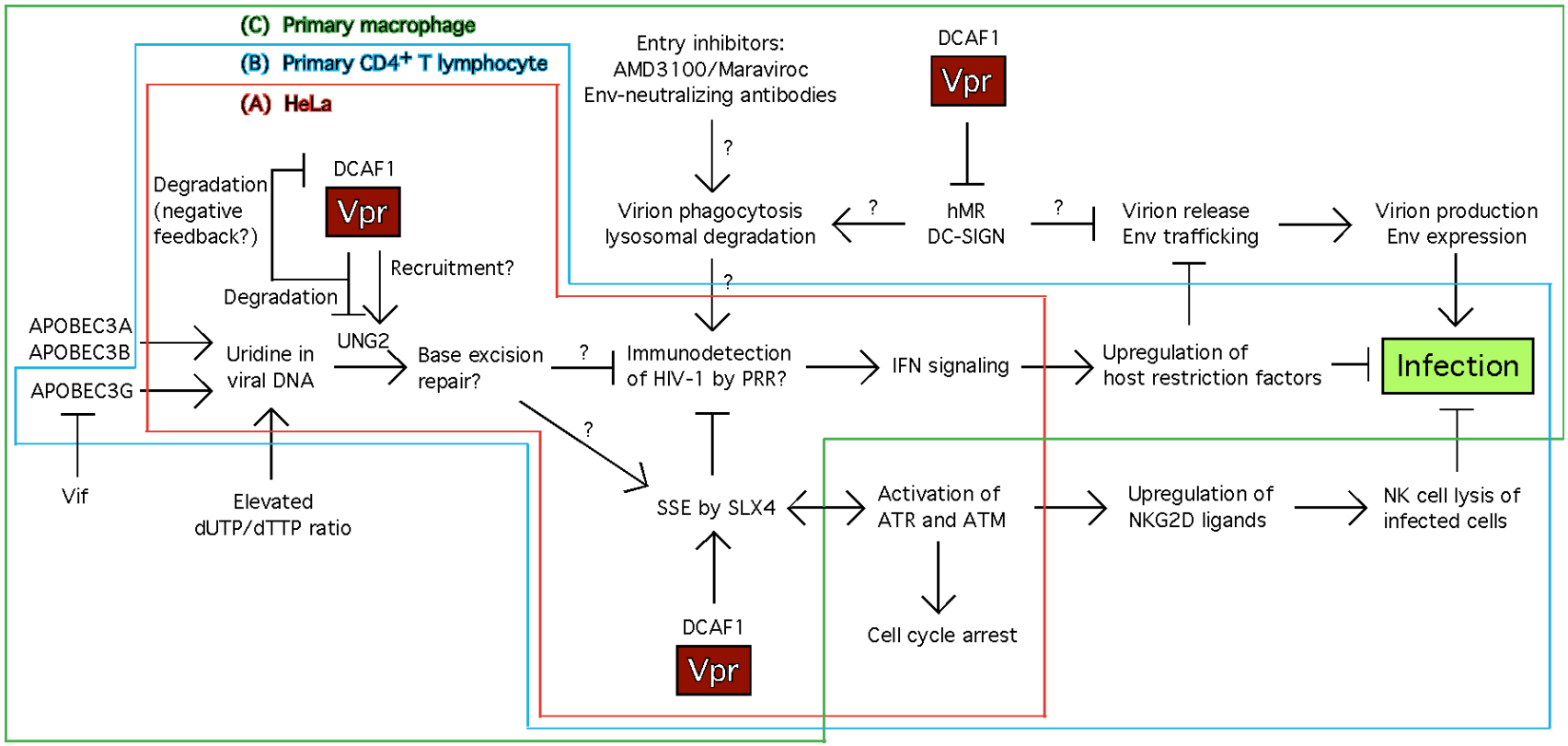
(C) Primary macrophage
(B) Primary CD4+ T lymphocyte
(A) HeLa
Entry inhibitors:
AMD3100/Maraviroc
Env-neutralizing antibodies
DCAF1
Vpr
?
Virion phagocytosis
lysosomal degradation
?
hMR
DC-SIGN
?
Virion release
Env trafficking
Virion production
Env expression
Degradation
(negative
feedback?)
DCAF1
Vpr
Recruitment?
Degradation
APOBEC3A
APOBEC3B
APOBEC3G
Uridine in
viral DNA
UNG2
Base excision
repair?
?
Immunodetection
of HIV-1 by PRR?
IFN signaling
Upregulation of
host restriction factors
Infection
?
Vif
Elevated
dUTP/dTTP ratio
SSE by SLX4
Activation of
ATR and ATM
Upregulation of
NKG2D ligands
NK cell lysis of
infected cells
Cell cycle arrest
DCAF1
Vpr

Figure 5.1. Proposed model for cell type-specific Vpr phenotypes. Arrows indicate activation and horizontal lines indicate inhibition. **(A)** In HeLa cells, Vpr binds to UNG2 and may recruit the uracil DNA glycosylase to repair uracilated HIV DNA. Vpr also degrades UNG2, which may reflect negative feedback following UNG2 utilization, or targeting of UNG2 as an antiviral factor. The interaction between Vpr and UNG2 may lead to base excision repair of HIV DNA, possibly avoiding immunodetection. ATR and ATM may be activated by repair of uracilated DNA, resulting in cell cycle arrest **(B)** Primary $CD4^+$ T lymphocytes express high levels of APOBEC3G, which increases uracilation of HIV DNA. Because primary T cells express low basal levels of NKG2D ligands, activation of ATM and ATR triggers the upregulation of NKG2D ligands, increasing the susceptibility of infected T cells to NK-cell lysis. **(C)** Primary macrophages contain an elevated dUTP/dTTP ratio and express APOBEC3A and APOBEC3B, conditions favoring uracilation of HIV DNA. MDM do not express ATR, do not divide, and so do not experience cell cycle arrest and may not upregulate NKG2D ligands like T cells. However, MDM are sensitive to interferon signaling and may upregulate the expression of a host restriction factor(s) that inhibit virion release and Env trafficking. Also, MDM express C-type lectin receptors that are downregulated by Vpr. C-type lectin receptors may also interfere with virion release or Env trafficking, and may enhance phagocytosis of virions, triggering an antiviral response.[1]

[1] This figure was produced by Michael Mashiba.

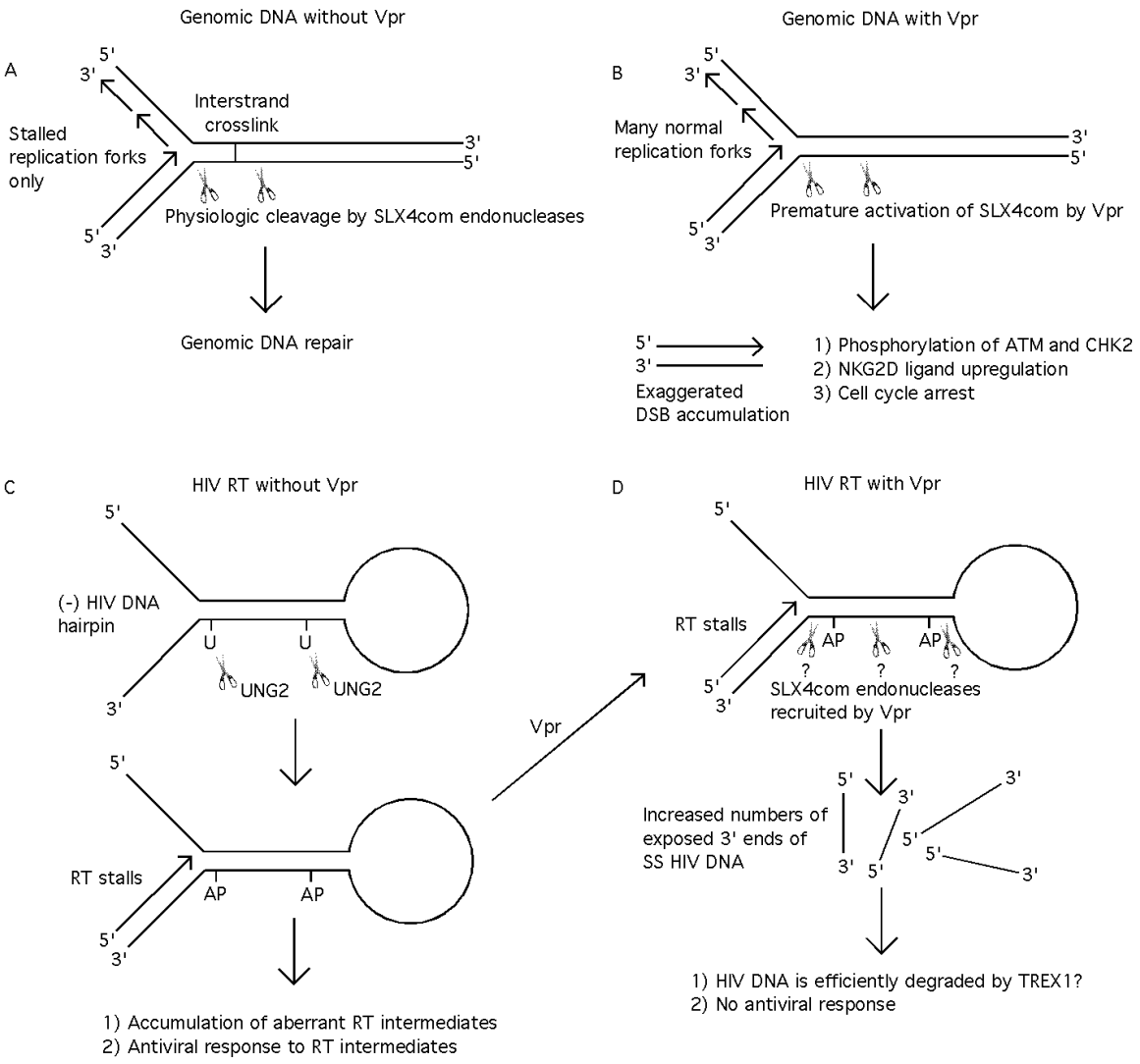

Figure 5.2. Proposed implications of Vpr activation of the SLX4 complex for genomic DNA synthesis and HIV reverse transcription (RT). **(A)** In the absence of Vpr, SLX4 complex (SLX4com) endonucleases such as MUS81-EME1 initiate repair of genomic DNA at stalled replication forks. Cleavage of 3' flaps is limited to specific DNA lesions such as interstrand crosslinks. **(B)** Vpr activates SLX4-associated endonucleases prematurely, which may result in non-specific nicking of normal replication forks, hypothetically generating massive amounts of double-stranded breaks (DSB) in genomic DNA. DSB result in ATM and CHK2 phosphorylation, possibly leading to NKG2D ligand upregulation and cell cycle arrest. **(C)** Several regions of negative (-) sense single stranded (SS) HIV DNA are palindromic and form hairpin structures (Cosa et al., 2004) that may resemble replication forks depicted in **A** and **B**. HIV DNA is uracilated by APOBEC3G on the (-) strand (Yu et al., 2004) or by dUTP misincorporation by RT(Kennedy et al., 2011). UNG2 excises uracil, generating abasic (AP) sites. RT stalls at the abasic site(Cancio et al., 2004). Canonical BER enzymes such as APE-1 may not efficiently excise AP sites from single-stranded HIV DNA. In the absence of Vpr,

the host may upregulate type I interferon in response to accumulating RT intermediates. **(D)** SLX4 endonucleases activated by Vpr such as MUS81-EME1 may nick HIV DNA around AP sites, which resemble stalled replication forks. Nicking increases the number of 3' ends of SS DNA, which are the preferred substrate for the TREX1 3'-5' exonuclease (Yang et al., 2007). Accumulated RT intermediates are degraded, avoiding immunodetection. [2]

[2] This figure was produced by Michael Mashiba.

