Vpr mediates immune evasion and HIV-1 spread

by

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Dedication

In loving memory of Sullivan James and Aiko Rin

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Abstract

The molecular mechanisms by which human immunodeficiency virus (HIV-1) evades immunity to cause persistent infection remain incompletely characterized. Viral protein R (Vpr) is conserved in all primate lentiviruses, including HIV-1. Previous studies have demonstrated that Vpr is required for maximal infection of T lymphocytes in vivo. However, Vpr does not enhance HIV-1 infection of T lymphocytes under standard in vitro infection conditions, and the mechanism of Vpr function is poorly understood. Our work demonstrates that Vpr prevents the induction of a type I interferon-stimulated antiviral response in macrophages that targets Env and Env-containing virions for lysosomal degradation. By preventing this response, Vpr promotes Env-dependent virological synapse formation and enables efficient spread of HIV-1 from macrophages to activated T lymphocytes. This mode of spread requires direct cell-to-cell contact and is highly resistant to neutralizing antibodies. These studies provide a mechanistic explanation for the evolutionary conservation and function of Vpr.

Chapter 1

Introduction[1]

Human immunodeficiency virus (HIV-1) has infected over seventy million people and caused nearly forty million deaths (WHO, 2015). By infecting immune cells directly, HIV-1 subverts immune control to establish persistent infections. Antiretroviral therapies (ART) are available that block HIV-1 replication and enable the survival of infected individuals when taken continuously; however, ART are not curative and infection quickly rebounds after discontinuation of treatment (Harrigan et al., 1999). Moreover, no vaccine exists to prevent infection and treatments are often prohibitively expensive in regions most affected by the virus. Thus, despite the availability of ART, HIV-1 remains the leading infectious cause of mortality worldwide, and more effective treatments are required to combat this global health crisis. Understanding how HIV-1 evades host immunity to establish persistent infections will inform future advances toward development of a vaccine and/or cure. This dissertation examines human immune cells infected by HIV-1, revealing a mechanism of immune evasion that enables HIV-1 to replicate and

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spread efficiently. This introductory chapter provides background on HIV-1 and lays the framework for the dissertation research.

HIV-1 replication

Entry and cellular tropism

HIV-1 is an enveloped virus with an RNA genome containing nine genes and two long-terminal repeats (LTR, **Figure 1.1**). The viral genome is encapsidated within a lattice composed of the viral structural protein Gag, and this viral capsid is surrounded by a cell-derived lipid envelope (**Figure 1.2**). On the surface of the viral envelope, glycoproteins encoded by the viral *env* gene form trimers that facilitate entry into target cells (**Figure 1.3**). The surface-exposed portion of Env, called gp120, mediates attachment to the HIV-1 receptor, CD4 (McDougal et al., 1986). Upon attachment, gp120 also interacts with a co-receptor, either CCR5 or CXCR4 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). These interactions induce a conformational change in Env that exposes a fusion peptide within the transmembrane domain, gp41 (Chan et al., 1997; Gallaher, 1987; Veronese et al., 1985). Exposure of the gp41 fusion peptide enables the viral lipid membrane to fuse with the target cell membrane via formation of six-helix bundle structures (Melikyan et al., 2000). Upon membrane fusion, the viral capsid is released into the cellular cytoplasm, where HIV-1 replication continues. Due to the specificity of Env attachment, HIV-1 infects cells expressing CD4 and either CCR5 or CXCR4: primarily $CD4^+$ T lymphocytes and macrophages. This tropism for cells of the immune system enables HIV-1 to

dysregulate immune function, evade immune control and ultimately cause immunodeficiency.

Reverse transcription and integration

The HIV-1 genome is replicated in a multi-stage process known as reverse transcription. The viral *pol* gene encodes an enzyme called reverse transcriptase (RT), which is packaged within the viral capsid with the genomic RNA and mediates reverse transcription (Baltimore, 1970; Temin and Mizutani, 1970). During reverse transcription, the genomic RNA is converted to double-stranded DNA in a stepwise manner (**Figure 1.4**; reviewed in (Goff, 1990)). First, a cellular tRNA primer binds to the primer binding site (PBS) within the 5' LTR. RT has an RNA-dependent DNA polymerase activity that catalyzes the synthesis of single-stranded complementary DNA. The first cDNA contains an untranslated region of the LTR, and is called strong stop cDNA. RT also has a ribonuclease activity that next catalyzes the removal of the 5' RNA within the cDNA-RNA hybrid created upon strong stop cDNA synthesis. Once the RNA is degraded in this region, the strong stop cDNA and its primer transfer to the opposite end of the viral genomic RNA (vRNA) where it binds to a repeated sequence in the 3' LTR in a process known as strand transfer. After strand transfer, RT polymerizes the remainder of the cDNA strand. The ribonuclease activity of RT then removes the remaining RNA template, leaving only a small polypurine region near the 3' LTR. In addition to its ribonuclease and RNA-dependent DNA polymerase activities, RT also functions as a DNA-dependent DNA polymerase, which catalyzes synthesis of the second DNA strand. Using the

polypurine RNA as a primer, RT polymerizes the 3' LTR region of coding strand DNA, including a region of the original primer. The original tRNA primer is displaced and the new strand undergoes another strand transfer event to bind complementary sequence at the 5' end of the cDNA strand. Finally, RT polymerizes the remainder of both strands, resulting in a complete viral genomic double-stranded DNA intermediate.

Reverse transcription is believed to occur within a semi-intact viral capsid in the cellular cytoplasm to protect the viral genome and its DNA intermediate from recognition by cellular sensors (reviewed in (Arhel, 2010)). During or after completion of reverse transcription, the viral capsid further uncoats at or near nuclear pores, where viral DNA (vDNA) and other factors comprising the pre-integration complex (PIC) enter the nucleus. Once in the nucleus, a PIC component also encoded by the *pol* gene, known as integrase (IN), facilitates nonspecific incorporation of vDNA into the cellular genomic DNA by a process known as integration (**Figure 1.5**). Integration of vDNA, now called a provirus, occurs relatively nonspecifically and enables sheltering and replication of HIV-1 using cellular machinery (reviewed in (Bushman and Craigie, 1991)).

Active and latent infection

Upon integration of vDNA into the host genome, the provirus may either continue its replication in an active infection or remain dormant in a process known as latency. Latent proviruses are mediated at least in part by histone modifications and other epigenetic factors that restrict transcriptional accessibility (reviewed in

(Hakre et al., 2011)). By remaining latent, HIV-1 can effectively survive in the presence of effective immune responses and ART. The pool of latently-infected cells within an individual is termed the latent reservoir. Latent reservoirs mediate HIV-1 persistence and enable viral rebound after discontinuation of ART (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997). Strategies to reverse latency in these reservoirs to render them susceptible to eradication are currently being investigated (Archin et al., 2012). In addition to latency reversal driven by therapeutic interventions, latency can be reversed by naturally occurring means through reactivation of viral transcription.

During active infection and reactivated latent infection, the cellular RNA polymerase machinery is recruited to the provirus by promoter elements in the LTR to transcribe viral genes into a single mRNA transcript. The viral mRNA is alternatively spliced to produce many variants, ultimately leading to translation of different viral gene products (Stoltzfus and Madsen, 2006). Among the earliest gene products are the viral regulatory proteins, Tat and Rev. Tat, a *trans*-activator of viral transcription, cooperates with cellular transcription factors to promote transcription of viral gene products (Ott et al., 2011). Rev mediates export of unspliced and partially spliced viral RNAs from the nucleus into the cytoplasm to enable translation of viral proteins and packaging of gRNA into nascent virions (Pollard and Malim, 1998).

HIV-1 assembly and release

Upon transcription and translation of viral genes during active infection, HIV-1 structural proteins Gag, Env and Pol are produced to facilitate virion assembly. Gag is the main driver of assembly. Gag is synthesized as a polyprotein known as pr55, which contains multiple distinct domains, including matrix (MA), capsid (CA), nucleocapsid (NC), and p6 (reviewed in (Freed, 1998)). Gag pr55 contains a myristoyl head group that mediates its interaction with lipid membranes, and Gag is specifically targeted to the plasma membrane by interactions between a highly-basic region in MA with phosphoinositol-(4,5)-bisphosphate (PIP_2) and RNA (Ono et al., 2004; Ono et al., 2000; Ono and Freed, 1999). At the plasma membrane, Gag multimerizes via interactions mediated by CA and NC (Ono et al., 2000). Multimerization enables Gag to drive membrane curvature and begin to bud from the cell surface (**Figure 1.6**). Interactions between NC and a packaging signal (*psi*) in the viral gRNA enable the viral genome to be packaged within the budding virion (Clever et al., 1995). Cellular ESCRT machinery interacts with Gag p6 at the plasma membrane, enabling budding virions to pinch off and release from the infected cell (von Schwedler et al., 2003).

Late during virion assembly or after release of nascent virions from infected cells, the HIV-1 protease (PR), encoded by *pol*, mediates proteolytic cleavage of Gag pr55 into its processed subunits, MAp17, CAp24, NCp7, and p6. This allows physical separation of the viral capsid core, formed by CAp24, from the viral lipid envelope; without being processed by PR, virions are unable to mediate infection of target cells because the capsid core cannot be released into the cytoplasm upon viral fusion (Peng et al., 1989).

The Env glycoprotein is synthesized as a precursor, gp160, which is processed into its gp120 and gp41 subunits by host furin protease in endoplasmic reticula (Hallenberger et al., 1992). The processed forms of Env interact to form trimers that are retained within cellular membranes by a transmembrane domain in gp41 (Liu et al., 2008). Incorporation of Env into virions is required for infectivity, but is not required for virion formation; Gag alone is sufficient for assembly of virus-like particles (Gheysen et al., 1989). Env trimers are incorporated into budding virions by a relatively poorly-defined process (Checkley et al., 2011). It is known that regions within Gag MA and Env gp41 cytoplasmic tail are required for Env packaging, but it remains unclear whether a direct or indirect interaction between Gag and Env mediates Env incorporation (Tedbury and Freed, 2014; Yu et al., 1992). Env trimers on the surface of infected cells may interact with CD4 on neighboring cells to initiate recruitment of Gag and assembling virions to intercellular contact sites enriched for Env (Jolly et al., 2004). This mechanism has direct implications for HIV-1 spread.

Cell-to-cell spread of HIV-1

In vivo, HIV-1 is believed to spread between cells primarily by a direct, contact-dependent mechanism, termed cell-to-cell spread (Sattentau, 2008). This mode of spread contrasts with cell-free infection, in which free-floating virions attach to and enter target cells. Direct cell-to-cell spread is believed to be more efficient than cell-free infection for several reasons.

Cell-to-cell spread of HIV-1 is mediated by a structure known as the virological synapse (VS, **Figure 1.7**). This structure is formed by contact between Env on the surface of infected cells and CD4 on the surface of target cells (Jolly et al., 2004). This Env-dependent VS formation leads to actin cytoskeleton rearrangements in both the producer and target cells and a recruitment of Env and CD4 to the contact site. Virions assemble and release efficiently at VS contact sites, enabling a high multiplicity of infection (MOI) transmission directly into the target cell (Del Portillo et al., 2011). Moreover, virions released at VS contact sites are resistant to immune recognition and neutralization by some antibodies (Schiffner et al., 2013), and this mode of spread is also relatively resistant to blockade by some antiretroviral inhibitors (Duncan et al., 2013; Sigal et al., 2011). For these reasons, cell-to-cell spread of infection is more efficient than cell-free infection and is believed to contribute significantly to HIV-1 spread in vivo.

HIV-1 pathogenesis and current treatments

Transmission

HIV-1 can transmit between individuals in several ways. The virus is spread through horizontal routes, in which HIV-1-containing bodily fluids contact blood or mucosal tissue of another person, as well as through vertical transmission from mother to neonate during childbirth or breastfeeding (Bryson, 1996). Horizontal transmission of HIV-1 frequently occurs between intravenous drug users who share needles (Chaisson et al., 1987). Before HIV-1 screening became prevalent, contaminated blood transfusions also contributed significantly to viral transmission,

especially in patients being treated for hemophilia with pooled plasma from many sources (Ragni et al., 1987). In addition to blood-to-blood contact, HIV-1 is also communicable through sexual intercourse. HIV-1 is shed in the semen and is capable of establishing infection through both vaginal and anal intercourse (Baggaley et al., 2010; Boily et al., 2009). Sexual transmission is enhanced by factors in the semen, including amyloid fibrils that promote efficient HIV-1 attachment to target cells (Munch et al., 2007). Concurrent infection by other sexually-transmitted pathogens, especially those that cause open sores or lesions, can also increase the risk of sexual transmission of HIV-1 (Cohen, 1998). Sexual transmission of HIV-1 can be effectively prevented by barrier methods, including condom use (Weller and Davis, 2002). Unfortunately, such practices are often impeded by societal or religious views in many areas most affected by HIV-1. Recent studies have shown efficacy of using antiretroviral drugs as pre-exposure prophylaxis, providing an additional means of prevention (Celum and Baeten, 2012). Moreover, ART-treated patients generally suppress viral replication to sufficiently low levels to prevent vertical and/or sexual transmission (Granich et al., 2010). Vertical transmission from mother to child can occur through various routes, but reduced transmission in Caesarean surgical births suggests that many vertical transmission events occur during vaginal childbirth (Dunn et al., 1994).

One of the major barriers to ending preventable HIV-1 transmission is that many individuals are unaware of their infection status (Hall et al., 2012). Upon initial infection, symptoms vary between individuals and are often generic, including fever, rash, swollen lymph nodes and other flu-like symptoms (Hecht et al.,

2002). It is easy to confuse the symptoms of HIV-1 infection with other, more common afflictions, and thus HIV-1 continues to spread even in areas in which testing, protections and treatments are prevalent.

The biological mechanisms of HIV-1 transmission remain incompletely characterized. For instance, it is unclear which cell type first becomes infected by HIV-1 upon transmission. One hypothesis is that macrophages are among the first infected cell type, and can recruit $CD4^+$ T lymphocytes through their antigen-presenting immune functions and transmit virus through cell-to-cell spread (Koppensteiner et al., 2012). Consistent with this hypothesis, vaginal macrophages are permissive to HIV-1 infection (Shen et al., 2009), and early studies suggested that macrophage infection during transmission is essential because transmitted viruses are almost always CCR5-tropic, and CCR5-tropic viruses are often observed to preferentially infect macrophages (Kedzierska and Crowe, 2002). Later on during HIV-1 infection, a variety of HIV-1 isolates can be obtained from a patient, many of which utilize CXCR4 as a co-receptor and can infect $CD4^+$ T lymphocytes but not macrophages (Regoes and Bonhoeffer, 2005). However, more recent evidence has challenged this hypothesis by demonstrating that many CCR5-tropic HIV-1 clones can also infect $CD4^+$ T lymphocytes, and that transmitted HIV-1 clones are often poorly macrophage-tropic (Ochsenbauer et al., 2012). Moreover, a recent study in SIV-infected macaques called into question whether mucosal tissue-resident macrophages are permissive to infection *in vivo* (Calantone et al., 2014). This study suggests that HIV-1 DNA isolated from macrophages may be attributable to phagocytosis of HIV-1-infected T lymphocytes. In contrast, another recent report

has shown that such phagocytosis actually enables productive HIV-1 infection of macrophages (Baxter et al., 2014). Thus, more research is needed to better define initial transmission events and the potential role of macrophages.

Viremia

During acute HIV-1 infection, the virus rapidly spreads through the body and establishes high titers in the bloodstream (Busch and Satten, 1997). During this phase of infection, $CD4^+$ T lymphocytes are rapidly depleted as a result of the spreading viral infection. The virus typically elicits a strong immune response from the infected individual that reduces viral loads substantially and enables a partial recovery of $CD4^+$ T lymphocyte counts (Moss and Bacchetti, 1989). In a rare population of HIV-1-infected people termed 'long-term nonprogressors' or 'controllers', this response is sufficient to control the infection indefinitely, and treatment interventions are not required to prevent disease or transmission in these individuals (Gea-Banacloche et al., 2000). The mechanisms governing effective immune control in these individuals are the subject of active investigation and may guide the development of an effective vaccine or functional cure. Another small subset of the population carries a mutation in CCR5 that renders cells impenetrable to HIV-1 (Huang et al., 1996). These individuals are able to be exposed to HIV-1 repeatedly without becoming themselves infected. However, aside from these rare groups of individuals, HIV-1 ultimately evades immune control to cause a persistent and progressive immunodeficiency. In the absence of treatment, spread continues to occur in the presence of immune responses, and

viremia continues to be detectable in the bloodstream (Trono et al., 2010). This spread is believed to primarily consist of cell-to-cell spread between T lymphocytes and from macrophages to T lymphocytes, which enable HIV-1 to evade antibody-mediated neutralization (Schiffner et al., 2013).