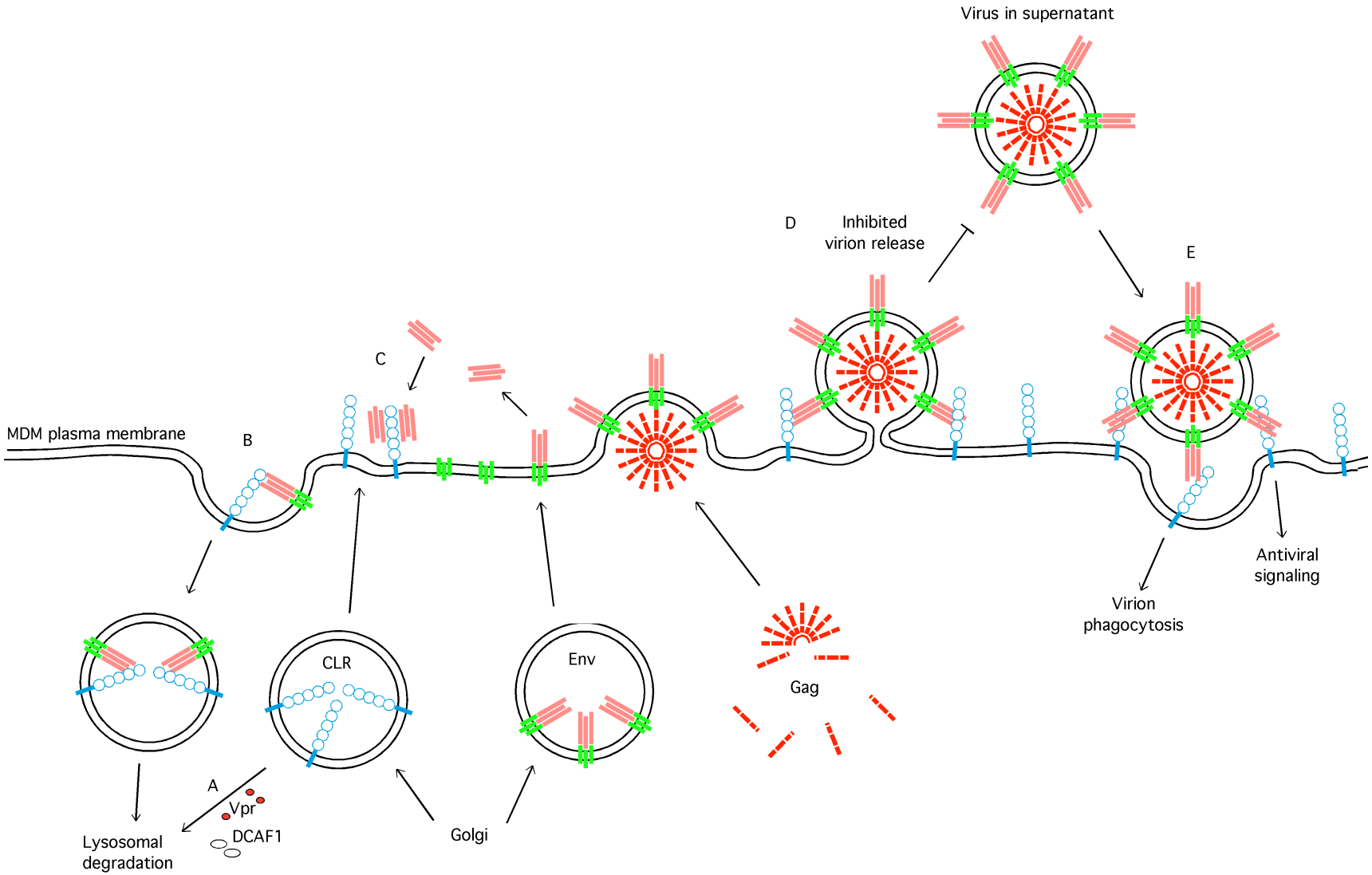
Virus in supernatant
D
Inhibited
virion release
E
C
MDM plasma membrane
B
CLR
Env
Gag
Virion
phagocytosis
Antiviral
signaling
A
Vpr
DCAF1
Lysosomal
degradation
Golgi

Figure 5.3. Proposed model for Vpr, Env and C-type lectin receptors (CLR) interactions. **(A)** Vpr downregulates CLRs such as hMR and DC-SIGN, possibly by trafficking them to the lysosome. **(B)** At the plasma membrane, the carbohydrate recognition domains (CRD) of CLRs bind glycosylated regions of Env, possibly mediating Env endocytosis and lysosomal degradation. **(C)** Env gp120 shed from the cell surface may further neutralize and downregulate CLRs. **(D)** CLRs might inhibit virion release by tethering particles coated with Env to the cell surface. **(E)** CLRs may mediate phagocytosis and degradation of virus in the supernatant of infected cells. CLRs may also initiate antiviral signaling pathways in response to virus in the supernatant or Env expressed at the cell surface. [3]

[3] This figure was produced by Michael Mashiba.

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Appendix:

Vpr may protect uracilated HIV-1 DNA in macrophages

Introduction

$CD4^+$ T cells and macrophages are the major targets of HIV *in vivo*. Macrophages express a number of unique antiviral factors, including the host cytidine deaminases APOBEC3A (A3A) (Berger et al., 2011; Koning et al., 2011) and APOBEC3B (A3B) (Peng et al., 2007), which deaminate deoxycytidine to deoxyuridine in HIV DNA. A3A has been shown to hypermutate HIV DNA (Koning et al., 2011) and restrict HIV infection in the target cell (Berger et al., 2011), but it is unknown whether A3B inhibits lentiviral infection. In addition, macrophages contain a low intracellular concentration of deoxunucleotide triphosphate (dNTP) substrates for reverse transcriptase (RT) (Diamond et al., 2004; Gavegnano et al., 2012; Kennedy et al., 2011), delaying reverse transcription relative to $CD4^+$ T cells (Spivak et al., 2011). Macrophages also contain a high intracellular concentration of deoxyuridine triphosphates (dUTP) relative to $CD4^+$ T cells, and studies suggest that RT misincorporates dUTP into HIV-1 cDNA (Norman et al., 2011; Priet et al., 2005; Yan et al., 2011b). Thus, replication in macrophages favors increased uracilation of HIV-1 DNA.

The host factor SAMHD1 restricts lentiviral replication in myeloid cells by depleting the intracellular pool of dNTPs (Lahouassa et al., 2012). However, the SIV Vpx protein enables reverse transcription by mediating degradation of SAMHD1 (Laguette and Benkirane, 2012; Laguette et al., 2012; Laguette et al., 2011). Replication of SIV Vpx-null virus in macrophages can be rescued by addition of exogenous doxynucleosides (dNs), which increase the intracellular dNTP concentration through the salvage pathway of dNTP synthesis (Lahouassa et al., 2012). Remarkably, HIV-1 infects macrophages, but does not encode a Vpx protein or counteract SAMHD1 (Lim et al., 2012).

Instead, HIV-1 encodes Vpr, a homologous, 14kDa accessory protein that does not facilitate SAMHD1 degradation. The possibility that Vpr enhances reverse transcription through a SAMHD1-independent mechanism has not yet been explored. However, exogenous dNs or Vpx enhance HIV-1 infection of macrophages in the presence of Vpr, suggesting that Vpr does not enhance intracellular dNTP pools as efficiently as Vpx (Lahouassa et al., 2012). Although the function of Vpr is unknown, several studies associate Vpr with the DNA damage response pathway. For example, Vpr upregulates the ATM and Rad3 related protein (ATR) (Roshal et al., 2003). ATR upregulation is a marker for the effect of Vpr on DNA repair, but is not required for HIV replication in cell lines (Zimmerman et al., 2006). Interestingly, macrophages do not express ATR or upregulate ATR during HIV-1 infection(Zimmerman et al., 2006), but Vpr enhances HIV replication most dramatically in this cell type(Chen et al., 2004b; Eckstein et al., 2001; Heinzinger et al., 1994; Subbramanian et al., 1998; Vodicka et al., 1998). Additionally, it was recently shown that Vpr suppresses the interferon response to HIV infection by recruiting the structure-specific endonuclease (SSE) regulator SLX4 complex to process unintegrated HIV DNA.

While the mechanism through which Vpr enhances macrophage infection is not known, it has been proposed to mediate degradation of a host restriction factor through the DCAF1-DDB1-Cul4A E3 ubiquitin ligase(Lim et al., 2012).

Several studies performed in 293T and HeLa cells indicate that Vpr mediates polyubiquitination and proteasomal degradation of UNG2, a host uracil DNA glycosylase that initiates the base excision repair (BER) pathway to repair uracilated DNA (Ahn et al., 2010; Wen et al., 2012). The substrate adaptor DCAF1 is required for UNG2 degradation via this mechanism(Ahn et al., 2010; Wen et al., 2012). One study proposed that UNG2 inhibits infection by initiating degradation of uracilated HIV DNA (Weil et al., 2013). While these data might suggest that UNG2 is degraded by Vpr because it counteracts HIV infection, silencing of UNG2 results in the production of non-infectious virions (Priet et al., 2005). Furthermore, Vpr associates with UNG2 and recruits UNG2 into viral particles (Chen et al., 2004a). Therefore, UNG2 potentially has proviral and antiviral activities.

This study begins to explore the effects of Vpr on HIV infection and HIV DNA uracilation through UNG2. Preliminary data confirms that Vpr mediates UNG2 degradation in 293T cells. Interestingly, we found that Vpr also enhances UNG2 degradation in primary MDM, possibly reflecting the ability of Vpr to address high levels of HIV DNA uracilation in this cell type. In addition, we have made the novel observation that Vpr mediates degradation of DCAF1, although the implications of this activity are unknown. We have also developed two assays to explore the hypothesis that Vpr counteracts downstream consequences of dUTP incorporation by RT, and the cytidine deaminase activity of A3B, respectively. Therefore, these preliminary experiments begin to link Vpr to uracilation of HIV DNA.