Persistence

HIV-1 establishes life-long infection in most infected individuals. Immune escape is critical to the ability of HIV-1 to persist *in vivo*. While the mechanisms of HIV-1 immune escape remain an area of active investigation, several pathways to persistence have been described in the literature. The HIV-1 reverse transcriptase is notoriously error-prone, allowing for the creation of a swarm of genetically diverse virus (Goodenow et al., 1989; Hubner et al., 1992). This diversity allows a subset of HIV-1 clones to persist despite the immune-mediated clearance of a majority of viral quasispecies. This is best evidenced by an array of common escape mutants that are selected for by HIV-specific cytotoxic T lymphocyte (CTL) responses (Leslie et al., 2004). Although such mutations often reduce the replication capacity of HIV-1, the virus typically persists to deplete immune cells and ultimately overcome antiviral responses to cause disease.

Even in persons whose HIV-1 is effectively suppressed by antiretroviral drugs, the virus quickly rebounds after cessation of treatment (Chun et al., 2010). Such persistence is attributed primarily to the latent reservoir, which prevents effective clearance of HIV-1 from the body. Consistent with this notion, the stable amount of virus present after insufficient immune control, known as the 'set point'

viral load, correlates with the size of the latent viral reservoir and with the speed of disease progression (Masel et al., 2000). Current high-priority research seeks to eliminate or reduce the latent reservoir in treated patients to afford a functional cure of persistent infections. However, a major barrier is that such reservoirs are incompletely defined. Resting memory $CD4^+$ T lymphocytes are believed to comprise a substantial proportion of the latent reservoir (Siliciano et al., 2003). Within this subset, effector memory and central memory cells have been shown to contribute to persistence through reactivation after cessation of treatment. In addition, recent evidence suggests that memory T lymphocytes with stem-cell like properties may also contribute to persistence through long-lived replenishment of T cell pools with integrated latent provirus (Buzon et al., 2014). Similarly, other groups propose that long-lived hematopoietic stem and precursor cells in the bone marrow may also contribute to long-term persistence (McNamara and Collins, 2011). It is also possible that low-level viral replication in cells and/or parts of the body with sub-optimal drug concentrations may contribute to persistence, at least in some patients (Zhang et al., 1999). For instance, infected macrophages, in addition to supporting long-term latent infection (Watters et al., 2013), are able to cross the blood-brain barrier and contribute to HIV-1 replication and neuropathological sequelae (Burdo et al., 2013). Macrophages require higher concentrations of antiretroviral drugs to effectively block low-level replication (Jorajuria et al., 2004; Perno et al., 1998), and may contribute to macrophage-to-T lymphocyte spread in the presence of antiretroviral treatment (Duncan et al., 2013). In sum, many cell types and mechanisms potentially contribute to viral persistence

and improved understanding of such mechanisms will facilitate improved treatment strategies.

Immunodeficiency

After years of persistent, untreated infection, HIV-1 causes gradual decline of $CD4^+$ T lymphocyte counts. Because $CD4^+$ T lymphocytes are critical mediators of adaptive immunity, depletion of these cells below a functional threshold renders one susceptible to an array of fatal opportunistic infections (Pantaleo and Fauci, 1996). These infections can be caused by microbes that are normally innocuous to individuals with intact immune systems, including commensal strains of bacteria, viruses and fungi. Such opportunistic infections were a defining hallmark of the AIDS epidemic as it emerged in the 1980s. Although $CD4^+$ T lymphocytes, the major target cell of HIV-1, are profoundly depleted by the infection, the virus continues to replicate even when very few T lymphocytes remain; macrophages and other target cell types are believed to contribute significantly to replication during this stage of infection (Orenstein et al., 1997). Despite the advent of antiretroviral drugs that prevent this late stage of HIV-1 disease, this immunodeficiency still affects many people in many parts of the world. In fact, HIV-1 is still responsible for over one million global deaths annually.

Antiretroviral therapy

Although there is no preventative vaccine or cure for HIV-1, antiretroviral drugs that target viral fusion, reverse transcription, integration, and protease-

mediated maturation exist and are used in combination therapies to block the spread of HIV-1 within infected individuals or, more recently, to prevent establishment of infection in uninfected, at-risk individuals. However, because of HIV-1 persistence as described above, HIV-infected people must take these drugs daily for life, and cessation of treatment leads to rebound of infection, progression to AIDS, and ultimately, death. This major shortcoming of currently available treatments is made worse by the serious side-effects of antiretroviral treatments, including lipodystrophy, heart disease, diarrhea, rash, nausea and vomiting (Carr and Cooper, 2000). For these reasons, it is imperative that scientists continue to study HIV-1 and elucidate the mechanisms of viral persistence and immune escape in order to develop better treatment alternatives.

HIV-1 accessory proteins counteract immunity

Nef adapts trafficking adaptors to evade CTL responses

To establish a successful infection, intracellular pathogens must evade CTLs, which recognize foreign antigens presented in association with host major histocompatibility complex class I (MHC-I). One way that HIV-1 achieves this goal is through the activity of the accessory protein negative effector factor (Nef). Nef enhances the survival of infected cells in the presence of CTLs by mislocalizing and degrading MHC-I (Collins et al., 1998; Schwartz et al., 1996). To accomplish this, Nef stabilizes an interaction between MHC-I and the clathrin adaptor protein-1 (AP-1), which regulates clathrin-dependent trafficking of proteins between the trans-Golgi network and endosomes. When stabilized in this complex by Nef, AP-1 directs MHC-

I to the endolysosomal pathway where it is degraded at an accelerated rate (Roeth et al., 2004). Biochemical and structural analysis have revealed that a critical tyrosine residue in the MHC-I cytoplasmic tail mediates the interaction with the tyrosine-binding pocket in the μ1 subunit of AP-1 (Jia et al., 2012; Le Gall et al., 1998; Wonderlich et al., 2008) (**Figure 1.8A**). While this tyrosine can weakly bind AP-1 in some cell types (Kulpa et al., 2013), a complex containing MHC-I and AP-1 is normally not detected in T lymphocytes. This is primarily because the MHC-I cytoplasmic tail tyrosine does not conform to a canonical AP-1 tyrosine signal in which there is a downstream hydrophobic amino acid (Yxxφ). Nef stabilizes the weak interaction between MHC-I and AP-1 by providing additional contacts with AP-1 and with the MHC-I cytoplasmic tail. Specifically, an acidic cluster in Nef forms an electrostatic interaction with positively charged residues of AP-1 μ1 (Jia et al., 2012). In addition, polyproline (PxxP) repeats in Nef lock the MHC-I cytoplasmic tail onto μ1 (**Figure 1.8A**) (Jia et al., 2012).

Interestingly, Nef also interacts directly with clathrin adaptor proteins AP-1, AP-2, and AP-3 through a canonical dileucine trafficking signal in Nef's C-terminal loop domain (Wonderlich et al., 2011). By simultaneously binding to host protein cytoplasmic tails and clathrin adaptor proteins, Nef facilitates the down-modulation of a number of host proteins from the cell surface, including CD4 (**Figure 1.8B**) (Leonard et al., 2011). Down-modulation of CD4, the main cellular receptor for HIV entry, enables HIV-1 to avoid Env-CD4–mediated retention of virions at the cell surface and promotes efficient virus release and dissemination (Lama et al., 1999; Ross et al., 1999).

Vif, Vpu and Vpr adapt ubiquitin ligase adaptors to counteract antiviral responses

Ubiquitination is a post-translational protein modification that regulates protein degradation and trafficking. Cellular E3 ubiquitin ligases facilitate the transfer of ubiquitin from E2 ubiquitin-conjugating enzymes to lysine, serine, or threonine residues on specific target proteins. E3 ligases often comprise multi-protein complexes that include a scaffold, an adaptor, and a target protein substrate. By serving as substrate adaptors that simultaneously interact with ubiquitin ligase adaptors and cellular target proteins, three HIV accessory proteins (Vif, Vpu, and Vpr) induce ubiquitination of host targets. This leads to proteasomal degradation and/or mislocalization of targeted host proteins. For example, viral infectivity factor (Vif), an accessory protein encoded by primate lentiviruses, including HIV-1, counteracts the antiviral activities of apolipoprotein B mRNA editing complex 3 (APOBEC3, or A3) proteins, especially APOBEC3G (A3G) (Sheehy et al., 2002). A3 deaminases, which attack single-stranded DNA converting cytidine to uridine, have broad antiviral functions (Malim, 2009). In the absence of Vif, A3G-mediated cytidine deamination results in uridination of the first strand of DNA synthesized by the viral reverse transcriptase. Guanosine-to-adenosine hypermutation results as uridine residues are paired with adenosine upon second strand synthesis. There is also evidence that A3G has a separate inhibitory effect on the processivity of reverse transcription (Malim, 2009). In HIV-1-infected T cells, A3G activity can induce a DNA damage response that stimulates up-regulation of natural killer (NK) cell-activating ligands on the surface of the infected cells and activates NK cell lysis of

infected cells (Norman et al., 2011). To evade A3-mediated responses, the HIV-1 Vif protein simultaneously binds A3G and the ubiquitin ligase adaptor EloBC, causing polyubiquitination by the Rbx2/Cullin5 E3 ubiquitin ligase complex (**Figure 1.9A**) (Yu et al., 2003). An additional cellular protein, core binding factor β (CBF-β), stabilizes the formation of this complex (Jager et al., 2012; Zhang et al., 2012). By driving the ubiquitin-dependent degradation of A3 family members, Vif enables viral escape from A3-mediated antiviral restriction. The critical importance of A3G as a cellular factor that restricts lentiviruses is evidenced by coevolution of Vif and A3G sequences (Compton et al., 2012).

Another example of this tactic is displayed by HIV-1 viral protein U (Vpu). This accessory protein promotes virus release by counteracting the antiviral activities of the interferon-induced restriction factor bone marrow stromal antigen 2 (BST-2/tetherin) (Neil et al., 2008; Van Damme et al., 2008). Vpu also down-modulates the HIV-1 receptor CD4 (Harris et al., 2012). In the absence of Vpu, CD4 and BST-2 inhibit the release of infectious viral particles. CD4 binds virions through interactions with Env glycoproteins, and BST-2 tethers virions by virtue of its unusual structure. The general consensus of a number of studies is that BST-2 is attached to membranes via its transmembrane domain at its N-terminus and via its C-terminal glycophosphatidylinositol anchor. By simultaneously binding to viral and cellular membranes, BST-2 tethers virions, preventing their release. To evade BST-2, Vpu acts as an adaptor that promotes an interaction between a ubiquitin ligase substrate adaptor [beta transducing repeat-containing protein (β-TrCP)] and target proteins. In this way, Vpu promotes ubiquitination of the target protein by

Skp1/Cullin1/F-box (SCF) ubiquitin ligase complex (**Figure 1.9B**) (Harris et al., 2012; Margottin et al., 1998). Recent studies have expanded the role of BST-2 to include viral sensing and signal transduction to activate NF-κB–dependent pro-inflammatory signals (Romani and Cohen, 2012). Like A3, the significance of BST-2–mediated restriction is illustrated by co-evolution of lentiviral genomes with species-specific variations in BST-2. In this regard lentiviruses have demonstrated remarkable flexibility. While HIV-1 utilizes Vpu to target BST-2, most primate lentiviruses use Nef for this purpose, and still others can use Env (Harris et al., 2012). In this way, BST-2 variation appears to serve as a barrier to cross-species infection.

Viral protein R (Vpr) is a pleiotropic lentiviral accessory protein that has been shown to activate the DNA damage response, up-regulate NK activating ligands, cause cell-cycle arrest, and promote infection of macrophages (Romani and Cohen, 2012). Like Vif and Vpu, Vpr adapts a substrate adaptor of a cellular ubiquitin ligase complex [damaged DNA binding protein 1-cullin 4-associated factor 1 (DCAF1)], promoting ubiquitination by a ubiquitin ligase complex (Rbx1/Cullin4A E3, **Figure 1.9C**) (Romani and Cohen, 2012). One protein targeted by Vpr is the cellular uracil DNA glycosylase 2 (UNG2) (Schrofelbauer et al., 2005). However, the precise role of UNG2 remains controversial as it has both positive and negative effects on HIV-1 replication (Mashiba and Collins, 2013). Because the interaction with UNG2 does not appear to explain all of Vpr's activities, it is likely that Vpr targets additional cellular proteins that have not yet been identified. A structurally related accessory protein, viral protein X (Vpx), which is encoded by HIV-2 and

some viruses of the simian immunodeficiency virus (SIV) family, but not by HIV-1, also interacts with the DCAF1 to promote the degradation of a cellular nucleotide triphosphate phosphohydrolase [SAM domain and HD domain-containing protein 1 (SAMHD1)] (Laguette et al., 2011). In the absence of Vpx, SAMHD1 inhibits reverse transcription by depleting the intracellular pool of deoxynucleoside triphosphates (Lahouassa et al., 2012). Vpx-mediated polyubiquitination of SAMHD1 induces its proteasomal degradation and allows viral replication in myeloid cells (Laguette et al., 2011). Like the other pairs of viral accessory protein and cellular targets described thus far, Vpx has co-evolved with SAMHD1 from different primate species, acquiring the capacity to utilize different SAMHD1 molecular interfaces to promote its degradation (Fregoso et al., 2013).

The role of Vpr in HIV-1 infection

The importance of Vpr for HIV-1 infection is evidenced by its conservation among all primate lentiviruses (Planelles et al., 1996; Stivahtis et al., 1997). Moreover, Vpr mutations have been associated with long-term nonprogressors (Lum et al., 2003) and studies using a simian animal model have demonstrated that Vpr is important for disease progression and maximal infection of T lymphocytes (Hoch et al., 1995; Lang et al., 1993). Despite its importance, the molecular mechanism underlying Vpr function has eluded researchers for decades (Guenzel et al., 2014). This is partially because Vpr is not required for infection of transformed cell lines in vitro. In fact, Vpr can be detrimental to replication in dividing cells and is rapidly selected against upon serial passage in cell culture (Balliet et al., 1994;

Planelles et al., 1995; Rogel et al., 1995). Given this, it is not inherently obvious how Vpr can enhance infection of T lymphocytes, a dividing cell type, in vivo. Indeed, infection of primary T lymphocytes in vitro is not enhanced by Vpr under standard infection conditions (Balliet et al., 1994). Despite the mystery surrounding Vpr's precise function and mechanism of action, many Vpr activities have been reported and described.

Vpr induces cell cycle arrest in dividing cells

The observation that Vpr arrests cell division was among the first reported Vpr activities. In the presence of Vpr, dividing cells stall in the G2/M phase of the cell cycle (He et al., 1995; Re et al., 1995). Vpr arrests cell division in both HIV-1-infected cell lines and primary T lymphocytes (Jowett et al., 1995). A predominant early model of Vpr function was that Vpr may enhance HIV-1 replication in these cells by arresting cell division in the stage at which HIV-1 replication is most efficient (Goh et al., 1998). However, Vpr is not required for HIV-1 replication in these cells and is rapidly lost during serial passage due to its inhibitory effects on cell growth (Rogel et al., 1995). Thus, cell cycle arrest cannot by itself explain the evolutionary conservation and in vivo function of Vpr. Despite this, the study of Vpr-mediated cell cycle arrest has illuminated many details regarding Vpr's molecular interactions. For instance, cell cycle arrest was shown to require a previously uncharacterized cellular factor, called Vpr binding protein (VprBP), which was later named DCAF1 (Belzile et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Wen et al., 2007). This series of biochemical and functional studies

identified that Vpr interacts with DCAF1 to alter the cellular ubiquitination machinery. More specifically, Vpr interacts with the Cul4 E3 ubiquitin ligase through DCAF1 to adapt this complex to other Vpr binding partners (**Figure 1.9**). In this way, Vpr causes the polyubiquitination and proteasomal degradation of its binding partners. More recent work has identified several of these target proteins, including the uracil glycosidases UNG2 and SMUG1 (Schrofelbauer et al., 2005), the small RNA processing enzyme Dicer (Casey Klockow et al., 2013), and the endonuclease complex component MUS81 (Laguette et al., 2014), among others. While the exact contributions of these targets to Vpr's in vivo function remain unclear, MUS81 degradation in particular has been shown to be required for Vpr-mediated cell cycle arrest in cell culture (Laguette et al., 2014).

Vpr promotes viral replication in nondividing cells

In addition to inhibition of cell division, it has been reported that Vpr enhances spreading infection of nondividing, terminally-differentiated macrophages (Connor et al., 1995). Early evidence suggested a model by which Vpr facilitates nuclear import of the HIV-1 PIC (Heinzinger et al., 1994; Popov et al., 1998). Vpr is packaged within virions via interaction with Gag p6, suggesting that Vpr acts early in the HIV-1 replication cycle (Paxton et al., 1993). Moreover, Vpr remains associated with the PIC and localizes to the nucleus (Heinzinger et al., 1994; Yao et al., 1995). However, further studies demonstrated that nuclear import in nondividing macrophages occurs efficiently even in the absence of Vpr, and that this function of Vpr is redundant with functions of integrase and Gag MA (Haffar et al.,

2000). Thus, enhanced nuclear import of the PIC does not explain Vpr-mediated enhancement of HIV-1 replication in macrophages. This activity of Vpr remains poorly understood in both mechanism and importance.

Vpr counteracts induction of innate immunity

It was recently described that Vpr-mediated degradation of MUS81 causes premature activation of the SLX4 endonuclease complex, which causes cell cycle arrest in dividing cells and also prevents type I interferon induction (Laguette et al., 2014). Type I interferons, which include interferon-alpha and -beta, are innate immune cytokines that function as global mediators of antiviral immune responses (Basler and Garcia-Sastre, 2002). Innate immune sensing of HIV-1 is poorly understood process, but recent evidence suggests that HIV-1 replication intermediates are sensed by IFI16 and cGAS, leading to signaling through STING and induction of antiviral immune responses, which are also poorly defined (Gao et al., 2013; Jakobsen et al., 2013). Cell-intrinsic immune responses against HIV-1 have been described, but Vpr-mediated evasion of type I interferons has not yet been implicated in protecting HIV-1 from such factors (Malim and Bieniasz, 2012; Yan and Chen, 2012). Interestingly, cell cycle arrest may be a consequential off-target effect of Vpr's primary immunoevasive action, and this activity may be of particular importance in nondividing cells of innate immune lineage, including macrophages, which may be primed to sense and respond to HIV-1 infection.

Summary of dissertation

The work presented in the following chapters addresses some critical gaps in our understanding of the mechanism and role of Vpr in HIV-1 pathogenesis. First, in Chapter 2, the mechanism by which Vpr enhances spreading replication of HIV-1 in primary monocyte-derived macrophages is elucidated. A series of experiments demonstrate that Vpr functions to counteract a macrophage specific, type I interferon-stimulated antiviral restriction that causes Env degradation and impairs virion production. Next, Chapter 3 extends this work to illustrate the mechanism by which Vpr enhances HIV-1 infection of $CD4^+$ T lymphocytes. Work presented in this chapter demonstrates that infection of T lymphocytes by cell-free infection is inefficient, but that HIV-1 efficiently spreads from macrophages to T lymphocytes in a largely Vpr-dependent manner. Vpr accomplishes this by preventing lysosomal targeting of Env-containing virions and inhibition of Env-dependent virological synapse formation. Chapter 4 provides in-depth discussion of these results, their relevance, and future experiments that will continue to illuminate molecular mechanisms of intrinsic and innate antiviral immunity as well as viral countermeasures.

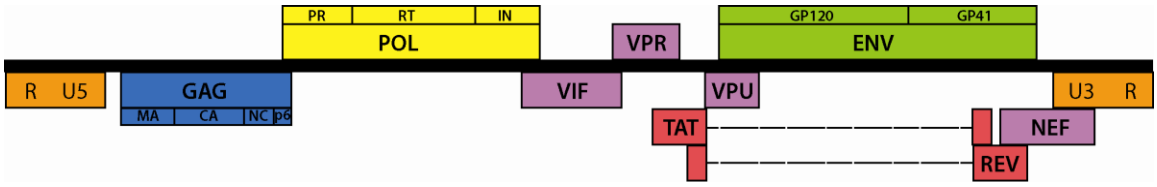

Figure 1.1. The HIV-1 genome. Graphical depiction of the relative organization of HIV-1 gene products. Black bar indicates continuous viral genomic RNA sequence. Regulatory genes are depicted in red and accessory genes in violet. U5, R, and U3 are regions of the long-terminal repeat.[2]

[2] This figure was created by David Collins.

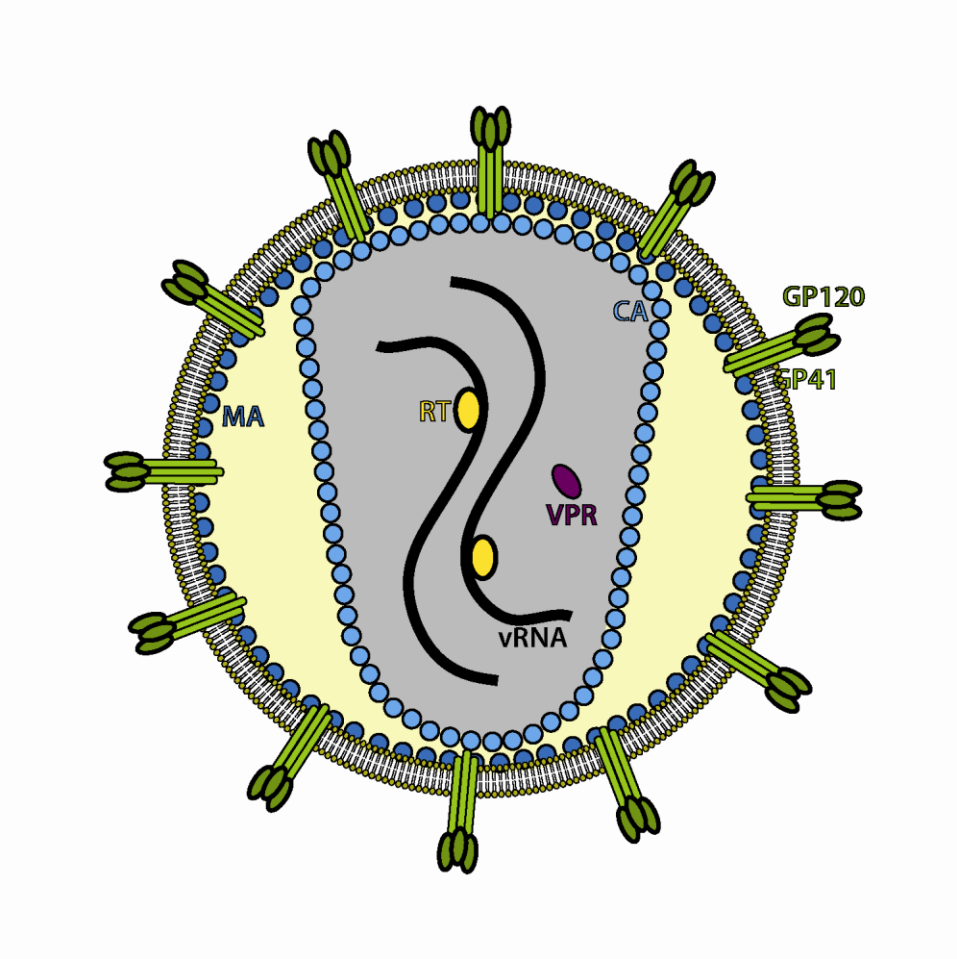

Figure 1.2. Structure of the HIV-1 virion. Graphical depiction of a typical HIV-1 virion. A cell-derived lipid envelope studded with viral Env glycoprotein trimers surrounds a capsid core formed by protease-mediated cleavage of Gag polyprotein into matrix (MA) and capsid (CA) components. Within the capsid core are two copies of the HIV-1 RNA genome (vRNA), as well as reverse transcriptase (RT) and Vpr, which packages into virions by association with Gag p6 (not shown).[3]

[3] This figure was created by David Collins.

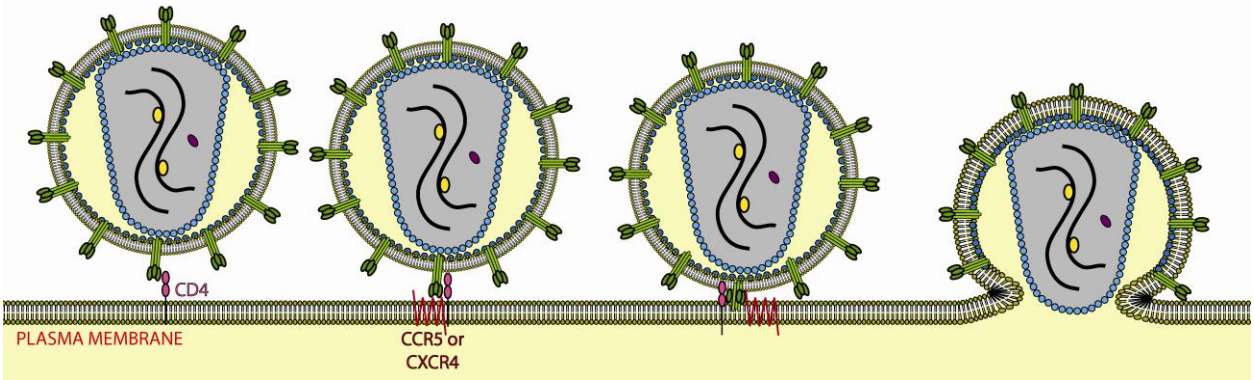

Figure 1.3. HIV-1 entry. Graphical depiction of the stages of HIV-1 entry, progressing from left to right. The first stage depicts attachment of Env gp120 (green) on the virion to CD4 (pink) on the cell surface. The second stage depicts Env gp120 further interacting with CD4 and an additional transmembrane chemokine receptor, either CCR5 or CXCR4 (Buchacher et al.). The third stage depicts a conformational change in Env that exposes the gp41 fusion peptide and enables formation of a six-helix bundle structure (not shown) that fuses the viral membrane with the cellular plasma membrane. The final stage depicts the viral capsid core being released into the cell cytoplasm.[4]

[4] This figure was created by David Collins.

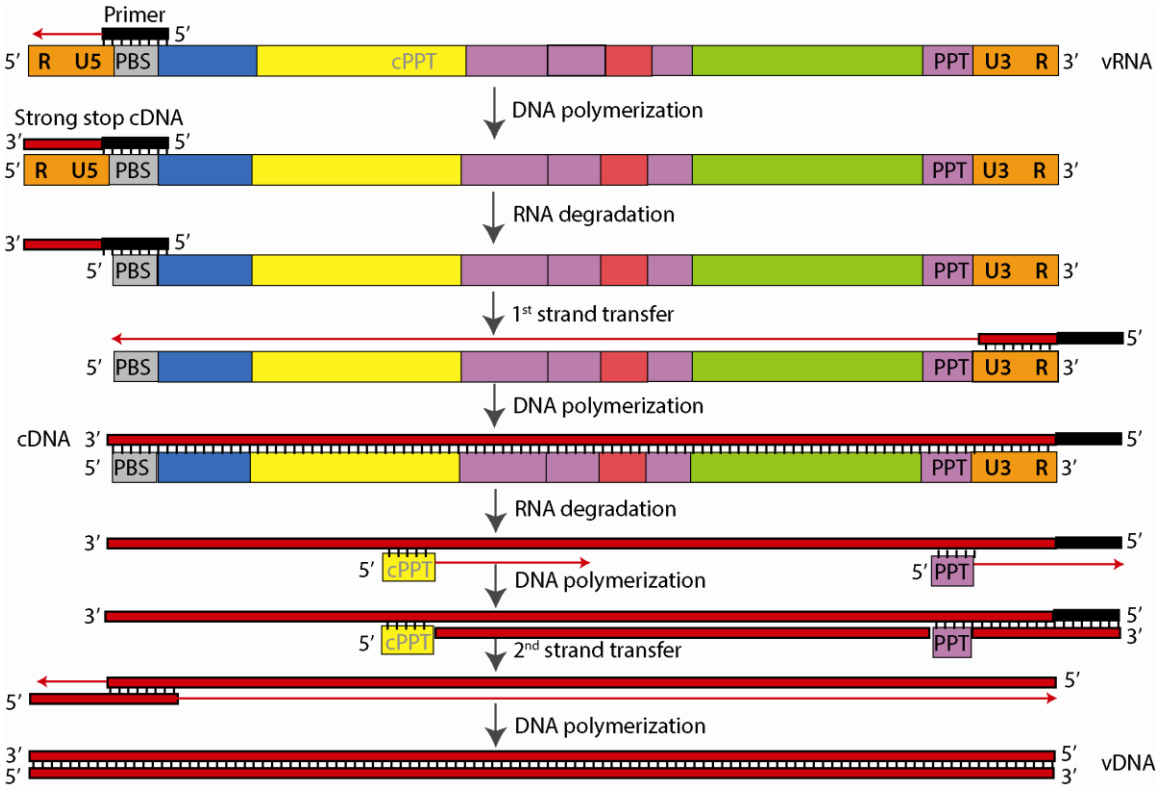

Figure 1.4. HIV-1 reverse transcription. Graphical depiction of the stages of HIV-1 reverse transcription, progressing from top to bottom, detailing the multi-step conversion from single-stranded viral genomic RNA (vRNA) to double-stranded viral DNA (vDNA, red strands). A tRNA primer binds to the vRNA primer binding site (PBS) and enables reverse transcriptase (RT, not pictured) to polymerize strong-stop cDNA from 5' to 3' using its RNA-dependent DNA polymerase activity. In the first strand transfer, the strong-stop cDNA anneals to the identical 3' LTR template sequence, allowing for polymerization of the full first-strand cDNA. The ribonuclease activity of RT degrades the vRNA template, leaving behind two polypurine-rich sequences (PPT and cPPT) that serve as primers for RT to polymerize a fragment of the second DNA strand via its DNA-dependent DNA polymerase activity. The second strand transfers to the 5' LTR cDNA sequence and allows RT to complete synthesis of the double-stranded vDNA.[5]

[5] This figure was created by David Collins.