Results

Deoxyuridine inhibits infection of MDM

We first aimed to confirm that uracilation of HIV DNA inhibits infection. It has been proposed that uracilated HIV DNA can be detected by host pattern recognition receptors, activating the innate immune response (Sire et al., 2008). dUTP is a non-canonical nucleotide that can be incorporated into HIV-1 cDNA during reverse transcription instead of dTTP (Weil et al., 2013). It has been demonstrated that macrophages have an elevated intracellular dUTP/dTTP ratio relative to T lymphocytes (Diamond et al., 2004; Gavegnano et al., 2012; Kennedy et al., 2011) and that addition of exogenous deoxyuridine (dU) can restrict HIV-1 replication (Kennedy et al., 2011). To confirm this finding, we treated MDM with dU, infected them with 89.6 and measured the infection rate by flow cytometry at four days post infection (dpi). As expected, dU treatment decreased the infection rate in MDM two to three fold compared to cells treated with water alone (**Figure A1 compare second and fourth panels**). These results suggest that dU treatment increases uracil incorporation into HIV DNA, which inhibits infection.

To rule out the possibility that non-specific dU toxicity inhibited infection in our system, we attempted to rescue infection of dU-treated MDM by addition of exogenous canonical deoxynuleosides (dNs). dNs, which include deoxyadenosine (dA), deoxythymidine (dT), deoxyguanosine (dG), and deoxycytidine (dC) increase the intracellular deoxynucleoside triphosphate (dNTP) concentration through the salvage pathway of dNTP synthesis (Lahouassa et al., 2012). We hypothesized that dN treatment could rescue the negative effect of dU on HIV replication by lowering the intracellular dUTP/dTTP ratio. Indeed, we found that MDM treated with both dU and dN were infected

at a level comparable to dN treatment alone (**Figure A1, third, fourth and fifth panels**). These data are consistent with a model in which dN treatment enhances infection of MDM by decreasing uracil incorporation into HIV DNA by RT, likely by lowering the dUTP/dTTP ratio.

The effect of Vpr on virion production and spread in macrophages is dependent on a high dUTP/dTTP ratio

Because SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase that inhibits lentiviral reverse transcription by restricting the intracellular canonical dNTP pool, infection by lentiviruses lacking *vpx* can be rescued with exogenous dN treatment (Goldstone et al., 2011; Lahouassa et al., 2012). The accessory protein Vpr, which is homologous to Vpx, does not degrade SAMHD1 (Lim et al., 2012), but it is not known whether dN treatment can complement an HIV-1 *vpr* mutant through an alternative mechanism. To test this hypothesis, we treated MDMs with dNs, infected them with wildtype HIV-1 89.6 or 89.6*vpr*$^-$, and measured the virus produced per infected cell by flow cytometry and ELISA at four dpi. To limit toxicity, medium containing exogenous dNs was replaced with fresh medium at 20 hours post infection (hpi), and viral spread beyond this time point was blocked by addition of the HIV entry inhibitors, AMD3100 and Maraviroc. As expected, without dN treatment, mutation of *vpr* decreased virus production per infected cell by greater than two fold (**Figure A2A**). Interestingly, dN treatment decreased the magnitude of this Vpr-mediated virus production phenotype from two fold to less than 1.5 fold (**Figures A2A and A2B**). These results suggest that the mechanism through which Vpr enhances virion production depends on the intracellular concentration of dNTPs.

Because it was shown previously that the effect of Vpr on virus production enhances spread of HIV-1 in macrophage cultures (**Chapter 3, Figures 3.2B-D**), we hypothesized that the effect of Vpr on spread would also depend on the cellular dNTP pool. To assess the effect of dN treatment on *vpr*-mediated spread of HIV-1 among macrophages, we treated MDMs with dNs, infected them with 89.6 or 89.6*vpr*$^-$, and measured the frequency of infected cells by flow cytometry at four days post infection. As expected, in the absence of dN treatment, mutation of *vpr* decreased the infection rate more than two fold on average, and as high as three fold in two donors (**Figures A2C-E**). Interestingly, dN treatment decreased the effect of *vpr* on infection of MDM from three fold to two fold at 2.5mM and 5mM concentrations (**Figure A2C**). Over multiple donors, dN treatment decreased the effect of Vpr on the infection rate from two fold to less than 1.5 fold (**Figure A2E**). These data suggest that the effect of *vpr* on spread of HIV-1 in MDM depends on the intracellular dNTP concentration.

Having discovered that raising the intracellular dTTP/dUTP ratio with dN treatment diminished the *vpr* dependency of HIV virion production and spread in MDM, we asked whether lowering that ratio with dU treatment would increase the effect of *vpr* on those phenotypes. dU treatment decreased the spread of both wild type 89.6 and 89.6*vpr*$^-$ HIV in MDMs (**Figure A2D**). The magnitude of the Vpr phenotype was slightly decreased in the presence of dU, although this difference was not statistically significant (**Figure A2E**). Thus, these results are consistent with the hypothesis that Vpr enhances HIV replication within the range of intracellular dUTP/dTTP found in macrophages.

A3B promotes Rev-inependent Gag expression in vitro

While uracilation of HIV DNA can result from dUTP incorporation during reverse transcription, host APOBEC proteins can also introduce uracil into HIV DNA through cytidine deamination(Malim, 2009). Primary macrophages express high levels of A3A (Berger et al., 2011) and A3B (Peng et al., 2007). To determine if A3B is sufficient to inhibit HIV gene expression, we developed a unique assay that examines a possible effect of A3B on HIV DNA transfected into 293T cells. The HIV Rev protein is necessary for the nuclear export of incompletely spliced viral transcripts containing inhibitory introns(Blissenbach et al., 2010). In our assay, we utilized a Rev reporter construct, pRRE (Dull et al., 1998), that contains such introns and expresses Gag protein only in the presence of Rev (**Figure A3A**). 293T cells were co-transfected with pRRE and either an A3B expressing plasmid (pA3B-HA) or a pβarrestin-HA negative control (**Figure A3B**). As expected, in the absence of Rev, Gag p24 expression was very low by western blot and flow cytometry (**Figure A3C and D**). Suprisingly, however, A3B expression induced significant Gag expression from pRRE in the absence of Rev (**Figure A3C and D**). This result was unexpected because A3B has no known role in nuclear export of unspliced RNA. We hypothesized that A3B deaminase activity may have mutated intronic regions within the pRRE plasmid, promoting the stability and nuclear export of Gag-expressing RNA.

A3B, like A3F and A3G, contains two cytidine deaminase (CDA) domains that can convert deoxycytidine in DNA to deoxyuridine (Bogerd et al., 2006). To further understand this novel action of A3B, we tested the activity of two A3B deaminase mutants in our assay (Bogerd et al., 2006). Whereas mutation of the N-terminal CDA (CS) had no effect on Rev-independent Gag expression compared to wild type A3B, mutation of the C-terminal CDA (EQ) resulted in dramatically lower levels of Gag despite being expressed at wildtype levels

(**Figures A3C and D**). Interestingly, the A3B CS mutant was found to retain DNA editing activity an *E. coli* reporter assay, but the A3B EQ mutant did not (Bogerd et al., 2006). These findings suggested that the ability of A3B to induce Rev-independent Gag expression was dependent on DNA editing activity. These data are also consistent with the hypothesis that Rev-independent Gag expression from pRRE resulted from mutation of sequences inhibiting RNA export within intronic regions of pRRE by A3B. We concluded that Rev-independent Gag expression from pRRE was a reporter system reflecting deamination of exogenous DNA by A3B.

Vpr inhibits A3B-mediated, Rev-independent Gag expression

Given that A3B is upregulated during macrophage differentiation and Vpr facilitates HIV replication in macrophages, we hypothesized that Vpr counteracts antiviral effects of A3B. To examine the effect of Vpr in our A3B deamination assay, we co-transfected a Vpr-expressing plasmid containing a GFP reporter into the Rev-independent Gag assay system (**Figure A3B**). Vpr decreased Rev-independent Gag expression compared to a GFP-only negative control (**Figures A3C and D**, six fold). Therefore, we concluded that Vpr counteracts the effects of A3B on exogenous DNA in our assay through an uncharacterized mechanism. This result is consistent with a model in which Vpr protects HIV DNA from the antiviral consequences of A3B deamination in primary macrophages.

Vpr downregulates UNG2 levels in MDMs

Having preliminary evidence that *vpr* counteracts the antiviral consequences of uracil incorporation by RT and deamination by A3B, we hypothesized that uracilated HIV

DNA represents a PAMP that can be detected by the innate immune system. UNG2 is a candidate pattern recognition receptor (PRR) because the uracil DNA glycosylase excises uracil incorporated into HIV-1 DNA, leaving an abasic site that might initiate a downstream antiviral state by activating DNA damage response pathways. If UNG2 were required to initiate host recognition of uracilated HIV-1 cDNA as foreign, we hypothesized that downregulation of UNG2 would permit HIV to avoid immunodetection. Indeed, Vpr has been shown in several cell lines to mediate UNG2 degradation through the DCAF1-DDB1-Cul4A E3 ubiquitin ligase (Wen et al., 2012). To confirm downregulation of UNG2 by Vpr in 293T cells, we generated a Vpr-null mutant (NLG*vpr*$^-$) of the single-round HIV reporter construct pNL4-3ΔE GFP (NLG). We transfected 293T cells with NLG or NLG*vpr*$^-$ and measured UNG2 levels by western blot. pVSV-G was also co-transfected into cells to permit entry of these HIV-1 Env-null viruses in a second round of replication and increase the percentage of cells expressing Vpr. Cells transfected with NLG expressed dramatically lower levels of UNG2 than mock-transfected cells or cells transfected with NLG*vpr*$^-$ (**Figure A4A**). Complementation of NLG*vpr*$^-$ with Vpr expression plasmids (pCDNA-89.6Vpr and pCDNA-NL4-3Vpr) confirmed that *vpr* genes from two molecular clones are sufficient to downmodulate UNG2 (**Figure A4A**). These observations are consistent with a model in which Vpr degrades UNG2 to avoid immunodetection of uracilated HIV-1 cDNA.