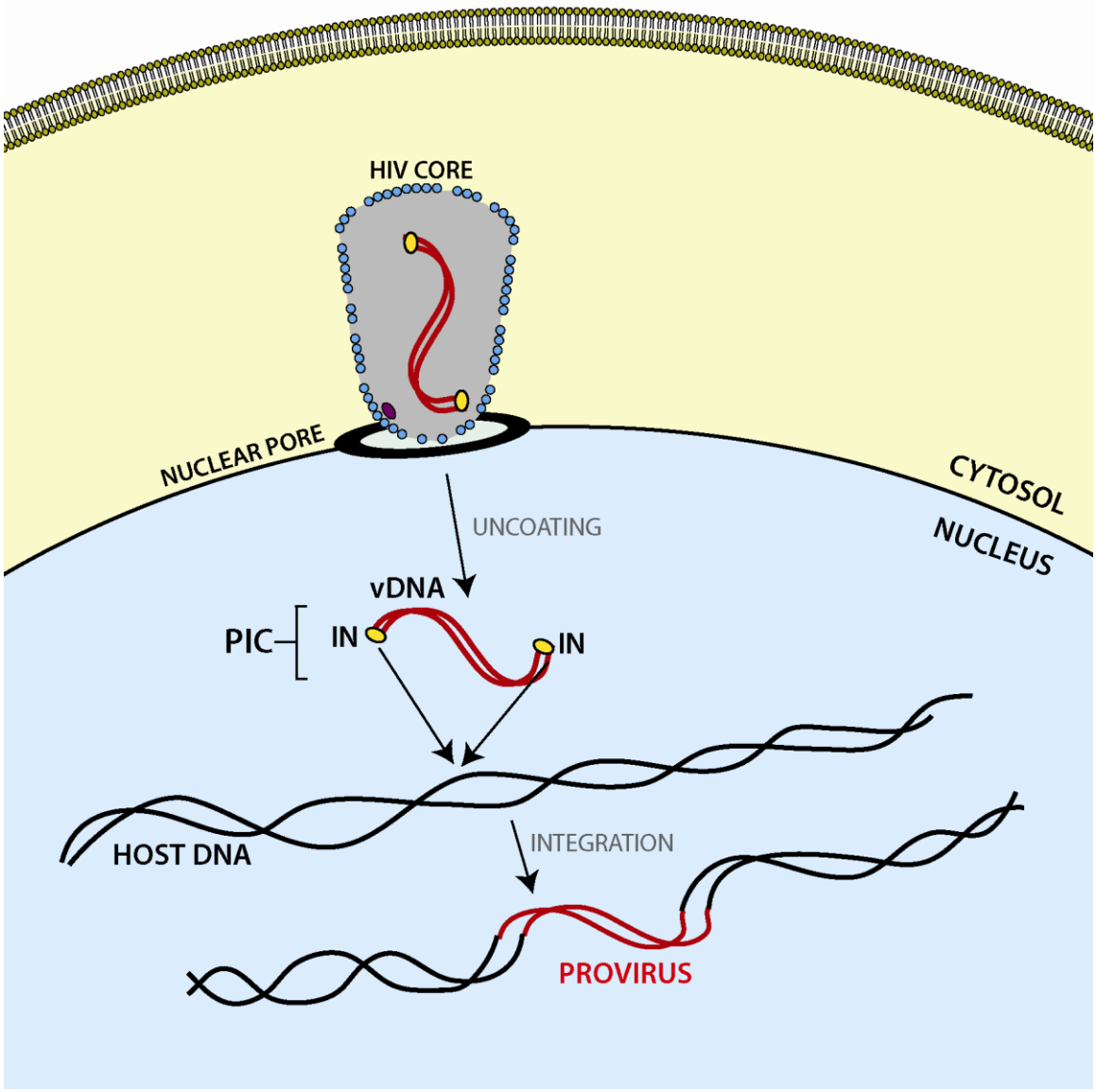

Figure 1.5. HIV-1 uncoating and integration. Graphical depiction of HIV-1 post-entry events that occur concurrent with and following reverse transcription, including viral uncoating, nuclear import, and viral integration, progressing from top to bottom. Upon entry of the capsid core into the cytoplasm, reverse transcription is believed to occur within a semi-intact core that enables protection from cellular antiviral factors. The viral capsid uncoats in the cytoplasm either before or during docking to the nuclear envelope. The viral DNA (vDNA), along with the integrase enzyme (IN), Vpr, and other factors, form a pre-integration complex (Leslie et al.) that enters the nucleus through a nuclear pore. Integrase catalyzes the insertion of vDNA into the host chromosomal DNA to form a provirus.[6]

[6] This figure was created by David Collins.

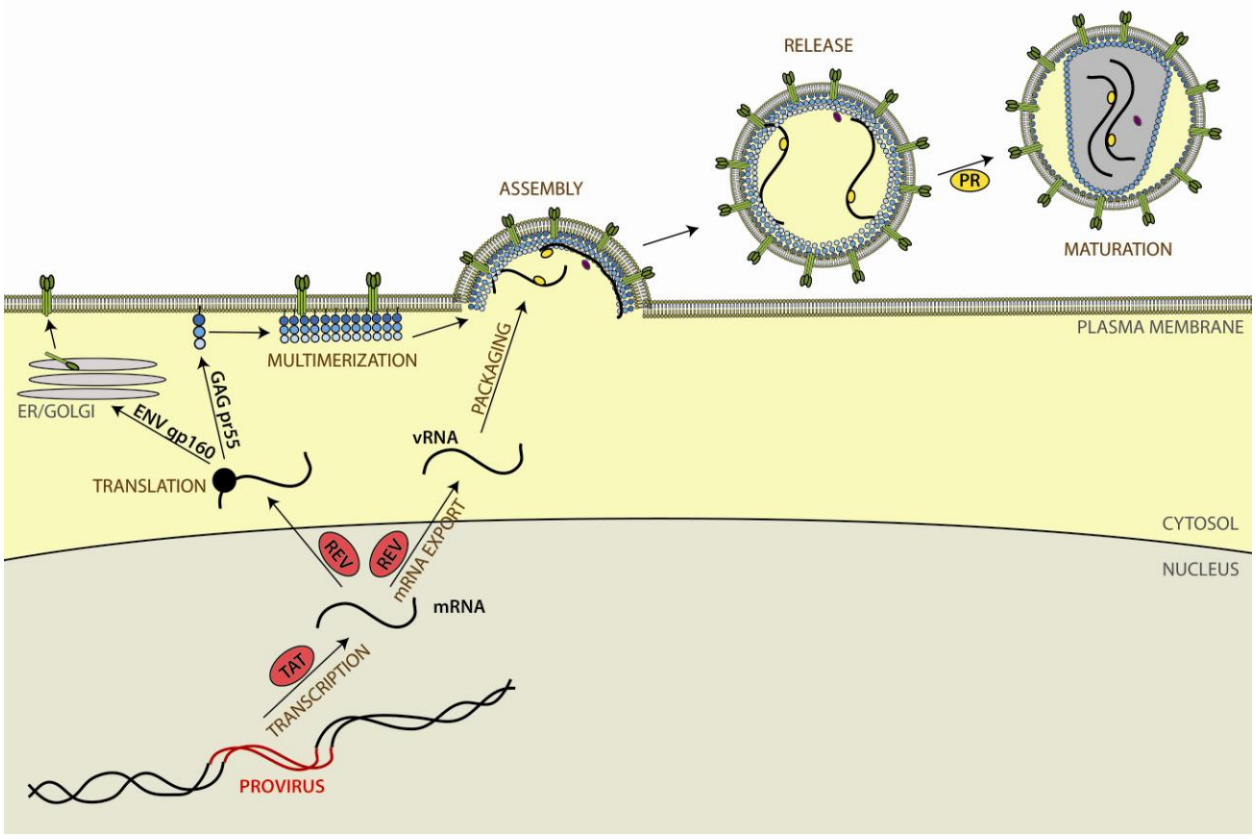

Figure 1.6. HIV-1 assembly. Graphical depiction of HIV-1 transcription, translation, viral assembly and release, progressing from bottom to top and from left to right. The provirus is transcribed in the nucleus by host machinery. The spliced mRNA enables translation of the viral regulatory proteins Tat and Rev. Tat enhances transcription of the provirus into RNA. Rev mediates nuclear export of unspliced and incompletely spliced mRNAs, which encode the HIV-1 structural proteins. In the cytoplasm, viral mRNAs are transcribed at cellular ribosomes to form viral proteins. Env is formed as a precursor (gp160) and cleaved by cellular proteases in the endoplasmic reticulum (ER) and Golgi apparatus to form mature heterodimeric trimers that can localize to the plasma membrane. Gag is also formed as a precursor polyprotein (pr55), which contains a myristoyl head-group that enables insertion into the plasma membrane. Domains within Gag pr55 enable multimerization, vRNA packaging, Env incorporation and membrane curvature. The immature virion releases from the cell surface via utilization of cellular ESCRT machinery (not pictured). Finally, the HIV-1 protease (PR) enzyme cleaves Gag pr55 into its mature proteins (maturation), enabling formation of the viral capsid within the viral lipid envelope.[7]

[7] This figure was created by David Collins.

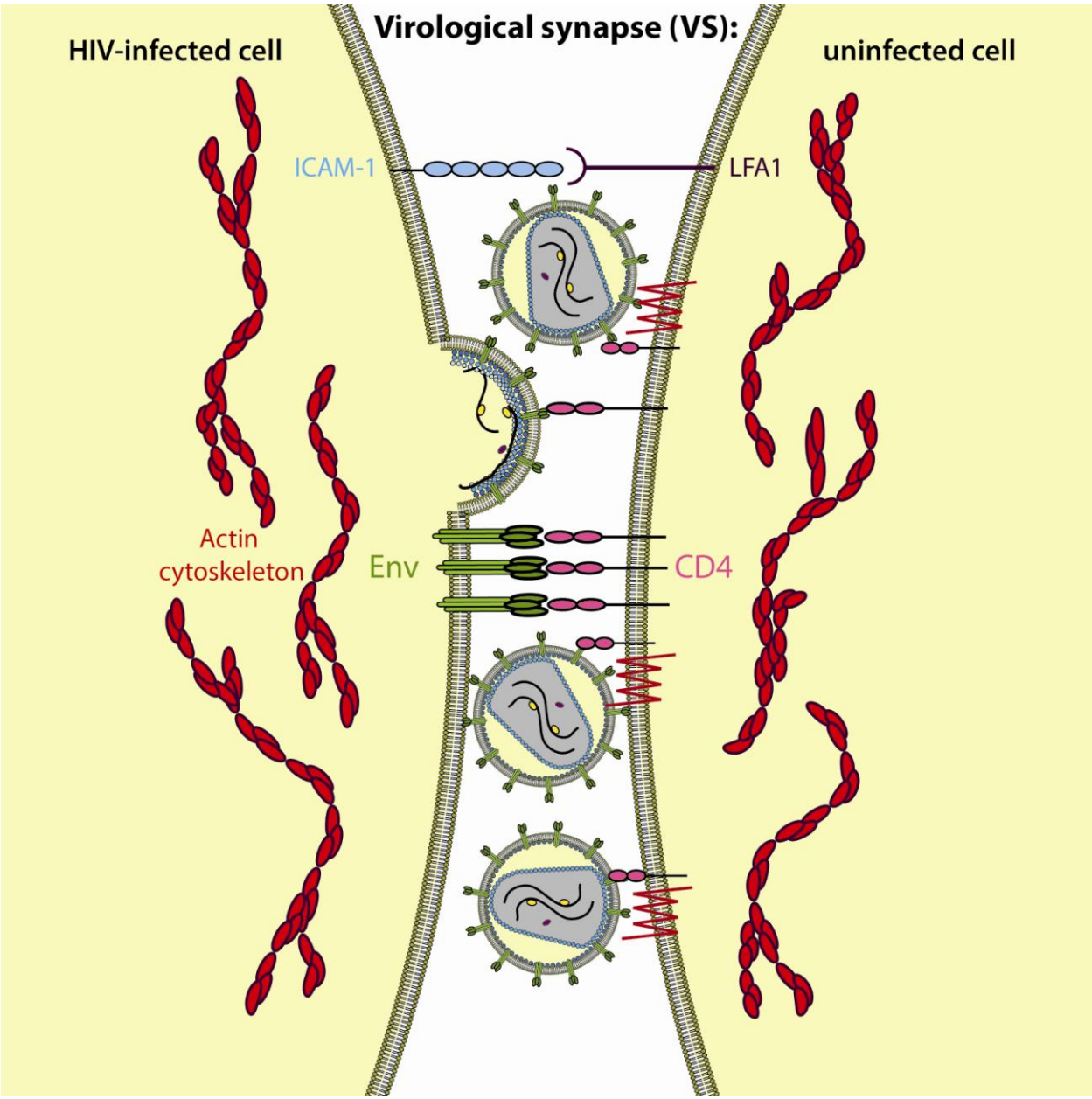

Figure 1.7. HIV-1 cell-to-cell spread. Graphical depiction of HIV-1 cell-to-cell spread across a virological synapse (VS), progressing from left to right. HIV-1 Env glycoproteins on the surface of an infected cell interact with CD4 on the surface of an uninfected cell to form a virological synapse. At this synapse, which is also stabilized by interactions between cell surface adhesion molecules ICAM-1 and LFA1, rearrangement of the actin cytoskeleton (red ovals) accompanies the efficient release of mature virions into the synapse, where high multiplicity spread of HIV-1 into the uninfected target cell is mediated by Env interactions with CD4 (pink) and chemokine co-receptors (red lines) to facilitate viral fusion and infection.[8]

[8] This figure was created by David Collins.

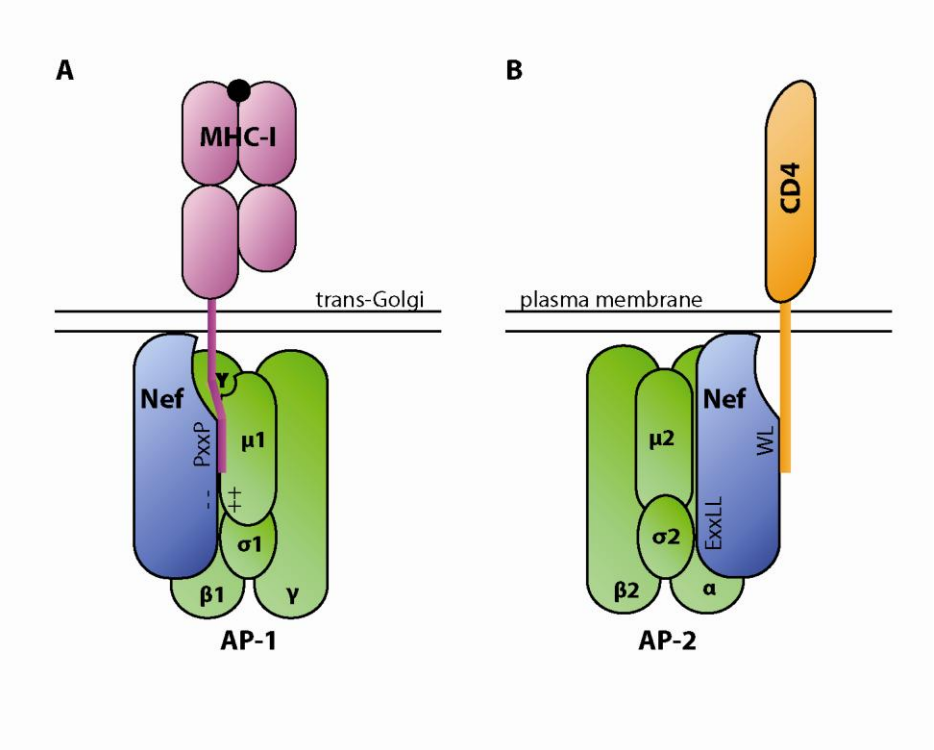

Figure 1.8. HIV-1 Nef adapts clathrin adapters. Graphical depiction of molecular complexes formed by HIV-1 Nef and cellular proteins. (**A**) The AP-1 μ1 subunit interacts with a tyrosine residue in the cytoplasmic tail of MHC-I via a tyrosine-binding pocket. This interaction is stabilized by electrostatic interactions between a poly-glutamic acid motif of Nef (--) and a positively charged patch in AP-1 m1 (++). A polyproline repeat of Nef (PxxP) further stabilizes the complex by forming a wall of the groove that contains the MHC-I tail. These interactions lead to downmodulation of MHC-I from the cell surface. (**B**) AP complexes interact with Nef via a dileucine motif (ExxLL) in the Nef C-terminal loop. AP complexes bind dileucine motifs at an interface between the AP complex σ and heavy chain subunits (α, β or γ in AP-2, AP-3 and AP-1 respectively). Nef utilizes the dileucine trafficking signal to downmodulate a number of host proteins including CD4.[9]

[9] This figure was created by David Collins.

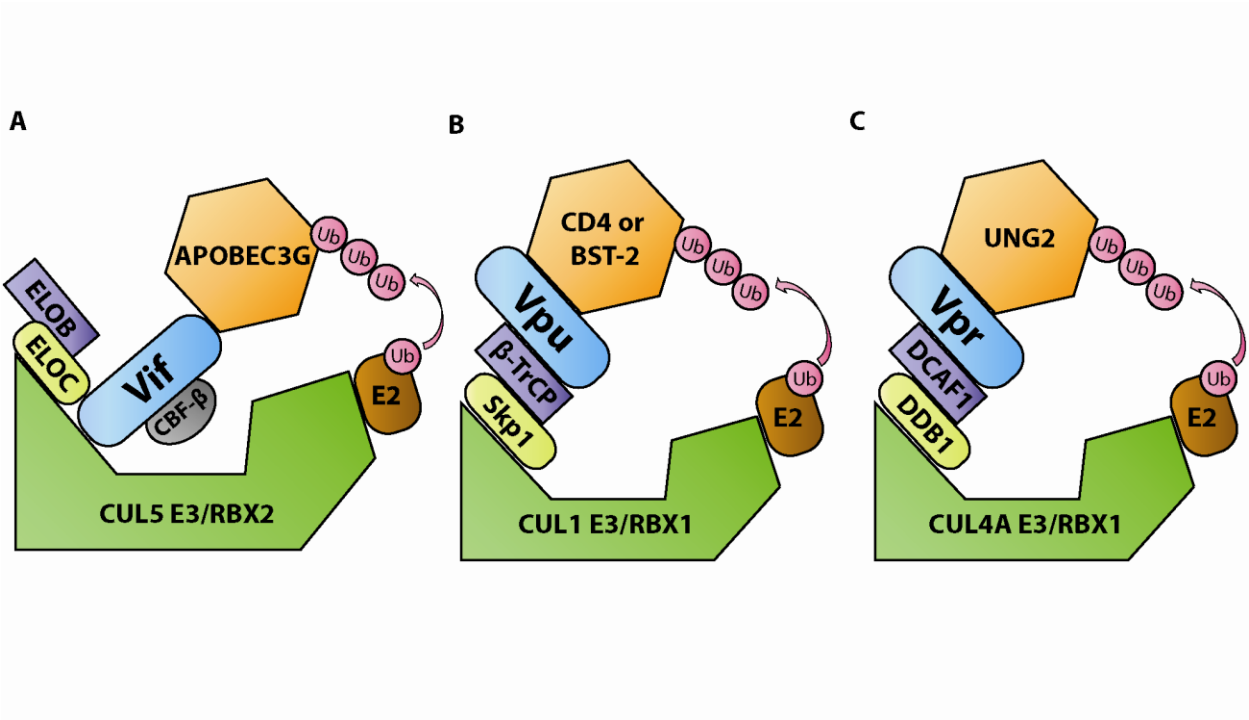

Figure 1.9. HIV-1 Vif, Vpr and Vpu adapt cellular ubiquitin ligase adapters. Graphical depiction of molecular complexes formed between HIV-1 Vif, Vpu or Vpr and cellular proteins. (**A**) Vif, in complex with and stabilized by cellular CBF-β, binds to the EloBC/Rbx2/Cullin5 E3 ubiquitin ligase complex and to APOBEC3G to induce its polyubiquitination and degradation. (**B**) Vpu interacts with the Skp1/Cullin/F-Box (SCF) ubiquitin ligase complex via β-TrCP, and with target proteins BST-2 or CD4 to induce their ubiquitination and mislocalization. (**C**) Vpr interacts with the DCAF1/DDB1/Rbx1/Cullin4A E3 ubiquitin ligase complex and with UNG2 to induce its polyubiquitination and degradation.[10]

[10] This figure was created by David Collins.

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Chapter 2

Vpr overcomes macrophage-specific restriction of Env expression and virion production[1]

Summary

The HIV-1 accessory protein Vpr enhances infection of primary macrophages through unknown mechanisms. Recent studies demonstrated that Vpr interactions with the cellular DCAF1-DDB1-CUL4 E3 ubiquitin ligase complex limit activation of innate immunity and interferon (IFN) induction. We describe a restriction mechanism that targets the HIV-1 envelope protein Env, but is overcome by Vpr and its interaction with DCAF1. This restriction is active in the absence of Vpr in HIV-1-infected primary macrophages and macrophage-epithelial cell heterokaryons, but not epithelial cell lines. HIV-1-infected macrophages lacking Vpr express more *IFN* following infection, target Env for lysosomal degradation, and produce fewer Env-containing virions. Conversely, Vpr expression reduces IFN induction, rescues Env expression, and enhances virion release. Addition of IFN or silencing *DCAF1* reduces the amount of cell-associated Env and virion production in wild-type HIV-1-infected

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primary macrophages. These findings provide insight into an IFN-stimulated macrophage-specific restriction pathway targeting HIV-1 Env that is counteracted by Vpr.

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-1 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 ubiquitylation 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 (MDMs) that have lower levels of SAMHD1 and APOBEC3A (Ayinde et al., 2010). Like Vpx, Vpr utilizes DCAF1 and the Rbx1/Cullin4A E3 ubiquitin ligase complex; however, some cellular targets of Vpr have only recently been identified, and their role in facilitating infection of restricted cell types is not well understood. Elegant studies performed in transformed cell-line systems demonstrated that Vpr activates the structure-specific endonuclease (SSE) regulator SLX4 complex through an interaction with DCAF1. 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 do not require Vpr for infection. Primary MDMs require Vpr for optimal spread, but the mechanism by which Vpr facilitates HIV-1 infection of macrophages has not yet been determined.

We characterized the molecular mechanism by which Vpr enhances HIV-1 infection in primary macrophages using three distinct HIV-1 molecular clones. In contrast to what is observed with Vpx-dependent SIV infection of immature monocytes, we found no effect of Vpr on the first round of infection. However, we noted a striking effect of Vpr on virions produced by infected MDMs, and we noted higher infection rates in subsequent rounds, particularly at low multiplicity of infection (MOI). Surprisingly, Vpr was needed for maximal virion production only when the HIV envelope protein (Env), which is incorporated into virions, was also expressed. Moreover, HIV-1-infected primary MDMs lacking Vpr had markedly reduced amounts of HIV-1 Env protein due to increased lysosomal degradation.

MDM-293T heterokaryons similarly restricted Env expression and virion production demonstrating the presence of a dominant restriction in macrophages that can act in *trans*. Based on studies using Vpr mutants and DCAF1 silencing, DCAF1 was required for Vpr to counteract this macrophage restriction. MDMs lacking Vpr produced more *IFN* RNA upon initial infection, and exogenous IFNα dramatically reduced Env expression and virion production. Thus, innate immune evasion promoted by Vpr impacts HIV-1 spread in macrophages by preventing the activity of a macrophage-specific intrinsic antiviral pathway that targets HIV-1 Env and that interferes with the release of Env-containing virions.

Results

Vpr is required for optimal spread of HIV-1 in macrophage cultures at low MOI

To explore the mechanism through which Vpr enhances HIV-1 infection of primary MDMs, we constructed a Vpr-null mutant of the 89.6 molecular clone (89.6*vpr*$^{-}$), which was isolated from the blood of an HIV-1-infected person with AIDS (Collman et al., 1992). As expected based on prior published studies, 89.6*vpr*$^{-}$ was not defective in permissive cell lines. 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 2.1A**). Additionally, equal mass amounts of 89.6 and 89.6*vpr*$^{-}$ virus stocks were equally infectious in CEMx174 cells (**Figure 2.1B**) as described previously (Balliet et al., 1994). Finally, HIV-1 89.6 and 89.6*vpr*$^{-}$ were similarly infectious in primary CD4^{+} T cells (**Figure 2.1C**, left).

In contrast, we noted striking differences in virion production by MDMs infected with the same viral stocks of wild-type and mutant viruses, particularly at low inoculum (**Figure 2.1D**). The impact of Vpr on virus production was most pronounced when the virus was allowed to spread through the culture for 18 days (up to 20-fold differences; **Figure 2.1C**, right three panels). Thus, 89.6*vpr*– is defective in infection of primary human MDMs, 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 (Chen et al., 2004; Connor et al., 1995; Eckstein et al., 2001).

Mechanism of Vpr-dependent enhancement of MDM infection

To determine whether the effect of Vpr we observed on the level of virus measured in the supernatant of infected MDMs resulted from a higher number of initially infected cells or from enhanced spread, we validated an intracellular HIV-1 Gag staining protocol to ensure that we could measure true infection events, rather than cell surface binding or endocytosis of viral particles by MDMs (**Figure 2.2A**). At 5 days post-infection (dpi), intracellular Gag staining and flow cytometry revealed a distinct population of cells that expressed HIV-1 Gag in a manner that was completely inhibited by HIV-1 reverse transcription and integration inhibitors azidothymidine (AZT) and raltegravir, respectively (**Figure 2.2A**). Thus, we concluded that this assay detected de novo infection of MDMs that was dependent on reverse transcription and integration.

At the earliest time points at which we could detect intracellular Gag (2 dpi), MDMs infected with an equal viral inoculum were equally infected plus or minus Vpr (**Figure 2.2B**, left). However by 4 dpi, we observed a higher frequency of infected MDMs with Vpr-containing viruses (2.8-fold, $p < 0.05$; **Figure 2.2B**, right). These results were confirmed using a PCR assay that detects HIV-1 DNA (**Figures 2.2C** and **2.3A**); Vpr did not stimulate the amount of cell-associated provirus detected at 2 dpi, but by 4 dpi, we detected 3- to 4-fold more provirus in Vpr-expressing HIV-infected MDMs (**Figures 2.2C** and **2.3A**).

In MDMs, reverse transcription and integration typically require 2–3 days for completion of the first round of infection (Spivak et al., 2011). Thus, at 4–5 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 MDMs at 2 dpi. We then harvested all the cells on day 4. In the absence of raltegravir, we again observed a 2.6-fold increase in the frequency of infected cells with Vpr-containing viruses (**Figure 2.2D**). However, in a side-by-side experiment using cells from the same donor, the addition of raltegravir abrogated this difference (**Figure 2.2D**). Thus, under the conditions of our assay, Vpr did not affect the initial infection of MDMs and primarily acted by stimulating spread of virus to new target cells.

Vpr facilitates spread in macrophages by increasing virion production

To better understand how Vpr promotes spreading infection of MDM cultures, we sought to distinguish effects of Vpr on different HIV-1 replication stages. We hypothesized that under conditions in which Vpr does not increase infection in the first round of HIV-1 replication (**Figure 2.2D**), it may affect the number of virions produced per infected cell. To examine this, we blocked spread of HIV-1 at 2 dpi using a concentration of raltegravir sufficient to fully inhibit new HIV-1 infection events and measured virion production under these conditions (**Figures 2.2A,D**). We found that Vpr increased virion production by MDMs infected with a Vpr-containing virus an average of 5-fold compared to HIV-1-infected MDMs lacking Vpr, which was statistically significant across multiple donors (**Figure 2.4A**). Of note, there were no significant differences in infected cell number that could explain these large differences in virion production (**Figures 2.4B** and **2.5A–E**).

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 2.4C,D**) and Gag DNA (**Figures 2.4E** and **2.3B**). Under these conditions of equivalent infection, Vpr increased virion production an average of 5-fold ($p < 0.01$; **Figure 2.4F**).

Similar results were observed when MDMs were infected with a T cell-tropic HIV-1 pseudotyped with a macrophage-tropic Env (NL4-3 pseudotyped with YU-2 Env). This virus is active for a single round of infection, but cannot spread in MDM cultures. Again, we observed that Vpr did not significantly modulate the number of infected MDMs (**Figure 2.6A**), but significantly increased the number of virions

released by MDMs infected with wild-type virus (**Figures 2.6B** and **2.4G**). The more modest phenotype observed in the single-round system is likely due to the use of a higher inoculum, which results in a reduced requirement for Vpr (**Figure 2.1D**), or to other differences between the NL4-3 and 89.6 viruses.

Vpr counteracts MDM-specific interference with release of Env-containing virions

Importantly, using the single-cycle infection system described above, we noted that Vpr did not alter the release of virions from MDMs infected with a mutant virus that did not express Env from the integrated provirus (NL4-3-ΔE-EGFP, pseudotyped with Env^{YU-2}) (**Figure 2.4G**). To confirm these results, we also examined virion production by MDMs infected with 89.6 Env-null and Env/Vpr-null mutants relative to wild-type and Vpr-null viruses. We again found that mutation of Env eliminated any significant effect of Vpr on virion production (**Figure 2.4A**). In contrast, Env and Vpr did not affect the release of virions by permissive cell lines (transfected 293T cells; **Figure 2.1A**). Because Env is incorporated into virions, these data support a model in which Vpr counteracts an MDM-specific factor or pathway that interferes with the release of Env-containing virions.

Vpr counteracts interference with Env expression by HIV-infected MDMs

Because our data strongly suggest that Vpr counters an MDM-specific factor that targets Env-containing virions, we asked whether MDMs also interfered with expression of cell-associated Env in the absence of Vpr. Indeed, we observed that the amount of HIV-1 Env protein detected in MDMs was Vpr dependent, whereas the level of Gag precursor (pr55) was not (**Figures 2.7A,B**). The effect of Vpr on Env

was MDM-specific because we observed no significant effect of Vpr on Env expression in 293T cell lines transfected with proviral DNA (**Figure 2.8A**). To more accurately assess the effect of Vpr on Env per infected cell, we examined serial dilutions of whole-cell lysate (WCL) and compared Env levels only for dilutions in which Gag pr55 expression was matched. Vpr consistently increased the expression of the processed gp41 form of Env (4- to 5-fold; **Figures 2.7A,B**). The gp160 precursor and processed gp120 forms of Env were also affected, but to a lesser extent than gp41 (2- to 4-fold; **Figures 2.7A,B**). Vpr also significantly increased the amount of processed Gag p24 in most donors (**Figures 2.7A,B**). However, this effect was more modest on average (about 2-fold) and varied considerably across donors.

To determine whether modulation of Env expression varied over the time course of infection, we analyzed Env expression in MDMs over time. To accurately compare the level of Env, we again performed serial dilutions and compared samples in which Gag pr55 expression was matched. We found that Vpr enhanced Env gp160 and gp120 expression even at 5 dpi (**Figure 2.7C**). However, the effect of Vpr on these forms of Env became more dramatic over time (**Figure 2.7C**). Indeed, by 20 dpi, Vpr increased Env gp160 and gp120 7- and 15-fold, respectively (**Figures 2.8B,C**).

To verify that these effects of Vpr were not specific to a single molecular clone of HIV-1, we performed similar experiments using HIV-1 molecular clone AD8 and a well-characterized AD8 Vpr-null mutant (Rey et al., 1998; Theodore et al., 1996). We again observed no defect in Env expression by the mutant virus in

transfected 293T cells (**Figure 2.8D**). However, MDMs infected with AD8*vpr*$^{-}$ displayed substantially lower levels of Env relative to Gag pr55 than MDMs infected with wild-type AD8 (**Figure 2.7D**). As with 89.6, this effect of Vpr was especially notable for Env gp41 where we observed an average of 7-fold more protein in MDMs infected with wild-type virus (**Figures 2.7D,E**). Again, we observed a more variable effect of Vpr on Env gp160 precursor (**Figures 2.7D,E**). Furthermore, we also observed effects of Vpr on NL4-3 Env when YU-2 Env-pseudotyped NL4-3 was used to infect MDMs (**Figure 2.6C**). Thus, the ability of Vpr to increase Env protein expression and virion production in MDMs is conserved among HIV-1 variants isolated from different HIV-1-infected people.

To determine whether the defect in cell-associated Env expression also led to diminished incorporation of Env into virions, we lysed virus from infected MDM supernatants and analyzed it by immunoblot. We found that Vpr significantly enhanced Env incorporation into virions by an average of 2- to 3-fold (**Figures 2.8E,F**). However, when MDMs were infected with equal mass amounts of MDM-derived virus containing or lacking Vpr, we observed equal numbers of infected cells (data not shown). Thus, MDMs express a factor or pathway that interferes with incorporation of Env into virions and that inhibits their release, but under the conditions of our assay, the magnitude of the interference was not sufficient to alter the infectivity of the residual recovered virions.

Vpr prevents lysosomal degradation of HIV-1 Env in MDMs

To determine the mechanism by which MDMs interfere with Env expression, we performed a pulse-chase analysis of Env protein synthesis and decay in infected MDMs at 10 dpi. Vpr did not affect the quantity of the precursor form of Env (gp160) synthesized within the 1-hour pulse (n = 8; **Figure 2.9A**; data not shown). However, Vpr increased the half-life of the processed form (gp120) from 3.3 to 7.7 hours ($p < 0.0001$; **Figures 2.9A,B**). In contrast, there was no significant effect of Vpr on the half-life of HIV-1 Gag pr55 or Gag p24 (**Figure 2.9A**; 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 2.9C,D**). In sum, these results indicate that Vpr increases Env protein expression by a posttranslational mechanism that prevents Env degradation.