Although Vpr downregulates UNG2 levels in cell lines such as 293T and HeLa, mutation of *vpr* does not affect virion production or virus proliferation in those cell lines(Chen et al., 2004b; Priet et al., 2005). Because *vpr* enhances virion production and virus proliferation in primary MDM (**Chapter 3**), we asked whether Vpr affects UNG2 levels in MDM. To this end, we infected MDM with 89.6 or 89.6*vpr*$^-$ and measured UNG2 levels by

western blot at ten days post infection. At this time point, flow cytometric analysis demonstrated that equal numbers of cells were infected by 89.6 or 89.6*vpr*-, and that greater than 80% of the cells were infected (data not shown). Also, intracellular levels of HIV-1 Gag pr55 and p24 were equal in the presence and in the absence of *vpr*, suggesting that Vpr did not affect the infection rate at this time point (**Figure A4B**). Because MDM expressed significantly lower levels of UNG2 than 293T cells, basal levels of UNG2 in MDM were near the limit of detection by western blot (**Figure A4B**). MDM infected with 89.6 contained slightly less UNG2 than mock-infected or 89.6*vpr*--infected cells (**Figure A4B**). Additionally, as has been reported in cell lines(Wen et al., 2012), the proteasomal inhibitor MG132 reversed downregulation of UNG2 by Vpr, suggesting that UNG2 is also degraded by the proteasome in MDM (**Figure A4B**). UNG2 recovered by MG132 treatment migrated at a slightly higher molecular weight (**Figure A4B**), possibly representing ubiquitinated forms. Therefore, we concluded that Vpr mediates proteasomal degradation of UNG2 in MDM, raising the possibility that UNG2 is a host restriction factor counteracted by Vpr in MDM.

Vpr packaged into virions mediates DCAF1 degradation

Because Vpr requires the DCAF1 substrate adaptor to mediate UNG2 degradation in 293T cells and MDM (Wen et al., 2012), we then explored whether Vpr regulates DCAF1 expression. Surprisingly, 293T cells transfected with NLG DNA expressed significantly lower levels of DCAF1 than cells transfected by NLG*vpr*- or mock transfected cells (**Figure A4A**). Additionally, complementation of NLG*vpr*- with Vpr expression plasmids also resulted in DCAF1 downregulation (**Figure A4A**). Therefore, while Vpr requires DCAF1 to

degrade UNG2, DCAF1 is also downregulated by Vpr, possibly through a negative-feedback mechanism.

We hypothesized that Vpr-mediated DCAF1 downregulation indicates utilization of the DCAF1-DDB1-Cul4A E3 ubiquitin ligase by Vpr, and that DCAF1 is degraded in the process of utilization. To determine if Vpr downregulated DCAF1 in primary macrophages, MDM were infected with equal mass amounts of wild type HIV-1 89.6 or 89.6*vpr*$^-$ and DCAF1 levels were assessed in five-day intervals. Cells were infected at a low multiplicity of infection (MOI) empirically determined to allow spread of the viruses to uninfected cells (data not shown). In wild type 89.6-infected MDM, we observed significant DCAF1 downregulation compared to mock-infected cells at ten and fifteen days post infection (**Figures A5A and A5B**), correlating with increased virion production at those time points (**Chapter 3, Figure 3.1**). At five days post infection, downregulation of DCAF1 by Vpr was less dramatic and a high molecular weight smear correlated with Vpr expression (**Figure A5A**), possibly representing polyubiquitinated forms. Additionally, MDM infected with 89.6*vpr*$^-$ expressed slightly more DCAF1 than mock-infected cells at five and ten dpi (**Figures A4B, A5A-B**). Therefore, we concluded that Vpr downregulated DCAF1 and that HIV-1 infection in the absence of Vpr upregulated DCAF1. These results show that primary MDM express DCAF1 and may reflect utilization of the DCAF1-DDB1-Cul4A E3 ubiquitin ligase by Vpr in MDM.

Given that Vpr regulates DCAF1 levels, we asked whether Vpr affects levels of the DDB1 substrate adaptor, another component of the DCAF1-DDB1-Cul4A E3 ubiquitin ligase. In MDM infected for ten days with either wildtype HIV-1 89.6 or 89.6*vpr*$^-$, we observed levels of DDB1 comparable to mock-infected cells (**Figure A5B**). Therefore, in MDM, Vpr

downregulated DCAF1 but not DDB1, another component of the Vpr-DCAF1-DDB1-Cul4A E3 ubiquitin ligase complex.

Because Vpr appeared to downregulate DCAF1 levels out of proportion to the expected infection rate at ten dpi and Vpr is packaged into virions(Bachand et al., 1999), we hypothesized that fusion of viral particles with target cells is sufficient to mediate Vpr-dependent DCAF1 degradation. To test this hypothesis, we incubated MDM with equal mass amounts of 89.6 or 89.6*vpr*⁻ and measured DCAF1 by western blot at five hours post infection, a time point known to precede the initiation of *de novo* HIV protein expression in the target cell at 24hpi (Spivak et al., 2011). A high MOI was chosen that was empirically determined to infect the majority of cells in culture (data not shown). DCAF1 was significantly downmodulated in MDMs infected with 89.6 relative to 89.6*vpr*⁻ (**Figure A5C**). Because *de novo* Vpr expression has not been initiated in target cells at this time point (Spivak et al., 2011), we concluded that Vpr packaged into particles is sufficient to cause DCAF1 degradation in MDM. These data are consistent with a model in which Vpr packaged into virions primes macrophages for infection by recruiting the DCAF1-DDB1-Cul4A E3 ubiquitin ligase to degrade a host restriction factor as early as five hpi.

We next examined the mechanism of Vpr-mediated DCAF1 degradation. Given that Vpu, Vpx, Vif and Vpr have all been demonstrated to promote proteasome-mediated degradation of host factors (Lim and Emerman, 2011; Lim et al., 2012; Malim and Emerman, 2008), we asked whether Vpr enhances DCAF1 degradation via the proteasome. MG132 treatment reversed Vpr-mediated DCAF1 degradation at five hours post infection (**Figure A5C**). These findings are consistent with the hypothesis that Vpr initiates proteasomal degradation of DCAF1 hours after entry to prevent UNG2 degradation.

Because DCAF1 is required for Vpr-mediated UNG2 degradation, we hypothesized that DCAF1 downregulation in the presence of Vpr represented a negative feedback response in which the substrate adaptor is degraded when its substrate, UNG2, is eliminated. To determine whether Vpr-mediated DCAF1 downregulation is caused by low UNG2 levels, we silenced UNG2 with siRNA in 293T cells and measured DCAF1 expression. DCAF1 levels remained unchanged after two or three days of UNG2 silencing (**Figure A5D**). Additionally, UNG2 levels did not change significantly following two or three days of DCAF1 silencing (**Figure A5D**). Therefore, while Vpr lowers the expression of DCAF1 and UNG2, the expression of each host factor did not depend on the level of the other under these conditions.

Discussion and future directions[1]

Macrophages contain a higher dUTP/dTTP ratio and increased levels of A3A and A3B compared to $CD4^+$ T cells and T cell lines (Eldin et al., 2014; Weil et al., 2013). These conditions favor uracilation of HIV DNA and may impact the stability or immunodetection of HIV DNA in macrophages. Preliminary data from our two assay systems suggests that Vpr may counteract the effects of dUTP incorporation and A3B expression in macrophages and 293T cells, respectively. However, these methods provide only indirect evidence that Vpr counteracts the effects of uracilation. Further experiments are needed to determine the mechanism through which deoxyuridine inhibits replication and the role of Vpr.

UNG2 may be an antiviral factor degraded by Vpr or a cofactor utilized by Vpr

[1] These data are discussed further in Chapter 5.

The uracil DNA glycosylase UNG2 likely plays a major role in the mechanism through which Vpr enhances HIV infection for several reasons. This and other studies(Eldin et al., 2014; Weil et al., 2013; Wen et al., 2012) have noted that Vpr mediates downregulation of UNG2. One hypothesis to explain this observation is that UNG2 prevents integration of uracilated HIV DNA by generating aberrant DNA structures downstream of base excision, promoting degradation or immunodetection of the provirus (**Figure A6A**). Several studies show that the uracilated provirus is capable of integrating in to the genome(Eldin et al., 2014; Weil et al., 2013). In fact, it has been proposed that uracilation increases the efficiency of integration into the host genome and decreases the likelihood of non-functional autointegration (Yan et al., 2011b). Thus, Vpr may promote HIV infection by targeting UNG2 for degradation (**Figure A6A**).

Other data support an opposing model in which Vpr may recruit UNG2 to promote repair of HIV DNA, leading to productive infection (**Figure A6B**). In support of this model, UNG2 has long been known to interact with Vpr, which is associated with the provirus (Selig et al., 1997). Two studies have demonstrated that UNG2 is required to maintain infectivity of HIV virions(Guenzel et al., 2012; Priet et al., 2005). Also, uracilated DNA is degraded more rapidly than non-uracilated DNA within a variety of cell types (Eldin et al., 2014; Sire et al., 2008; Weil et al., 2013). Aberrant HIV DNA structures downstream of base excision might be repaired by the base excision repair pathway or Vpr recruitment of the SLX4 complex (Laguette et al., 2014). Thus, uracil excision by UNG2 could lead to HIV DNA repair and productive infection (Laguette et al., 2014; Norman et al., 2011). In this model, degradation of UNG2 in the presence of Vpr may result from negative feedback following UNG2 utilization (**Figure A6B**), as has been reported for the SLX4-associated

endonucleases MUS81 and EME1(Laguette et al., 2014). Therefore, future investigation will be required to understand the significance of UNG2 degradation by Vpr.

Although these models propose viral and antiviral roles for UNG2, they may not be exclusive and UNG2 may enhance or inhibit the replication cycle alternatively at different stages or locations. Thus, while it is well established that UNG2 is recruited into virions by Vpr and is necessary in the producer cell to form infectious virus (Guenzel et al., 2012; Priet et al., 2005), it is not known whether UNG2 expression in the target cell might inhibit or enhance infection of restricted cell types. Because macrophages maintain a high dUTP/dTTP ratio and express A3A and A3B, we hypothesize that UNG2 may play a larger role in primary macrophage target cells than in $CD4^+$ T cells or T cell lines.

Vpr may enhance HIV infection by processing uracilated HIV DNA

Whether UNG2 promotes or inhibits HIV infection and whether Vpr recruits or counteracts UNG2 have been longstanding questions (**Figures A6A and B**). Here, we present two assay systems that may provide insight into those questions because they evaluate the effects of uracil incorporation into HIV DNA (**Figures A7A and B**). The first assay demonstrated that the efficiency of HIV infection was inversely proportional to the dUTP/dTTP ratio, which was raised or lowered with exogenous deoxynucleosides (**Figure A7A**). Although Vpr does not degrade SAMHD1(Lim et al., 2012), dN treatment partially rescued virus production and spread in macrophages infected by the Vpr-null virus. Further experiments will be necessary to understand the mechanism behind this observation. The simplest possibility is that Vpr enhances dNTP pools or reverse transcription through a SAMHD1-independent mechanism. However, an alternative

hypothesis is that Vpr minimizes degradation or detection of HIV DNA through its interaction with UNG2 (**Figures A6A-B and A7A**). Thus, modulation of the dUTP/dTTP ratio might alter Vpr's effect on macrophage infection by increasing or decreasing uracil substrates for UNG2. To test this hypothesis, it would be interesting to examine the effect of dU or dN treatment on HIV infection of macrophages following UNG2 silencing. UNG2 knockdown might reveal whether UNG2 inhibits HIV infection or is recruited by Vpr to enhance infection.