To determine which degradative pathways affected Env in MDMs infected with 89.6*vpr*$^{-}$, we treated cells that were pulse labeled for 1 hour and chased with non-labeled media for 8 hours 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 2.9E,F**). In contrast, ammonium chloride treatment did not significantly affect Gag pr55 levels or Gag p24 over the 8-hour time course of this assay (**Figures 2.9E,G**). These data demonstrate that HIV-1 Env is targeted to lysosomes in HIV-1-infected cells lacking Vpr.

Vpr counteracts a dominant restriction of Env and virion production in MDMs

To determine whether Env expression is diminished in MDMs because of a negative restriction factor that is counteracted by Vpr or because MDMs lack a positive cofactor that Vpr provides, we examined Env expression and virion production in heterokaryons of restricted (MDM) and permissive (293T) cells. Heterokaryons were generated using Newcastle disease virus (NDV) fusion proteins (F and HN) and HIV-1, which promotes cellular fusion via Env on transfected 293T cells and CD4 on MDMs. In these experiments, 293T cells were transfected equally by HIV-1 89.6 and 89.6*vpr*$^{-}$, and spread of HIV-1 to MDMs was prevented by the addition of raltegravir (**Figures 2.10A,B**). Similar to previously published work (Kaushik et al., 2009; Sharova et al., 2008), we observed that NDV fusion proteins and HIV-1 enhanced the fusion of transfected 293T cells to uninfected MDMs, and fewer than 10% of 293T cells were unfused at the time of harvest (**Figure 2.10C**). Remarkably, we found that MDM-293T heterokaryons restricted Env expression and virion production, whereas 293T homokaryons did not (**Figures 2.10D,E**). Moreover, the heterokaryon-specific restriction was counteracted by Vpr (**Figures 2.10D,E**). Collectively, our data indicate that Vpr counteracts a macrophage-specific restriction factor that targets Env for lysosomal degradation and impairs the release of virions.

DCAF1 Is required for Vpr to counteract restriction of Env and virion production in MDMs

Studies performed in permissive cells have shown that HIV-1 Vpr interacts with a cellular ubiquitin ligase complex through the adaptor protein DCAF1. Because lentiviral accessory proteins commonly utilize ubiquitin ligase substrate

adaptors to target restriction factors for degradation, we examined whether DCAF1 is required for Vpr to overcome the restriction we observed in MDMs. Interestingly, we observed that a mutant Vpr (Vpr^{Q65R}) that is defective at DCAF1 interactions, DCAF1-dependent cell cycle arrest, and SLX4 complex activation (DeHart et al., 2007; Hrecka et al., 2007; Laguette et al., 2014) was similarly defective at enhancing Env expression and virion production (**Figure 2.11**).

To more directly demonstrate a causal relationship between DCAF1-Vpr interaction and Vpr-mediated increases in virion production and Env expression, we silenced *DCAF1* expression in primary human MDMs. To accomplish this, we used an shRNA-expressing lentiviral construct that had been optimized to maximize silencing while limiting antiviral responses in MDMs (Pertel et al., 2011). Using this system we reproducibly achieved efficient silencing of *DCAF1* expression (**Figure 2.12A**). Remarkably, we observed that *DCAF1* silencing dramatically reduced Env expression (**Figure 2.12B**) and virion production (**Figure 2.12C**) in wild-type HIV-1-infected MDMs. Indeed, without DCAF1 expression, Env expression was similar between wild-type and Vpr-null viruses (**Figure 2.12B**). Thus, these studies indicate that Vpr requires interaction with the DCAF1-DDB1-CUL4 E3 ubiquitin ligase to overcome restriction of virion production and Env expression in HIV-1-infected MDMs.

Vpr disrupts a pathway that leads to induction of type I IFN

Recent studies have shown that inducible Vpr expression in a HeLa cell line activates the SSE 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 ubiquitylation and turnover of MUS81-EME1 endonucleases (Laguette et al., 2014) in a manner that requires the DCAF1-DDB1-CUL4 E3 ubiquitin ligase complex. We demonstrate here that this pathway is intact in primary human MDMs and $CD4^+$ lymphocytes (**Figures 2.13A,B**). Compared to infected cells lacking Vpr, wild-type HIV-1 infection led to the accumulation of PLK-1 and increased the turnover of MUS81 and DCAF1 in primary MDMs (**Figure 2.13A**). 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 MDMs, the levels of UNG2 were not assayable due to lower expression levels (data not shown). Interestingly, DCAF1 was diminished as early as 5 hours post-infection (hpi) in MDMs, consistent with its utilization by Vpr (**Figure 2.13C**). Vpr-dependent downmodulation of DCAF1 at 5 hpi was completely reversed by the addition of the proteasome inhibitor MG132 (**Figure 2.13C**), indicating that virion-associated Vpr utilizes DCAF1 at very early time points prior to the establishment of productive infection.

Because activation of the DCAF1-SLX4 pathway diminishes viral sensing and limits type I IFN responses in HeLa cells (Laguette et al., 2014), we examined IFN responses in infected MDMs. Remarkably, we found that wild-type HIV-1-infected MDMs exhibited lower *IFNA1* gene expression compared to Vpr-null-infected MDMs at 12 hpi (**Figure 2.14A**). Furthermore, the magnitude of the difference in *IFNA1* response correlated with the degree to which Vpr enhanced virion production at 10 dpi (**Figure 2.14B**).

To directly assess whether type I IFN may contribute to restriction of Env and virion production in MDMs, we treated infected MDMs with IFNα at 7 dpi and harvested at 10 dpi (**Figure 2.14C**). Because we observed that IFNα completely inhibited new infections when added at day 0, we added raltegravir to established infections at the time of IFNα addition to eliminate confounding effects due to differences in spread. Under these conditions, we observed that IFNα treatment strikingly reduced HIV-1 Env gp41, gp120, and gp160 protein levels relative to Gag pr55 in multiple donors (**Figure 2.14D**). Additionally, IFNα treatment suppressed virion production per infected cell (**Figure 2.14E**). These data support a model in which Vpr facilitates Env expression and release of Env-containing virions by limiting induction of IFNα production upon HIV-1 infection of MDMs.

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 (Lim and Emerman, 2011). 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: from Dr. Peter Cresswell.

Viral constructs

p89.6, pNL4-3 and pNL4-3ΔEGFP were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalog numbers 3552, 114 and 11100 from Dr. Ronald G. Collman, Dr. Malcolm Martin and Dr. Robert Siliciano respectively (Adachi et al., 1986; Collman et al., 1992; Zhang et al., 2004). 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 AD8VprX) were obtained from Vicente Planelles, PhD. (Zimmerman et al., 2006). pYU2*env* was obtained from Joseph Sodroski (Sullivan et al., 1995). p89.6*vpr*-and pNL4-3ΔE-EGFP*vpr*- were generated by filling in and re-ligating the Afl II site within the *vpr* open reading frame in p89.6 and pNL4-3-ΔE-EGFP respectively. 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*-. p89.6vprQ65R was constructed by substituting synthetic mutant oligonucleotides into the EcoRI and SalI sites in p89.6. pNL4-3*vpr*- was generated by ligating the SpeISalI fragment from pNL-PI*vpr*- (Norman et al., 2011) into pNL4-3. Virus stocks were obtained by transfection of 293T cells with provirus expression

plasmids using polyethylenimine, as described (Hrecka et al., 2007). Pseudotyped virus was produced by co-transfecting 293T cells with provirus and Env expression plasmid, as described (Carter et al., 2011). 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). Peripheral blood mononuclear cells (PBMCs) 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 (Salter et al., 1985).

$CD14^+$ monocytes were isolated by positive selection with an EasySep magnetic sorting kit (STEMCELL Technologies). MDMs were obtained by culturing monocytes in R10 (RPMI-1640 with 10% fetal bovine serum [Gibco, Invitrogen], penicillin [10 U/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). MDMs were incubated with HIV-1 for 4 hr, washed with PBS, and cultured in fresh medium, as described (Peeters et al., 2002). For IFNα treatment experiments, cells were treated with 500 U/ml recombinant human IFNα (Calbiochem) as described in **Figure 2.14**.

Flow cytometric analysis

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 (TreeStar).

Western blotting

MDM WCL preparation: MDMs infected with HIV-1 were washed with PBS before being lysed in Blue Loading Buffer (Cell Signaling Technology). WCLs were sonicated with a Misonix Sonicator (QSonica) and clarified by centrifugation at 13,000 rpm.

Viral lysate preparation: Supernatant from infected cells was passed through a 0.45 μm filter, and virions were pelleted by ultracentrifugation at 25,000 rpm, as described (McCall et al., 2008). The virus-containing pellet was lysed in Blue Loading Buffer and clarified by centrifugation at 13,000 rpm.

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

Virion quantitation

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). Antibody to HIV-1 CAp24 antibody (clone 183-H12-5C) was bound to Nunc MaxiSorp plates. Lysed

samples were captured for 1–2 hours and incubated with biotinylated antibody to HIV-1CAp24 (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). CAp24 concentrations were measured by comparison to recombinant HIV-1 CAp24 standards (ViroGen). 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.

Preparation of $CD4^+$ lymphocytes

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

Quantitative PCR

DNA was isolated from 4 to 5 × 10^5 MDMs using the DNeasy Blood & Tissue Kit (Qiagen). HIV-1 DNA and *ACTB* 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). Dilutions of ACH-2 DNA (Clouse et al., 1989; Folks et al., 1989) were used to calculate *gag* copy number and relative DNA input.

Quantitative RT-PCR

RNA was isolated from MDMs using RNeasy Kit (Qiagen) with on-column DNase I digestion. RNA was reverse transcribed using AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies) with 8.5 ng/µl oligo dT and 1.5 ng/µl random nonamers. cDNA was amplified with QuantiTect SYBR Green PCR Kit (Qiagen) on an Applied Biosystems 7300 Real-Time PCR System using commercially available *IFNA1* primers (Prime PCR, qHsaCED0020782, Bio-Rad) or with TaqMan Gene Expression Master Mix with *β-actin* primers and FAM-MGB probes (TaqMan Gene Expression, Hs99999903_m1, Life Technologies) (Applied Biosystems). Reactions were quantified using ABI Sequence Detection software compared to serial dilutions of a single-stranded DNA oligo spanning the *IFNA1* amplicon, or cDNA from mock-treated cells. Calculated copies from the no-RT controls were subtracted from the calculated copies of the cDNA samples, then normalized for input measured by *β-actin*.

Radioimmunoprecipitation assay

Metabolic labeling of HIV-1-infected MDMs was performed as described (Ono and Freed, 1999). MDM infected with 1 µg CAp24 for ten days were labeled with 125µCi [35S]Met/[35S]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, 1995a). 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 35S 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 35S in the gp120 band to calculated 35S signal from gp120 at time zero (T0). The calculated 35S 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 35S signal at each chase time from the actual 35S signal measured in gp160 at T0.

RNA Interference

Short hairpin RNA-mediated silencing 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 20 μg VSV-G-pseudotyped lentivirus containing an shRNA cassette targeting *luciferase* (control) or *DCAF1*. Following an overnight incubation, the cells were cultured for 3 days in fresh medium before addition of 10 μg/ml puromycin for 3 additional days prior to infection.

Generation of 293T-MDM heterokaryons

293T cells transfected with p89.6 or p89.6*vpr*$^-$ and paramyxovirus fusion protein expression plasmids (pCAGGS-NDV-HN and pCAGGS-NDV-F, Dr. Trudy Morrison, University of Massachusetts Medical School; (McGinnes, 2006 #378) were stained with Vybrant DiO (Invitrogen). Uninfected MDMs prepared as described above were stained with Vybrant DiD (Invitrogen). Transfected 293T cells were cultured alone or with MDMs (3:1, 293T:MDM) for 4 days in media containing 2 μM raltegravir and were trypsinized prior to harvest.

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Author Contributions

Michael Mashiba, David R. Collins and Kathleen L. Collins designed the studies and analyzed the data presented in this chapter. Michael Mashiba and David R. Collins performed most of the experiments; Valeri H. Terry performed and analyzed RT-PCR experiments. Michael Mashiba, David R. Collins, Valeri H. Terry and Kathleen L. Collins wrote and edited the manuscript from which this chapter was transcribed. Kathleen L. Collins supervised the research.

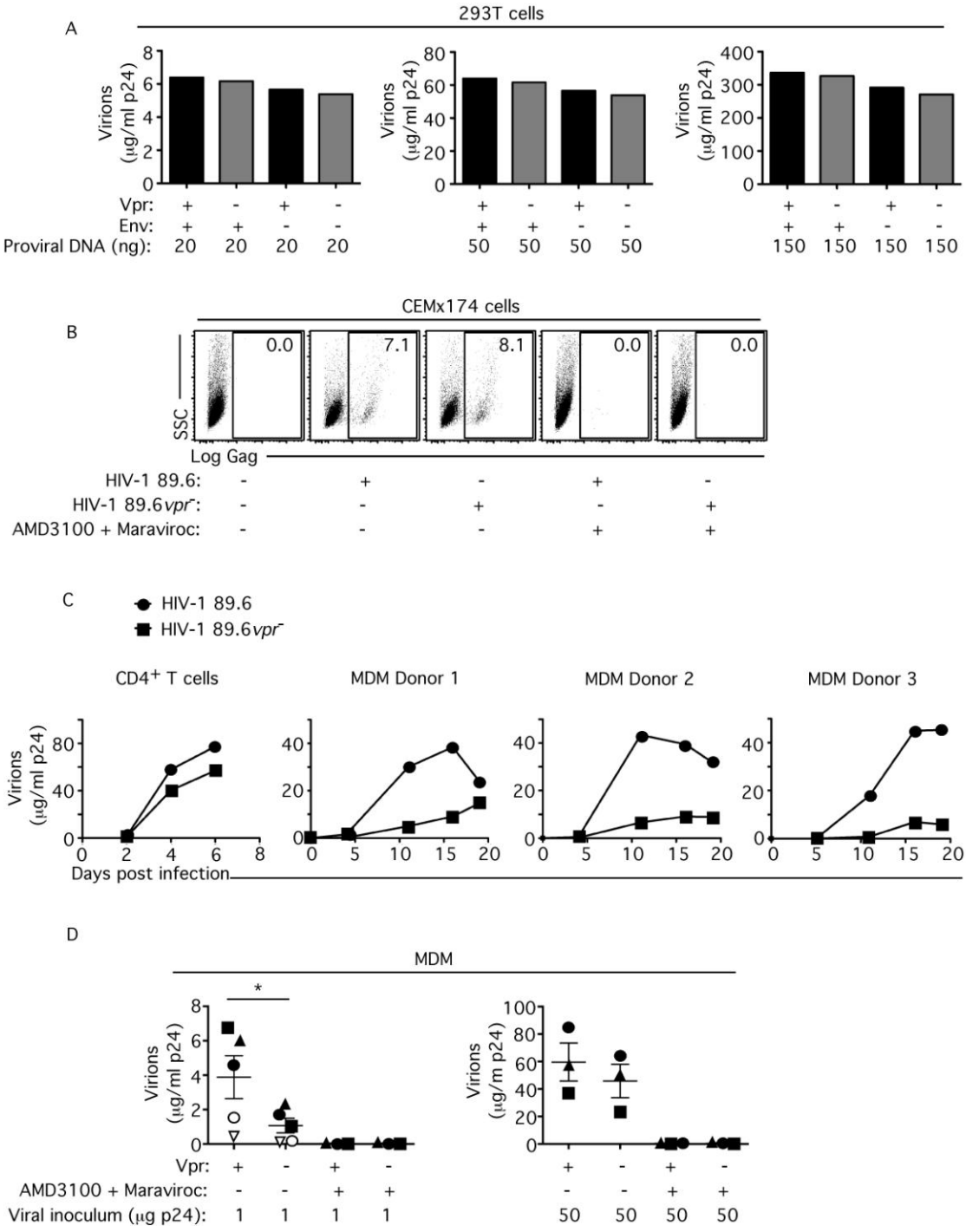

Figure 2.1: Vpr increases virion production in HIV-1-infected MDM but not 293T or CD4+ T cells. (**A**) Virion production by 293T cells transfected with HIV genomic DNA plasmids expressing the indicated HIV-1 proteins. Virion production was quantified using a CAp24 ELISA of the supernatant 2 days after 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 1

μg of the indicated virus for 6d or 18d, respectively. (**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. Error bars represent the standard error of the mean (Spruit et al.). *$p < 0.05$ (two-tailed paired *t*-test).[2]

[2] This figure was created by Michael Mashiba.

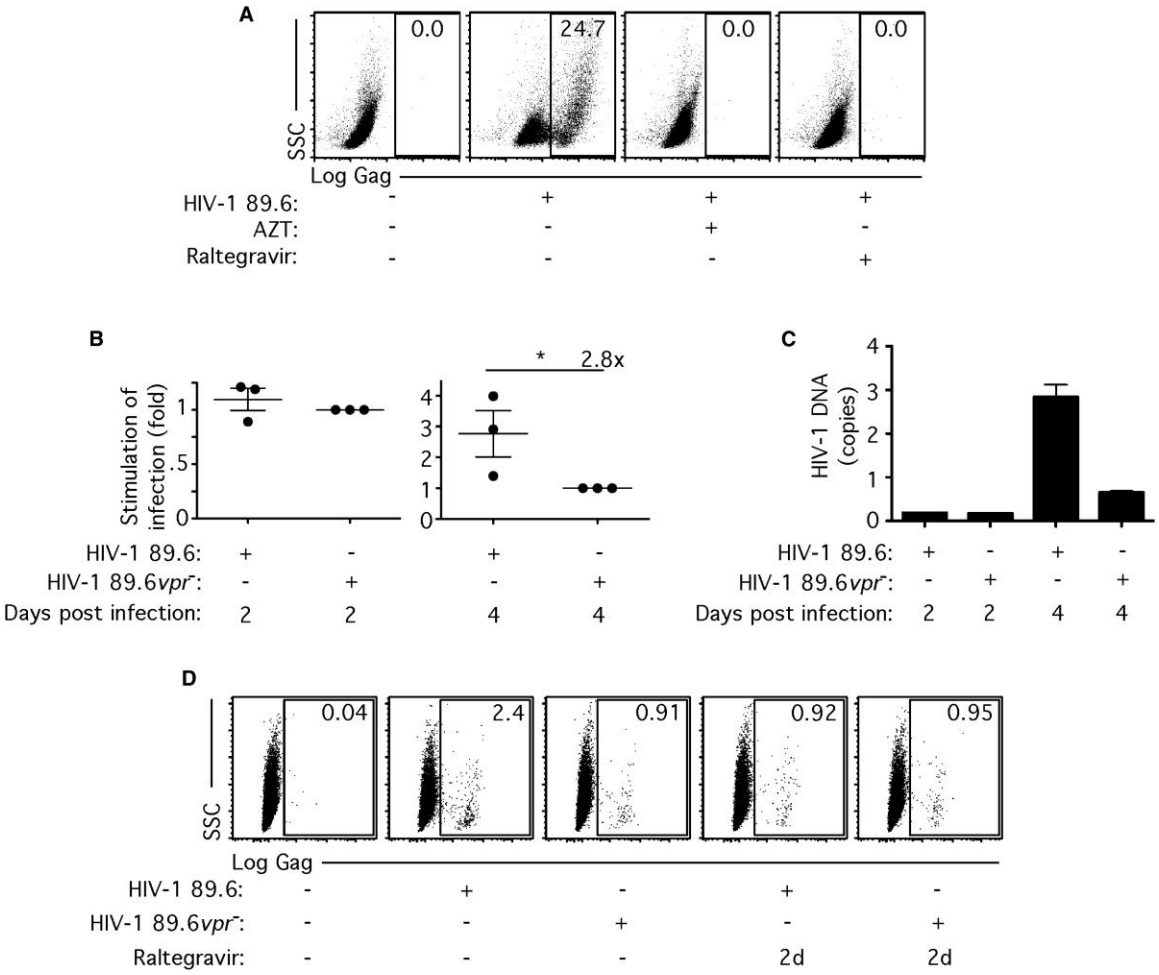

Figure 2.2: Vpr does not increase the first round of infection in primary human MDM cultures. (**A**) Flow cytometric analysis detecting bona fide infection of MDMs by intracellular HIV-1 Gag p24 stain. Cells were infected for 5 days with 50 µg of HIV-1 89.6 and treated with 20 µM azidothymidine (AZT) or 2 µM raltegravir where indicated. (**B**) Summary graph showing the fold difference in infection frequency observed in MDMs treated with 1 µg of wild-type or Vpr mutant virus. By definition, the mutant virus normalizes to 1.0. (**C**) qPCR analysis of *gag* DNA in MDMs infected with the indicated viruses. Levels of *gag* DNA were normalized for *β-actin* (*ACTB*) DNA levels to account for differences in cell number (Figure 2.3A). Each bar represents the average of three replicates from the same donor. Error bars represent SEM. $^{*}p < 0.05$, two-tailed paired t test. (**D**) Flow cytometric analysis of intracellular HIV-1 Gag p24 expression in MDMs infected with 1 µg of the indicated viruses and analyzed at 4 dpi. Cells were treated with 2 µM raltegravir at 2 dpi where indicated.[3]

[3] This figure was created by Michael Mashiba.

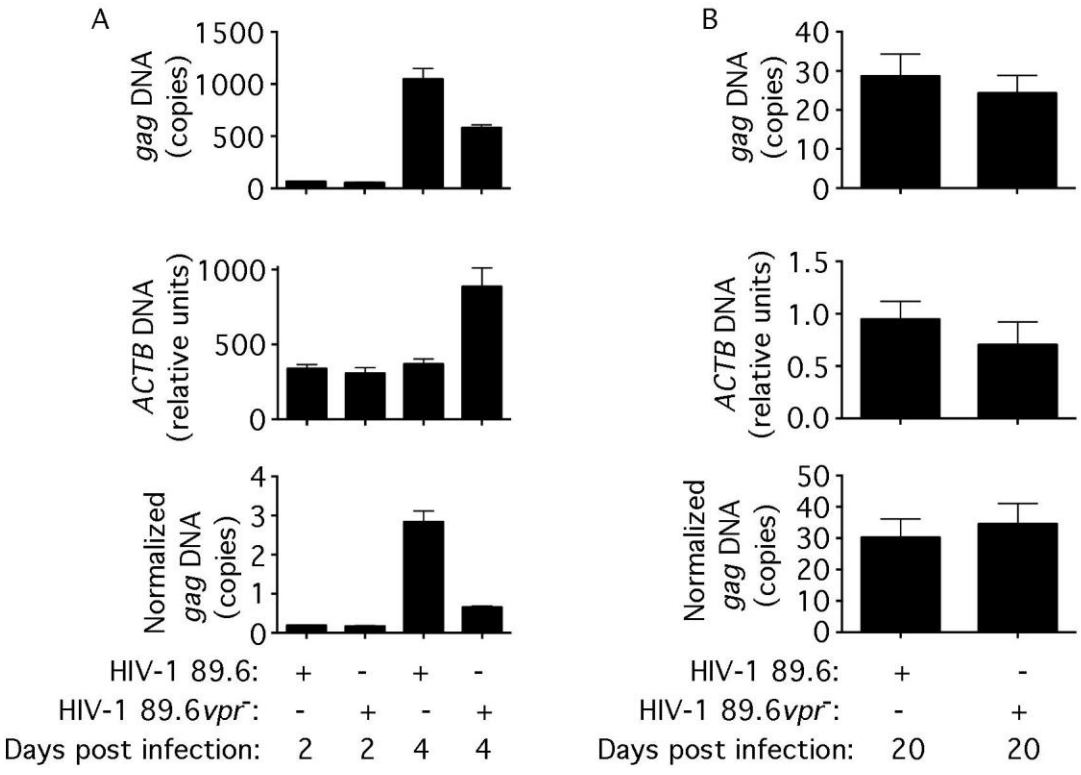

Figure 2.3: Calculation of normalized *gag* DNA in HIV-infected MDM. (**A**,**B**) qPCR analysis of the indicated genes in MDM infected with 1 μg of wild type or Vpr mutant virus. Normalized *gag* DNA (bottom graphs) was adjusted for total cell number by dividing measured *gag* DNA (top graphs) by total *ACTB* DNA (middle graphs). Normalized *gag* DNA in bottom graphs in A and B are shown in Figures 2.2C and 2.4E, respectively. Each bar represents the mean of three replicates from one experiment from a single donor. Error bars represent the SEM.[4]

[4] This figure was created by Michael Mashiba.

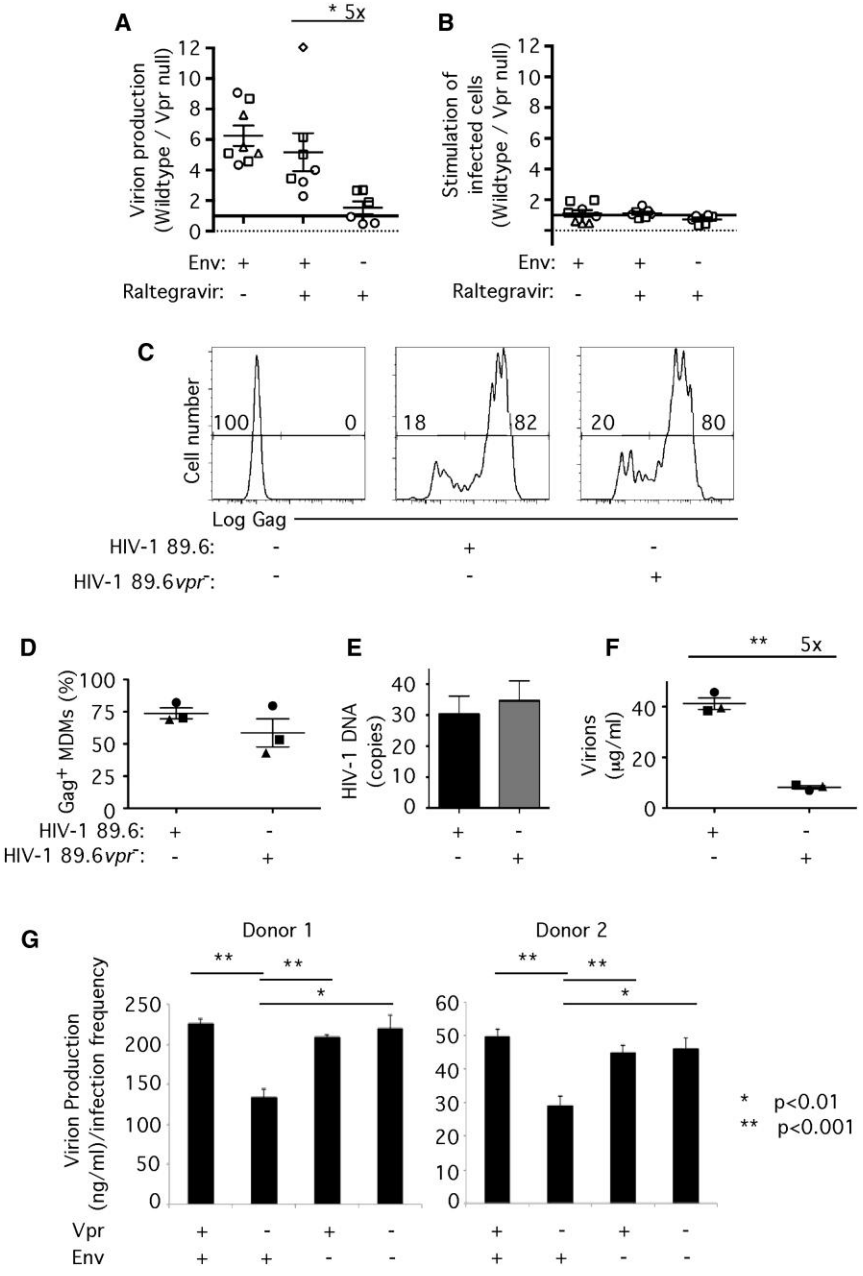

Figure 2.4: Vpr increases virion production in HIV-1-infected primary MDMs. (**A**) Summary graph of the effect of Vpr on virion production, calculated as the fold change in virion production by MDMs infected by wild-type 89.6 relative to 89.6*vpr*$^{-}$. Virion production was normalized for the absolute 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 MDMs infected by wild-type 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 SEM. (**C**) Flow cytometric analysis of HIV-1 Gag p24 expression in MDMs infected for 20 days with 1 µg of the indicated viruses. (**D**) Compilation of flow cytometry experiments staining for

intracellular HIV-1 Gag p24 in MDMs infected as in (C). (**E**) qPCR analysis of *gag* levels in MDMs from a duplicate well from the experiment in (C). Levels of *gag* DNA were normalized for *β-actin* (*ACTB*) DNA levels to account for differences in cell number. (**F**) Virion production based on HIV-1 Gag p24 levels in the supernatant of MDMs from three donors for which infection rates were shown in (D). Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, two-tailed paired t test. In (D) and (F) each symbol represents a different donor (n = 3). (**G**) Summary graph of virion production by MDMs 4 days post-infection with YU-2 envelope-pseudotyped NL4-3 wild-type or containing the indicated mutations. The mean ratio of viral production/infection frequency is shown ± SD. $^{*}p < 0.01$, $^{**}p < 0.001$, two-tailed paired t test, n = 3 replicates from each donor.[5]

[5] This figure was created by Michael Mashiba and David Collins.

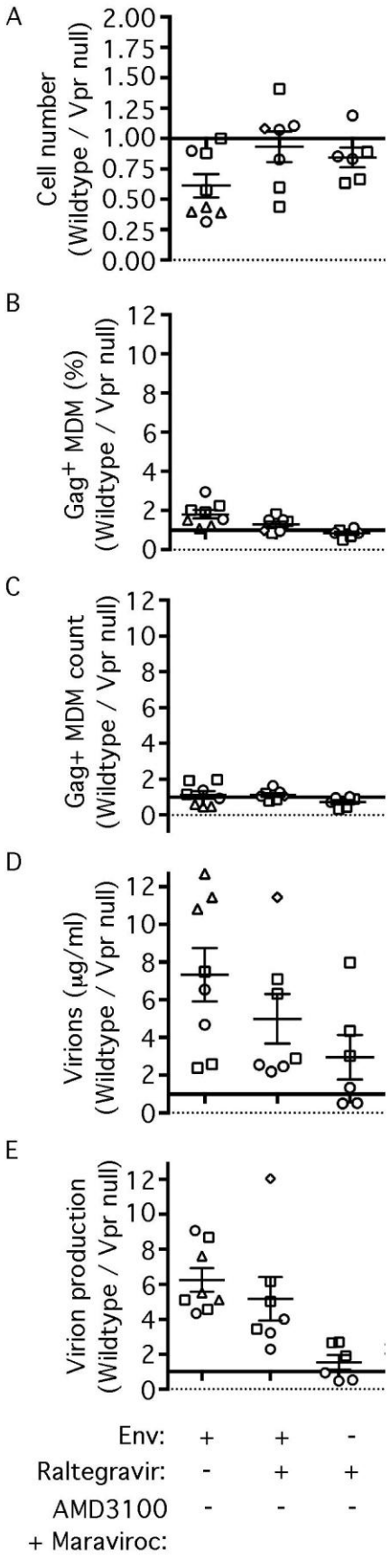
A
Cell number
(Wildtype / Vpr null)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
B
Gag+ MDM (%)
(Wildtype / Vpr null)
12
10
8
6
4
2
0
C
Gag+ MDM count
(Wildtype / Vpr null)
12
10
8
6
4
2
0
D
Virions (µg/ml)
(Wildtype / Vpr null)
12
10
8
6
4
2
0
E
Virion production
(Wildtype / Vpr null)
12
10
8
6
4
2
0
Env: + + -
Raltegravir: - + +
AMD3100 - - -
+ Maraviroc:

Figure 2.5: Calculation of virion production per infected MDM. (**A**) Summary graph of the effect of Vpr on the number of cells acquired by flow cytometry within a fixed interval of time, shown as the fold-change in cell number for MDM infected by wildtype 89.6 relative to 89.6*vpr*-. (**B**) Summary graph of the effect of Vpr on the infection rate, plotted as the fold-change in the infection rate for MDM infected by wildtype 89.6 relative to 89.6*vpr*-. (**C**) Summary graph of the effect of Vpr on the number of infected cells, calculated as the fold change in the number of infected cells determined from multiplying the number of total cells by the infection rate in A and B. (**D**) Summary graph of the effect of Vpr on virions measured in the supernatant of cells by p24 ELISA. Data were plotted as the fold change in virion production by MDM infected by wildtype 89.6 relative to 89.6*vpr*-. (**E**) Summary graph of the effect of Vpr on virion production per infected cell, calculated as the fold-change in virion production by MDM infected by wildtype 89.6 relative to 89.6*vpr*-. Virion production from D was normalized for the absolute number of Gag + cells acquired by flow cytometry within a fixed interval of time shown in C. Each shape in A-E represents a replicate from one experiment from a single donor. Error bars represent the SEM.[6]

[6] This figure was created by Michael Mashiba.