Although exogenous dNs completely rescue SIV*vpx*$^-$ infectivity to wild type levels(Lahouassa et al., 2012), dN rescue of HIV*vpr*$^-$ infectivity was partial in our system. One possible explanation is that dN treatment does not remove dUTP from macrophages, but merely increases the denominator of the dUTP/dTTP ratio. Toxicity limited further dilution of dUTP with higher concentrations of exogenous dNs. Another possibility is that Vpr might enhance HIV infectivity in macrophages through an additional mechanism that cannot be rescued by dNs, such as nuclear import of the preintegration complex (Caly et al., 2008; Jacquot et al., 2007). Altogether, differences in the extent of dN-mediated rescue between SIV*vpx*$^-$ and HIV*vpr*$^-$ may suggest that Vpx and Vpr enhance infection through separate mechanisms.

As has been reported in a previous study (Lahouassa et al., 2012), we found that dN treatment increased the infectivity of wild type HIV in our assays. Thus, Vpr and UNG2-mediated DNA repair may not be sufficient to overcome all DNA lesions inflicted in macrophages. In support of this idea, it has been demonstrated that the majority of HIV DNA does not integrate (Yamamoto et al., 2006; Yan et al., 2011a), raising the possibility that most HIV DNA contains lesions downstream of uracilation that cannot be repaired.

Because modulation of the dUTP/dTTP ratio impacted the effect of Vpr on virion production, we hypothesize that Vpr protects HIV DNA from the downstream consequences of uracilation. By promoting processing of uracilated HIV DNA as has been proposed(Laguette et al., 2014), Vpr could prevent the early upregulation of a host restriction factor that would otherwise inhibit virion production. For example, tetherin, an interferon-inducible host factor that restricts viral particle release, is upregulated by HIV infection, but the magnitude is small (Homann et al., 2011). Future studies are required to understand the mechanism through which Vpr enhances virion production, and whether effects of Vpr on the expression of host proteins might affect virion release.

SAMHD1 may also promote dUTP incorporation into HIV DNA

SAMHD1 is believed to restrict HIV infection by limiting the concentration of canonical dNTP substrates for reverse transcription in macrophages (Lahouassa et al., 2012). However, macrophages also contain a high concentration of dUTP and express APOBEC proteins that can deaminate deoxycytidine to deoxyuridine (Berger et al., 2011). Rather than by increasing dNTP substrates for reverse transcription, we hypothesize that dN treatment enhances HIV infection by lowering the dUTP/dTTP ratio, decreasing uracil incorporation into viral cDNA. Additionally, a dN-mediated increase in the velocity of reverse transcription might decrease the window of opportunity for APOBEC-mediated deamination. Therefore, our results raise the possibility that the true mechanism of dN-mediated enhancement of HIV replication is decreased uracilation of HIV DNA, protecting the provirus from degradation or immunodetection (**Figure A7A**). Because addition of exogenous dNs alters both the dUTP/dTTP ratio and the total dNTP concentration, our

assay does not favor one model over the other. In future studies, it may be possible to examine the effects of SAMHD1 or dN treatment on HIV infection following depletion of intracellular dUTP by an exogenous dUTPase, as has been done previously(Kato et al., 2014a; Kato et al., 2014b).

Vpr may protect uracilated HIV DNA from degradation or detection through UNG2

In our second assay, we examined the interaction between Vpr and A3B. Given that A3B is upregulated during macrophage differentiation (Peng et al., 2007), we hypothesized that Vpr protects HIV DNA uracilated by A3B from degradation or immunodetection. This model would predict that Vpr counteracts downstream effects of A3B deamination, such as the Rev-independent Gag expression we observed *in vitro*. A3B-dependent, Rev-independent Gag expression from the Rev reporter construct, pRRE requires an active C-terminal cytidine deaminase (CDA) domain, which is necessary for A3B-mediated hypermutation *in vitro* (Bogerd et al., 2006). In contrast, mutation of the putative N-terminal CDA, which is not required for hypermutation, had no effect on Gag expressed from pRRE. Further investigation is required to identify changes within transfected pRRE that permitted Rev-independent nuclear export of *gag* transcripts. Several inhibitory sequences within introns, for example, are known to prevent CRM1-dependent export of incompletely spliced transcripts (Askjaer et al., 1999; Fornerod et al., 1997). Nevertheless, our preliminary results suggest that Rev-independent Gag expression is a downstream consequence of A3B deamination of pRRE.

It is unclear why a host cytidine deaminase increases HIV gene expression in our assay. However, the conservation among lentiviruses of the Rev-dependent timing of early

and late gene expression suggests that the ordered expression of viral proteins provides a replication advantage *in vivo*. Viral factors that enhance viral gene expression *in vitro* may be selected against *in vivo* because they trigger an antiviral immune response. For instance, it has been suggested that HIV-1 does not encode Vpx because efficient infection of dendritic cells (DCs) induces cytokine secretion (Lim and Emerman, 2011; Lim et al., 2012). Moreover, it has been shown that higher Rev activity increases the sensitivity of infected cells to CTL killing (Bobbitt et al. Immunity 2003). Perhaps A3B enhances immunodetection of HIV-infected macrophages by promoting a premature display of viral antigens on the cell surface.

Finally, we discovered that Vpr inhibited A3B-mediated, Rev-independent Gag expression from the Rev reporter pRRE. If we assume that our assay measured A3B-mediated hypermutation, these data suggest that Vpr counteracted a downstream consequence of A3B deamination of pRRE. Such findings would be consistent with a model in which Vpr-mediated UNG2 degradation inhibits base excision repair, which could lead to the loss of inhibitory intronic regions within HIV DNA and allow HIV mRNA export independent of Rev (**Figure A7B**). To test this hypothesis, it would be interesting to examine the effects of A3B and Vpr on Rev-independent Gag expression following UNG2 silencing. UNG2 may be required to initiate excision of uracil leading to the deletion of inhibitory introns from HIV DNA (**Figure A7B**). An UNG2 silencing experiment might also distinguish between a role for UNG2 as an antiviral factor and a role for UNG2 as a mediator of HIV DNA repair.

The expression of A3B in relevant targets of HIV is currently debated. Although one study reported that A3B is upregulated during macrophage differentiation (Peng et al.,

2007), others have not detected A3B in macrophages (Koning et al., 2009). Inconsistencies may arise from variations in differentiation protocols between macrophage models. Also, A3B is expressed at a much higher level in our model system than would be found in any known target of HIV. Thus, further experiments are needed to link this Vpr activity to more physiologic systems. We hypothesize that Vpr protects HIV DNA uracilated by any source, including other APOBEC3 proteins that are abundant in macrophages and $CD4^+$ T cells such as A3A and A3G. In future experiments, it would be interesting to determine if other APOBEC3 proteins induce Rev-independent Gag expression in our assay.

Summary

In summary, our preliminary data demonstrates that Vpr mediates degradation of UNG2 in primary MDM. We hypothesize that Vpr either degrades UNG2 as a restriction factor, or recruits UNG2 to repair HIV DNA. We have also developed two assays that suggest Vpr counteracts the downstream consequences of uracil incorporation or cytidine deamination in macrophages. Macrophages contain a high dUTP concentration and upregulate A3B, conditions that may account for the observed replication advantage of Vpr-expressing lentiviruses in this cell type. Further study of the interaction between Vpr and UNG2 may lead to a deeper understanding of the role of uracil repair in HIV replication.

Materials and Methods

Antibodies and cell lines

Antibodies to CAp24 [KC57 conjugated to phycoerythrin (PE) or fluorescein isocyanate (FITC)] were obtained from Beckman Coulter. Antibodies to the following

proteins were used for immunoblot analysis: DCAF1 (11612-1-AP Proteintech), DDB1 (39-9901, Invitrogen), UNG2 (EPR4371, Epitomics), Tubulin (T5168, Sigma), Hemagglutinin (HA.11 MMS-101R, Covance), GFP (8362-1, Clontech), Gag CAp24, Vpr, and Nef AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog Numbers 4250, 3951, and 2949 from Dr. Michael Phelan (Hatch et al., 1992), Dr. Jeffrey Kopp, and Dr. Ronald Swanstrom, respectively.

Cell culture and viral infection

Leukocytes isolated from anonymous donors by apheresis were obtained from New York Blood Center Component Laboratory (Long Island City, NY). Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll density gradient separation, as described(Norman et al., 2011).

$CD14^+$ monocytes were isolated by positive selection with an EasySep magnetic sorting kit (StemCell Technologies). Monocyte derived macrophages (MDM) were differentiated and infected as described in **Chapter 3** (Lahouassa et al., 2012). MDMs were infected with HIV-1 for 4h, washed with phosphate buffered saline (PBS) and cultured in fresh medium, as described(Peeters et al., 2002).

Viral constructs and virus stocks

p89.6, pNL4-3 and pNL4-ΔE-EGFP (pNLG) were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalog numbers 3552, 114 and 11100 from Dr. Ronald G. Collman(Collman et al., 1992), Dr. Malcolm Martin and Dr. Robert Siliciano. p89.6*vpr*$^-$ was described in **Chapter 3**. pNLG*vpr*$^-$ was generated by ligating the SpeI-SalI

fragment from NL-PI*vpr*$^-$(Norman et al., 2011) into pNLG. pCDNA-89.6Vpr and pCDNA-NL4-3Vpr were generated by PCR amplification of the Vpr open reading frame from p89.6 or pNL4-3 with the forward primer:
5'-TCAGGATCCACCATGGAACAAGCCCCAGAAGACC-3', and the reverse primer:
5'-TGATCTAGACTAGGATCTACTGGCTCCATTTC-3' (89.6) or
5'-TGATCTAGACTAGGATTTACTGGCTCCATTTC-3' (NL4-3) and ligation of the PCR products into the BamHI/XbaI sites of pCDNA3.1$^+$.

Virus stocks were produced by transfection of 293T cells with provirus expression plasmids using polyethylenimine, as described(McNamara et al., 2012). Viral supernatants were collected at 48h and centrifuged at 1500 rpm to remove cell debris. Virus was stored at -80°C and quantified by p24 ELISA, as described in **Chapter 3** (Salmon and Trono, 2007).

Flow cytometry staining and antibodies

Intracellular staining for Gag CAp24 expression was performed as described previously {**Chapter 3** and (Carter et al., 2010)}. Data was analyzed using FloJo software (Cytek).

Western blotting

MDM whole cell lysate was prepared and analyzed by immunoblot as described previously (**Chapter 3**). Lysates were analyzed by immunoblot using autoradiography and protein levels were quantified using Adobe Photoshop as described(Norman et al., 2011).

Canonical deoxynucleoside and deoxyuridine treatment

Deoxycytidine, deoxythymidine, deoxyguanidine, and deoxyadenine (dNs), or deoxyuridine (dU) were obtained from Sigma, resuspended in water and adjusted to pH 7.4. MDMs were infected with 5μg of 89.6 or 89.6*vpr*⁻ and treated with between 1.25mM and 5mM dNs or dU from two hours pre- to twenty hours post-infection. At 20hpi, fresh medium was added containing Maraviroc (20μM) and AMD3100 (20μg/ml). Cells and supernatant were analyzed by flow cytometry and ELISA at four days post infection.

A3B deaminase assay

pA3B-HA, pA3B CS-HA, pA3B EQ-HA and pβarr-HA were obtained from the laboratory of Dr. John Moran(Bogerd et al., 2006). The Rev reporter pRRE was obtained from the laboratory of Dr. Luigi Naldini(Dull et al., 1998). pHIV-Vpr was generated by ligating the PCR-amplified *vpr* open reading frame from NL4-3 into the NheI and BamHI sites of pHIV 7c IresGFP (pHIV-GFP). 4 x 10^5 293T cells were transfected with 1μg pRRE, 1μg pA3B HA, pA3B CS-HA or pA3B EQ-HA, and 4μg pHIV-Vpr or pHIV-GFP. One day post transfection, cells were analyzed for intracellular Gag expression by flow cytometry and Gag, GFP, Vpr, HA and tubulin expression by western blot.

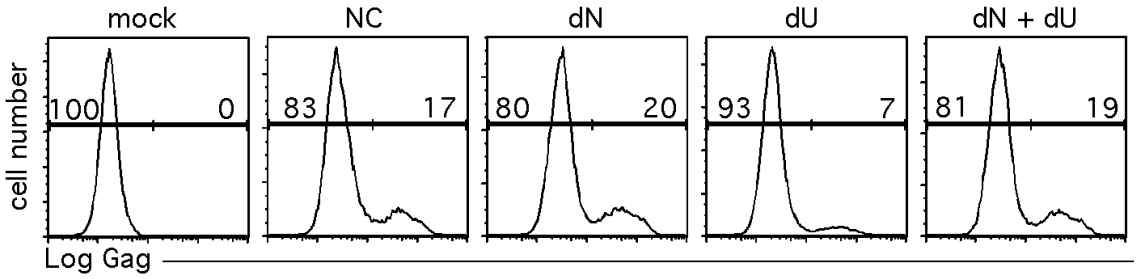

Figure A1. Deoxynucleoside (dN) treatment overcomes the negative effect of deoxyuridine (dU) on the HIV infection of macrophages. Flow cytometric analysis of intracellular Gag in MDMs infected with 5μg of 89.6 and either 2.5mM dN, 2.5mM dU, or 2.5mM dN + 2.5mM dU from -2 to 20hpi. Negative control (NC) cells were treated with water. Cells were analyzed at 4dpi. [2]

[2] The data in this figure was produced by Michael Mashiba.

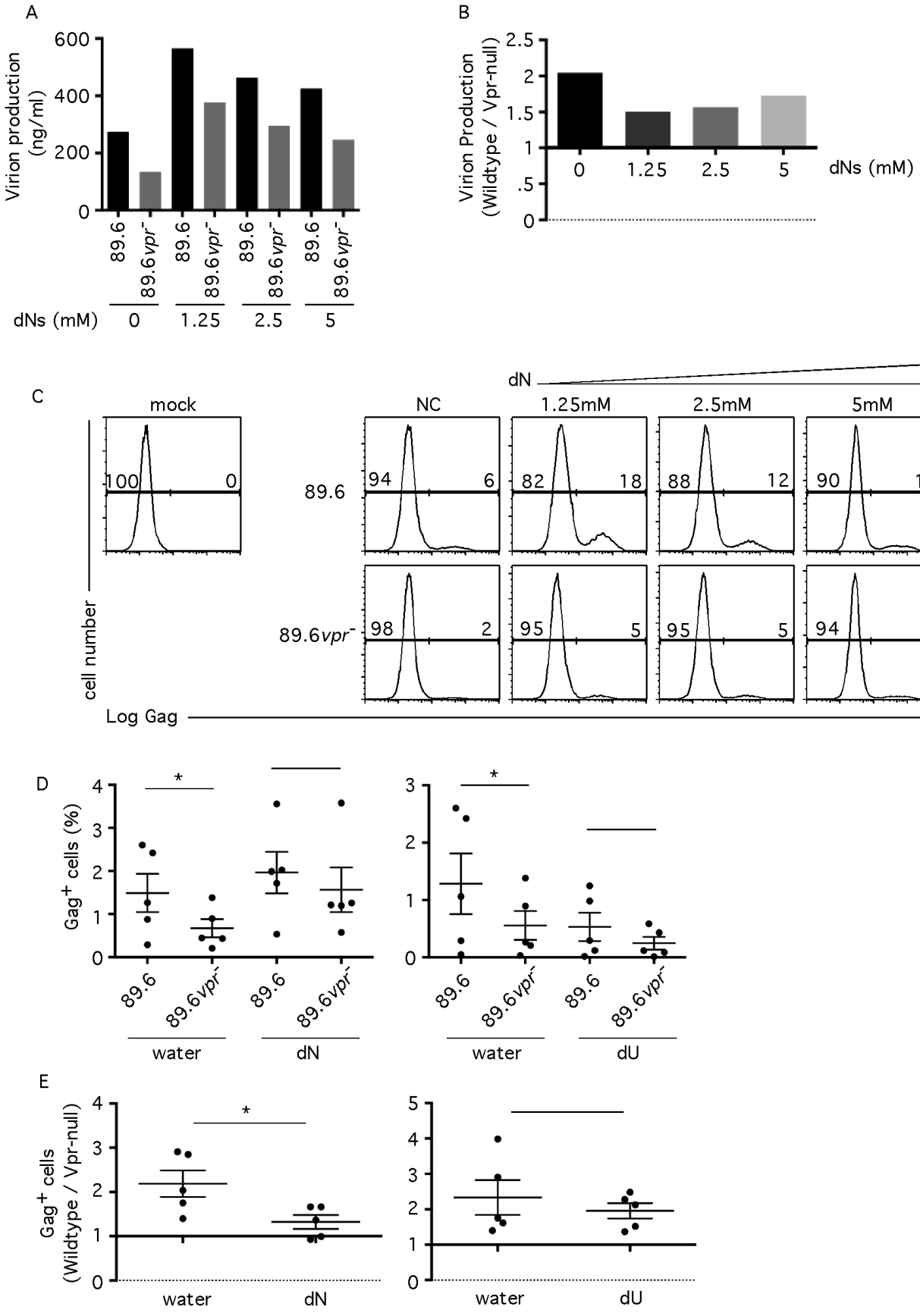
A
Virion production (ng/ml)
600
400
200
0
89.6
89.6vpr-
dNs (mM)
0
1.25
2.5
5
B
Virion Production (Wildtype / Vpr-null)
2.5
2
1.5
1
.5
0
dNs (mM)
C
dN
mock
NC
1.25mM
2.5mM
5mM
89.6
89.6vpr-
100
0
94
6
82
18
88
12
90
10
98
2
95
5
95
5
94
6
cell number
Log Gag
D
Gag+ cells (%)
water
dN
dU
E
Gag+ cells (Wildtype / Vpr-null)
water
dN
dU

Figure A2. Increasing the cellular pool of canonical dNTPs partially restores virus production and spread of HIV-1 89.6*vpr*- in macrophages. **(A)** Virion production from MDMs infected with 5μg of the indicated viruses and treated with the indicated concentrations of dN from -2 to 20 hpi. At 20 hpi, media was replaced with fresh media containing AMD3100 (20μg/ml) and Maraviroc (20μM). Virus production was normalized for the number of infected cells by dividing the concentration of virus in the supernatant determined by CAp24 ELISA by the infection rate determined by flow cytometry. **(B)** The effect of Vpr on virion production in the experiment in **A**, calculated as the fold change in virion production by MDMs infected by wildtype HIV-1 89.6 relative to 89.6*vpr*- at the indicated dN concentrations. **(C)** Flow cytometric analysis of intracellular Gag in MDMs infected with 5μg of the indicated viruses and treated with the indicated concentration of dN or untreated (NC) from -2 to 20hpi. Cells were analyzed at 4dpi. **(D)** Summary graphs of the infection rates measured as in **C** for MDMs infected with the indicated viruses and treated with 2.5mM dN, 2.5mM dU or water from -2 to 20hpi. Cells were analyzed at 4dpi. Each dot represents an experiment performed on cells from a different donor. **(E)** Summary graphs of the effect of Vpr on the infection rates measured in the experiments in **C,** calculated as the fold change in the infection rate of MDMs infected by wildtype 89.6 relative to cells infected by 89.8*vpr*-. *$p < 0.05$ (paired *t* test).[3]

[3] The data in this figure was produced by Michael Mashiba.

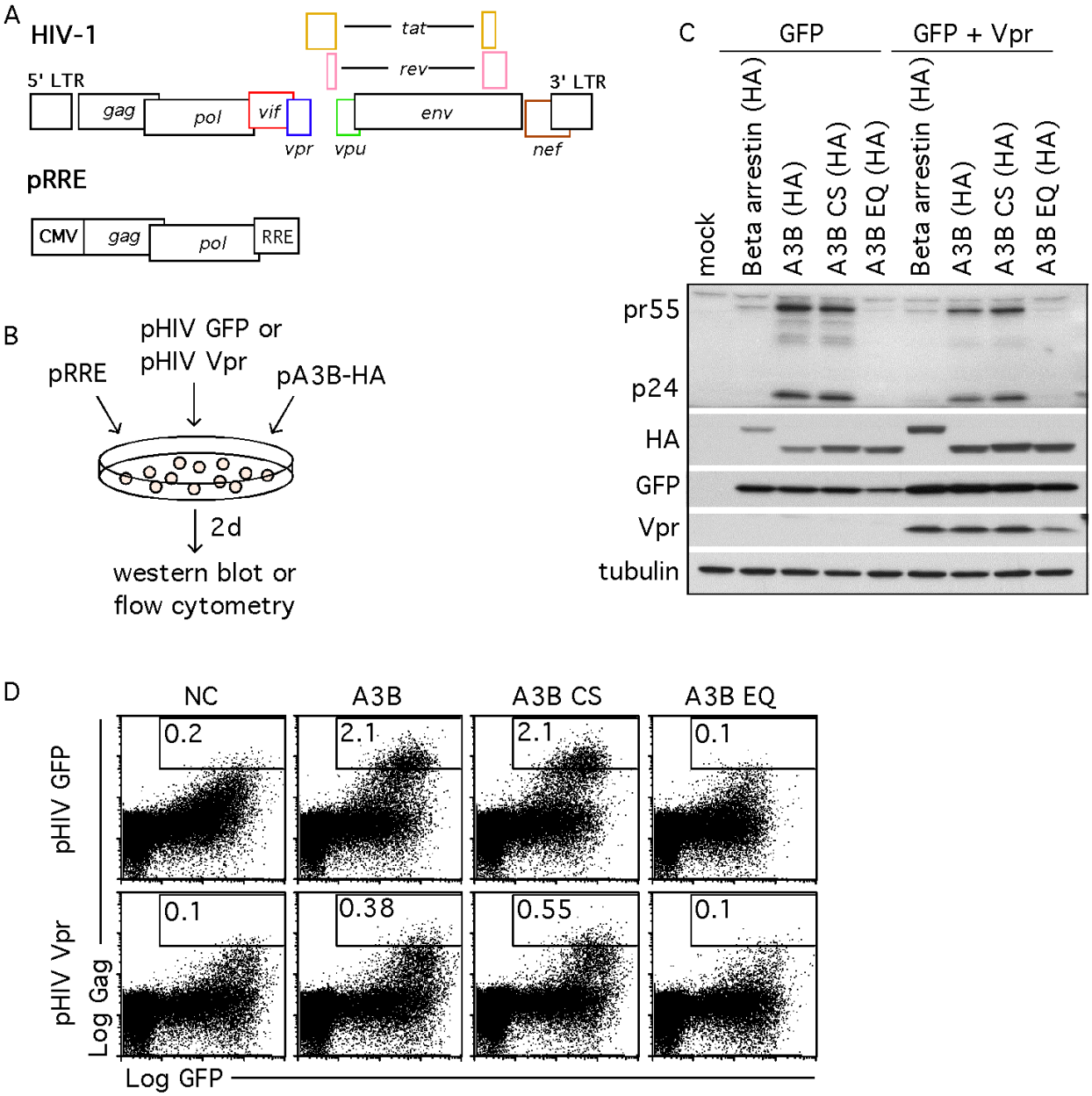

Figure A3. Vpr inhibits A3B-mediated, Rev-independent Gag expression. **(A)** Schematic of the HIV-1 genome and pRRE. **(B)** Graphical representation of the transfection experiment to assess the effect of Vpr on A3B-mediated, Rev-independent Gag expression in 293T cells. **(C)** Western blot analysis of Gag pr55 and p24, HA, GFP, Vpr and tubulin (loading control) in 293T cells transfected with the indicated plasmids and lysed at two days post transfection. **(D)** Flow cytometric analysis of GFP and Gag expression in cells shown in **C**.[4]

[4] The data in this figure was produced by Michael Mashiba.

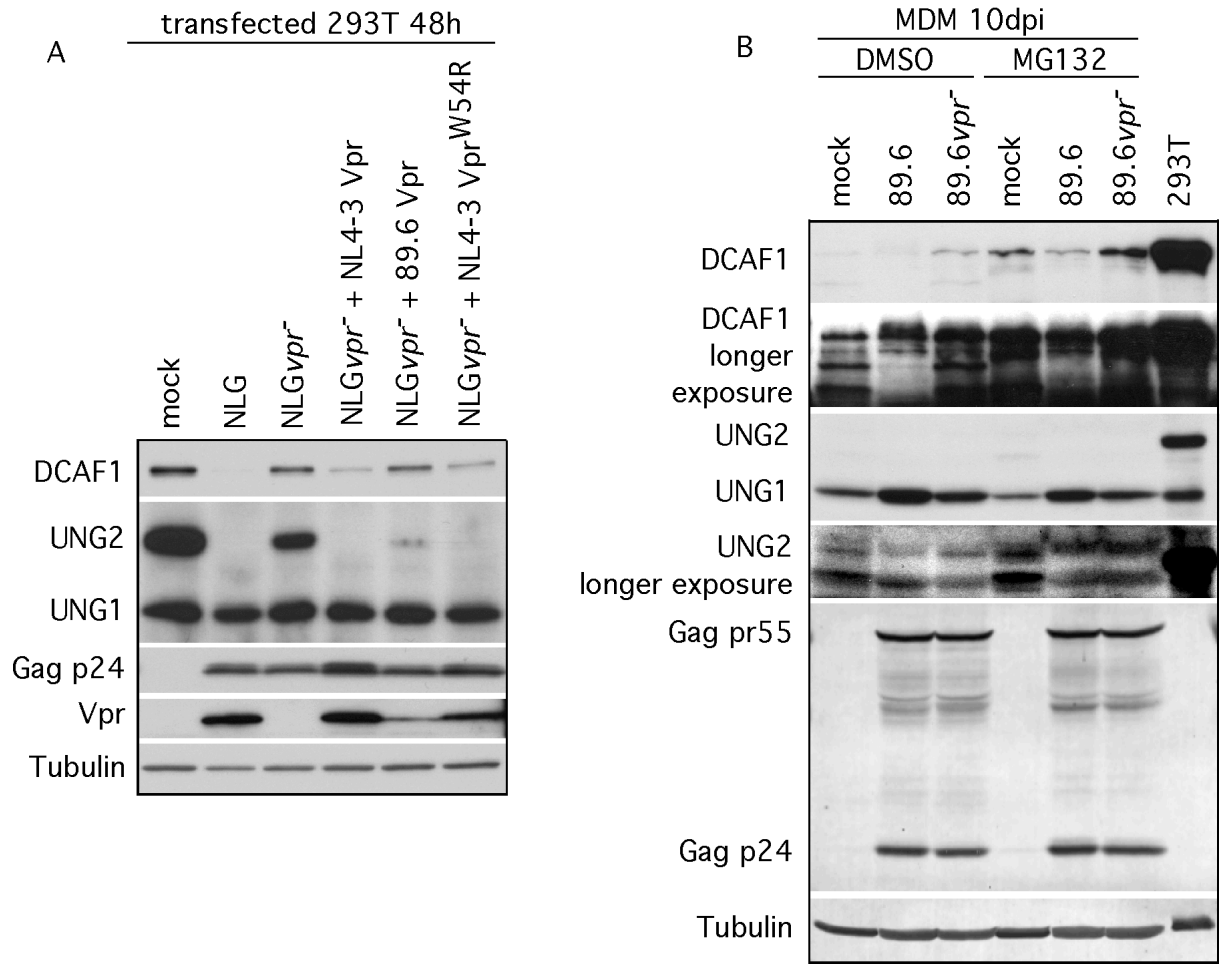

Figure A4. Vpr enhances degradation of UNG2 in primary macrophages. **(A)** Immunoblot analysis of DCAF1, UNG1, UNG2, p24, Vpr and tubulin (loading control) in whole cell lysates (WCLs) from 293T cells transfected with the indicated plasmids and pVSV-G. Cells were lysed at 48h post transfection. **(B)** Immunoblot analysis of DCAF1, UNG2, pr55, p24 and tubulin in WCLs from MDMs infected with the indicated viruses for 10d or from uninfected 293T cells. Cells were treated with 2.5 μ M MG132 or DMSO as indicated for 12h before lysis. [5]

[5] The data in this figure was produced by Michael Mashiba.

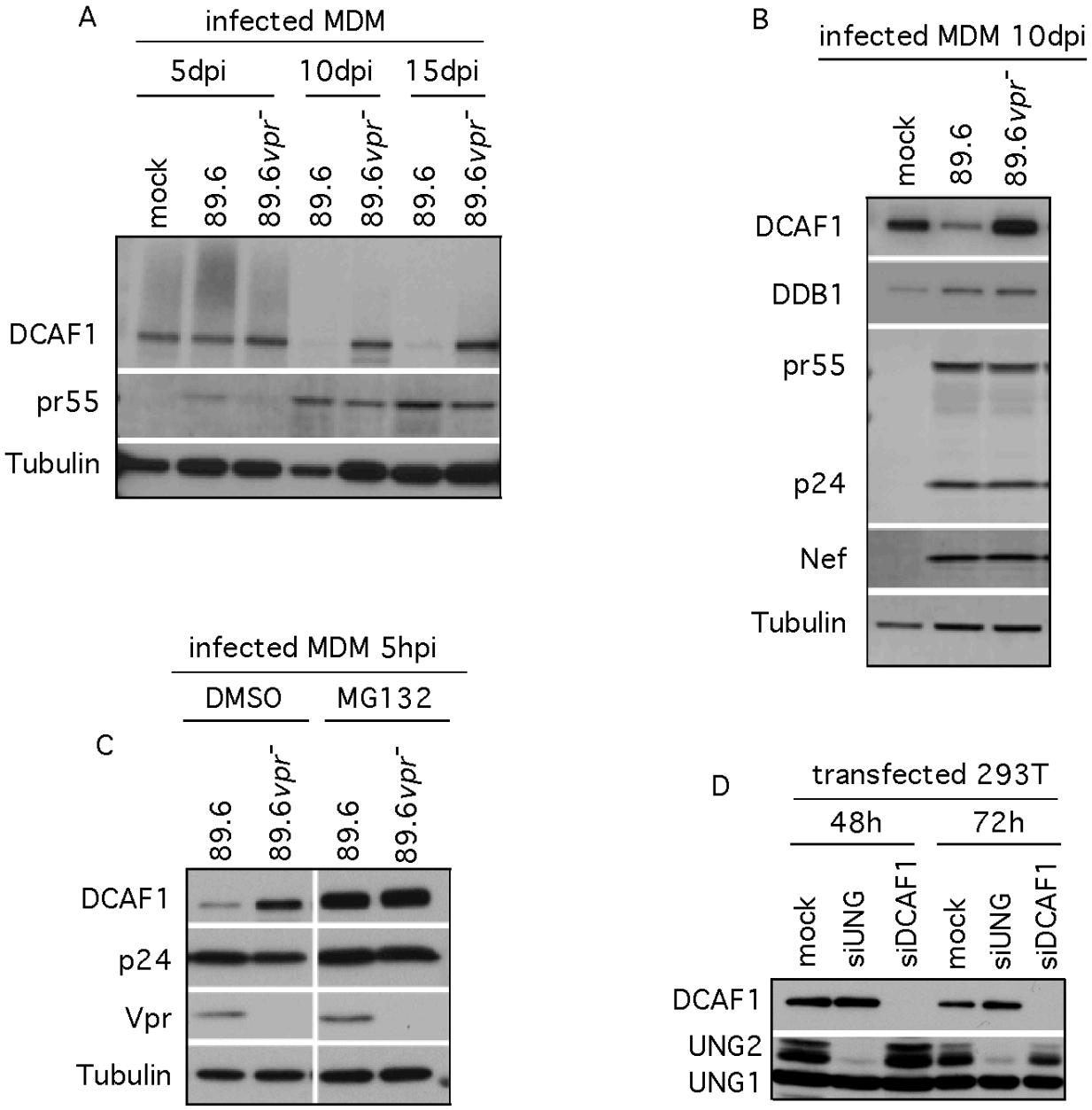

Figure A5. Vpr in viral particles is sufficient to mediate degradation of DCAF1 but not DDB1. **(A)** Immunoblot analysis of DCAF1, pr55 and tubulin in WCLs from MDMs infected with 1μg of the indicated viruses and lysed at the indicated times post infection. **(B)** Immunoblot analysis of DCAF1, DDB1, pr55, p24, Nef, and tubulin (loading control) in whole cell lysates (WCLs) from MDMs infected with 1μg of the indicated viruses for 10 days. **(C)** Immunoblot analysis of DCAF1, p24, Vpr, and tubulin in WCL from MDMs infected with 100μg the indicated viruses for five hours. Cells were treated with 2.5μM MG132 or DMSO as indicated from -2 to 5hpi. **(D)** Immunoblot analysis of DCAF1, UNG1 and UNG2 in WCLs from 293T cells transfected with the indicated siRNAs for the indicated periods. [6]

[6] The data in this figure was produced by Michael Mashiba.

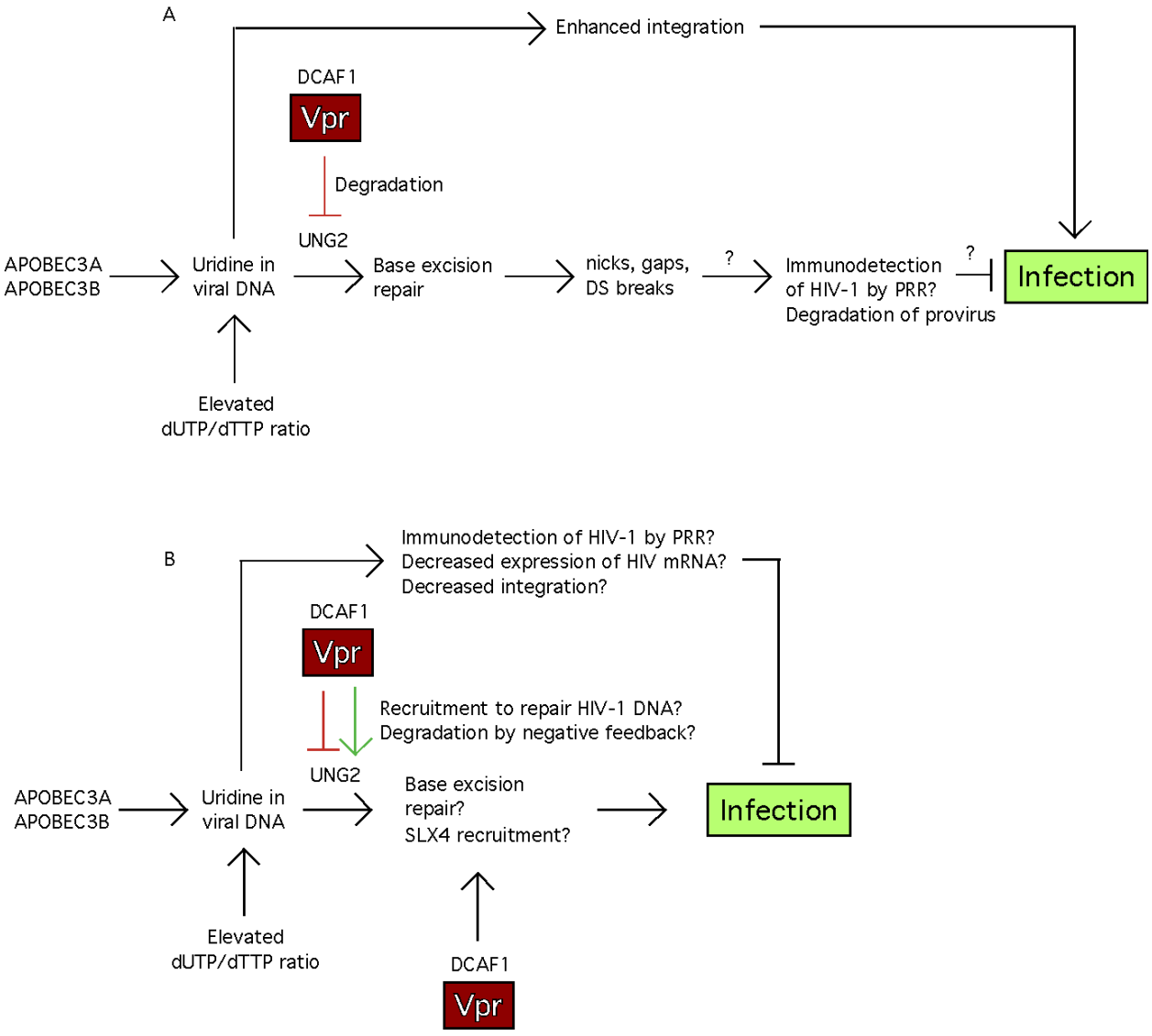

Figure A6. Possible roles for UNG2 in the early stages of HIV-1 replication. **(A)** UNG2 may be an antiviral factor degraded by Vpr. Uracil excision from HIV-1 DNA leads to the formation of aberrant DNA structures that are degraded or detected as pathogenic. Degradation of UNG2 permits uracilated HIV DNA to integrate into the genome. Uracilation may favor integration by preventing the suicidal autointegration pathway. **(B)** Alternatively, Vpr may recruit UNG2 to repair uracilated HIV DNA, permitting integration. SLX4 complex recruitment by Vpr may facilitate repair of intermediates downstream of uracil excision. UNG2 may be degraded following utilization as negative feedback. [7]

[7] The data in this figure was produced by Michael Mashiba.

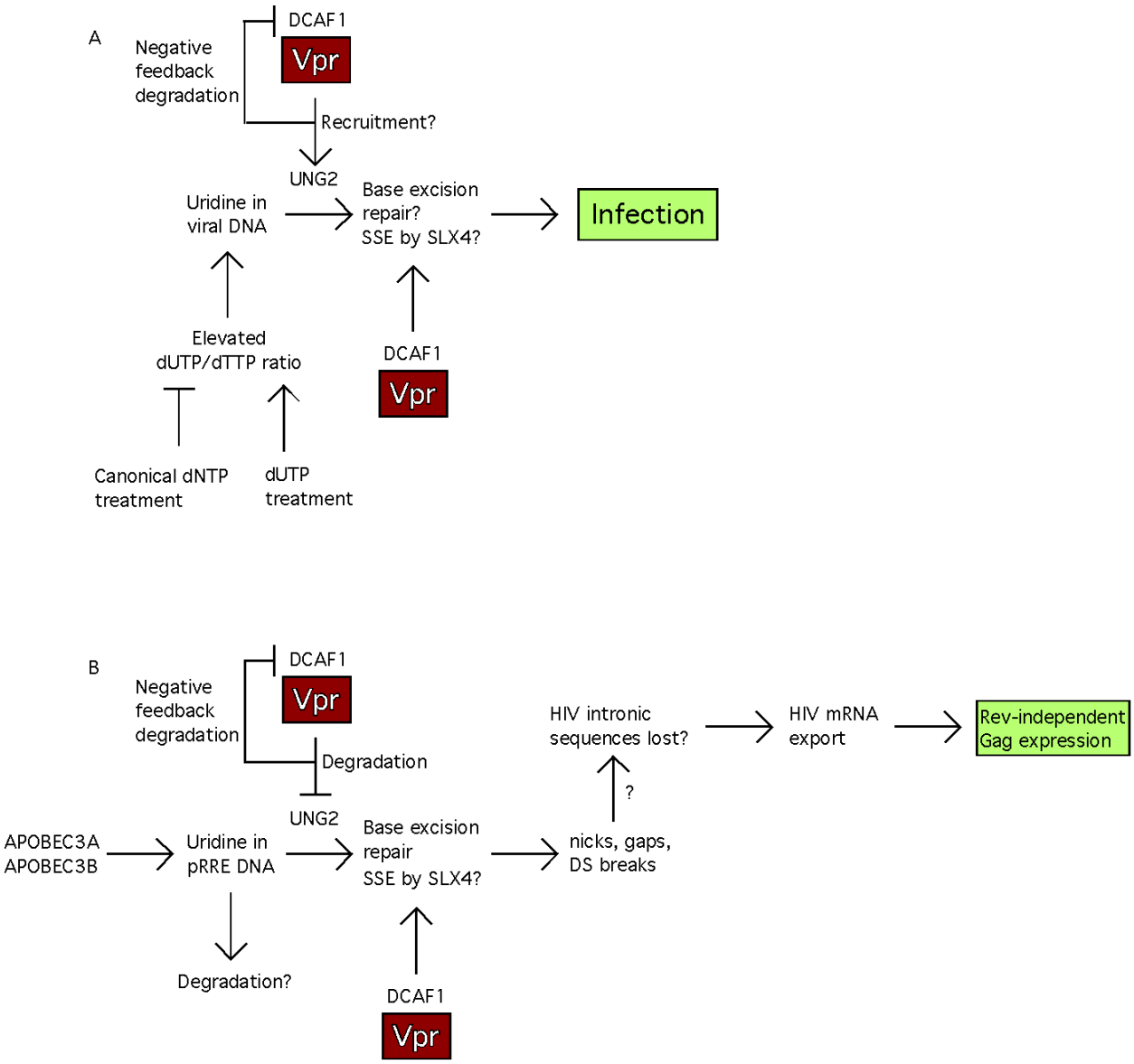

Figure A7. An elevated dUTP/dTTP ratio and APOBEC3B reveal a requirement for Vpr. Arrows indicate activation and horizontal lines indicate inhibition. **(A)** Manipulation of the intracellular dUTP/dTTP ratio through exogenous nucleoside treatment or use of MDM, which maintain a high endogenous dUTP/dTTP ratio, leads to incorporation of uridine into viral DNA by reverse transcriptase. By recruiting UNG2, Vpr promotes repair of HIV DNA, leading to infection. Vpr may also promote repair of these aberrant DNA intermediates by activating the SLX4 complex. Rather than being degraded or detected as pathogenic, uracilated HIV DNA becomes integrated into the host genome. **(B)** Cytidine deamination by APOBEC3B-transfected 293T cells or by APOBEC3A and APOBEC3B in macrophages may increase uracilation of pRRE DNA. In 293T cells in the absence of Vpr, uracilated pRRE DNA is repaired, possibly leading to aberrant DNA structures and the loss of intronic

sequences within pRRE that inhibit the export of Gag mRNA. In the absence of inhibitory introns, Gag mRNA is exported from the nucleus, leading to Rev-independent Gag expression. Vpr degrades UNG2, possibly preventing excision of uracil and the loss of inhibitory introns. In the absence of UNG2, uracilated pRRE DNA may be degraded or may activate an antiviral response. Vpr also recruits SLX4, which may facilitate repair of intermediates downstream of uracil excision. Nevertheless, degradation of UNG2 by Vpr likely limits the accumulation of HIV DNA molecules that lack inhibitory introns. Thus Vpr may inhibit Rev-independent Gag expression *in vitro* by degrading UNG2.[8]

[8] The data in this figure was produced by Michael Mashiba.

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