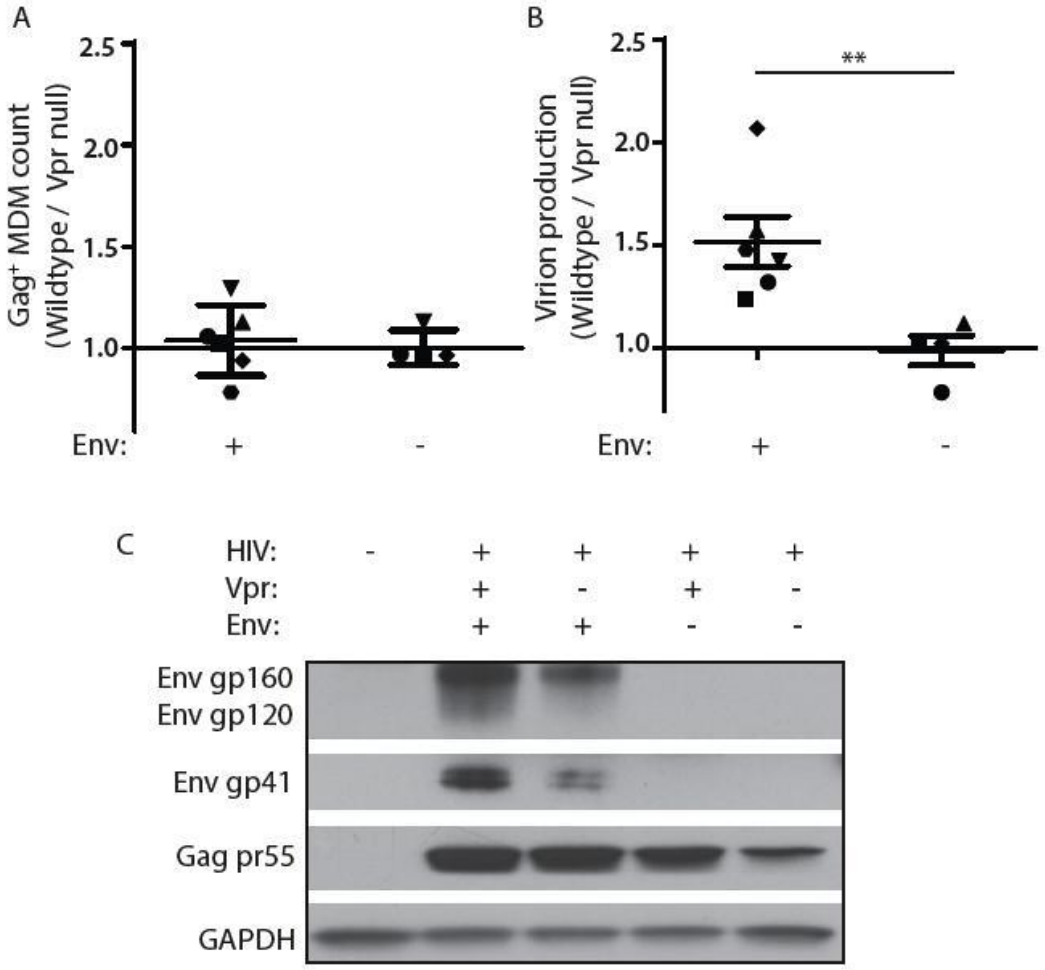

Figure 2.6: Vpr increases virion production in the presence of Env in single cycle (non-spreading) MDM infection. (**A**) Summary graph of infected cell number determined as described in Figure 2.5C 4 days post-infection with 50 μg YU-2 envelope-pseudotyped NL4-3 wild type or mutant virus. Data is expressed as a ratio of wild type to Vpr-null and is compiled from a total of six donors. Error bars represent the SEM. (**B**) Virion production from MDM infected as in A and calculated as described in Figure 2.5E. Data are displayed as a ratio of wild type to Vpr-null. (* $p<0.05$, ** $p<0.01$) (**C**) Western blot analysis of cells prepared as described for parts A and B.[7]

[7] This figure was created by David Collins.

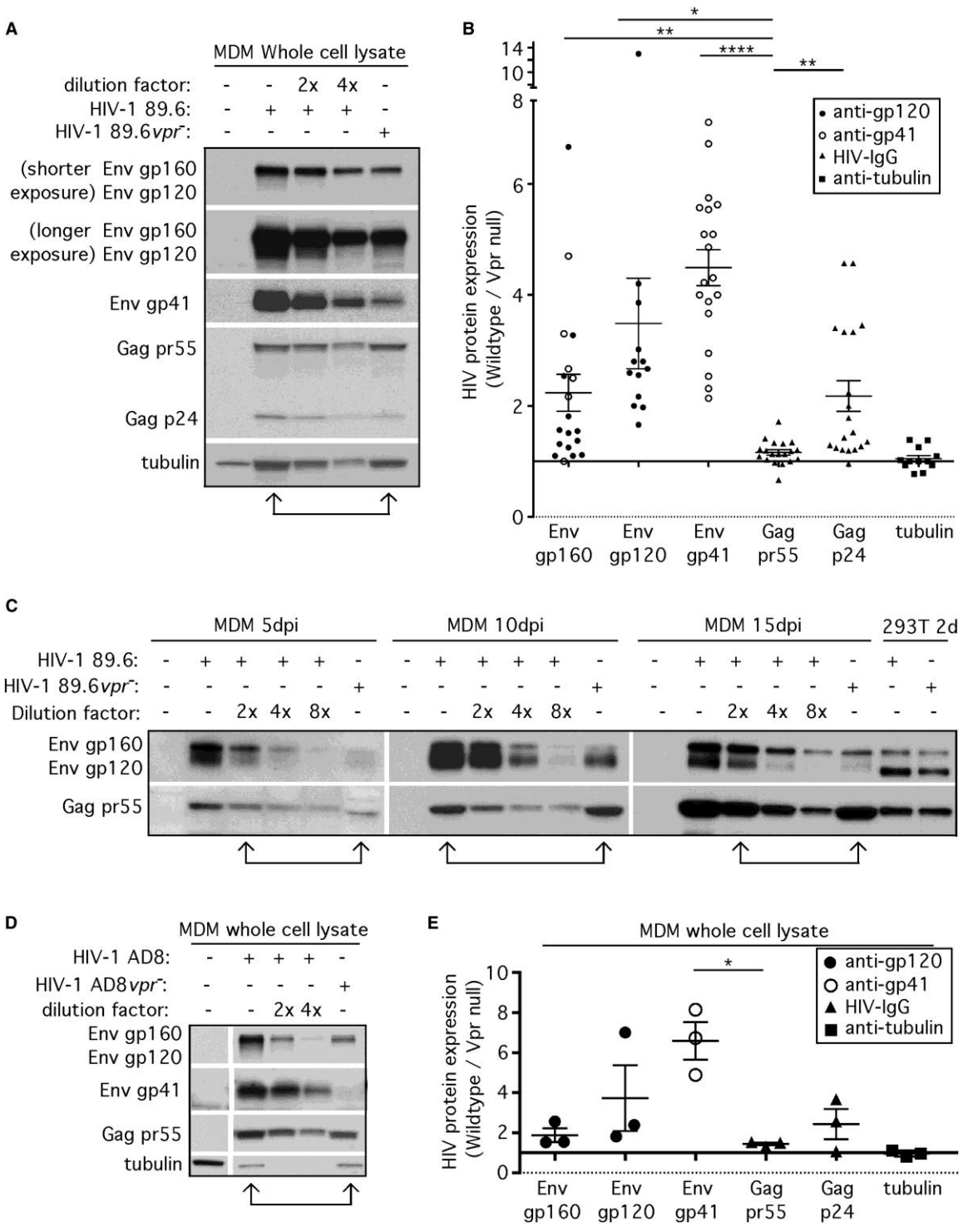

Figure 2.7: HIV-1 Vpr increases Env expression in MDMs. (**A**) Immunoblot analysis of HIV-1 protein expression in whole-cell lysates (WCLs) from MDMs infected for 10 days with 1 µg of the indicated viruses. Env gp160 and gp120 were detected with anti-gp120 antibody, gp41 was detected by anti-gp41 antibody, and Gag pr55 and p24 were detected with HIV-Ig. WCLs from MDMs infected with wild-type 89.6 were serially

diluted as indicated. Arrows denote samples containing comparable levels of Gag pr55 in the presence or absence of *vpr*. (**B**) Summary graph quantifying the fold increase in expression of the indicated HIV-1 proteins in MDMs infected with wild-type 89.6 relative to 89.6*vpr*$^{-}$ as in (A). Each symbol denotes the antibody used to detect the indicated protein and represents a separate experiment performed in a different donor (n = 19). Error bars represent SEM. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{****}p < 0.00005$, two-tailed paired t test. (**C**) Immunoblot analysis of viral protein levels in WCLs from MDMs infected for the times indicated with 1 μg of the indicated viruses and from 293T cells transfected for 2 days with the indicated HIV-1 genomic DNA plasmids. WCLs from MDMs infected with wild-type 89.6 were serially diluted as indicated. (**D**) Immunoblot analysis of viral protein levels in WCLs from MDMs infected for 6 days with 1 μg of wild-type AD8 or AD8*vpr*$^{-}$. Lysates from MDMs infected by wild-type AD8 were serially diluted in loading buffer as indicated. (**E**) Summary graph of the effect of Vpr on expression of the indicated HIV-1 proteins, calculated as fold change in protein levels in MDMs infected for 6 days with 1 μg of wild-type AD8 relative to AD8*vpr*$^{-}$. Each symbol denotes the antibody used to detect the indicated protein and represents a separate experiment performed in a different donor (n = 3). Error bars represent SEM. $^{*}p < 0.05$, two-tailed paired t test.[8]

[8] This figure was created by Michael Mashiba and David Collins.

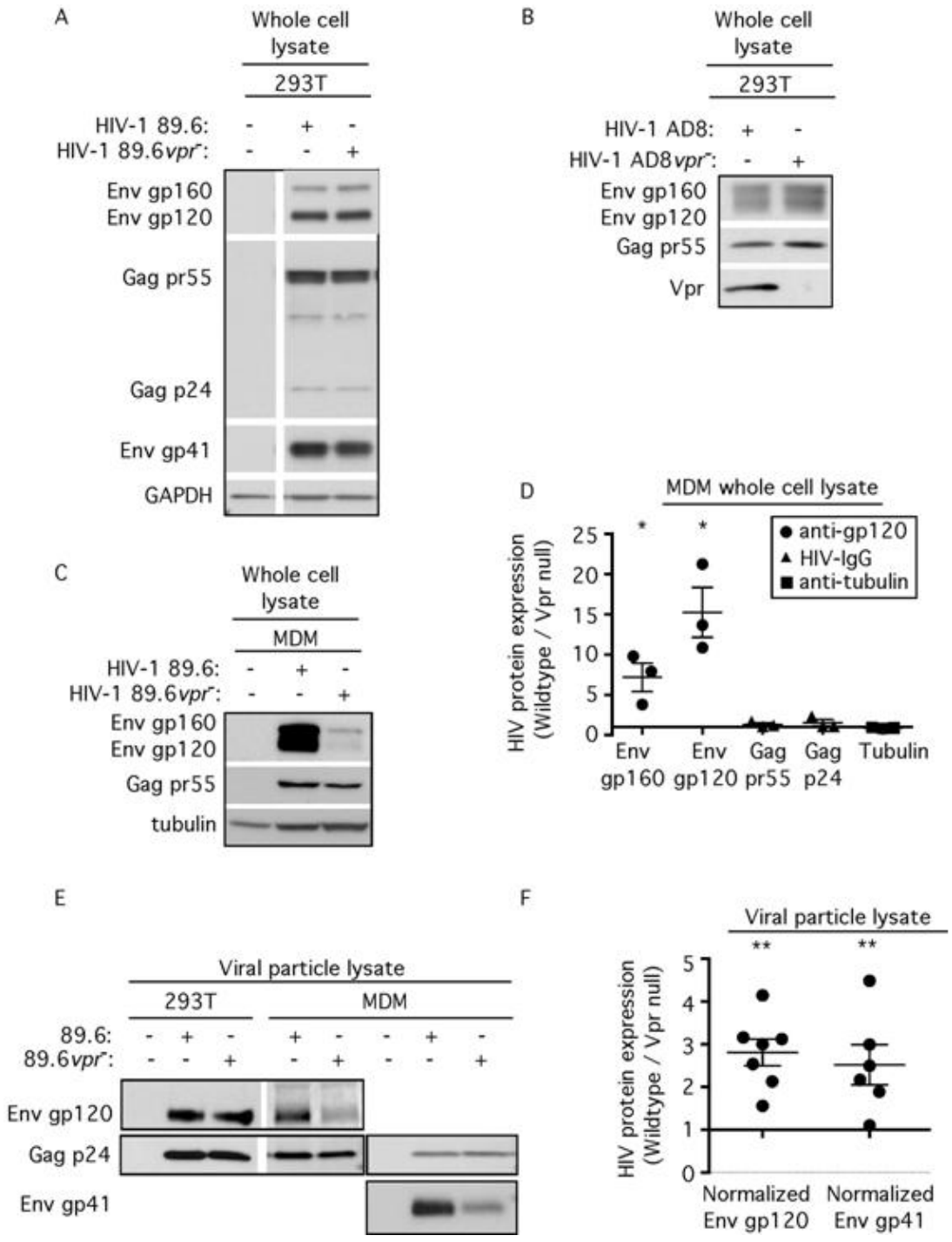

Figure 2.8: Vpr enhances levels of cell and virion associated Env expression in restricted MDM but not permissive 293T cells. (**A**) Immunoblot analysis of HIV protein expression in (whole cell lysate) WCL from 293T cells transfected for 2d with the p89.6 and p89.6*vpr*- genomic DNA plasmids. Env gp160 and gp120 were detected with anti-gp120 antibody, Env gp41 was detected with anti-gp41 antibody, and Gag pr55 and p24

were detected by HIV-IG. (**B**) WCL from 293T cells transfected for 2d with pAD8 and pAD8 *vpr*- genomic DNA plasmids. Env gp160 and gp120 were detected with anti-gp120 antibody, Env gp41 was detected with anti-gp41 antibody, and Gag pr55 was detected with HIV-IG. (**C**) Immunoblot analysis of HIV protein expression in WCL from MDM infected for 20d with 1 μg of the indicated viruses. (**D**) 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 B. Env gp160 and gp120 were detected with anti-gp120 antibody and Gag pr55 and p24 were detected by HIV-IG. Error bars represent the SEM. * $p < 0.05$ (two-tailed paired *t*-test). (**E**) Immunoblot analysis of HIV protein levels in viral lysates (VL) collected at 10dpi from cells infected with 1 μg wild type or mutant viruses. Lysates were adjusted to load equal Gag p24 based on p24 ELISA. Env gp120 was detected with anti-gp120 antibody, Env gp41 was detected with anti-gp41 antibody, and Gag p24 was detected by HIV-IG. (**F**) Summary graph quantifying the fold increase in expression of the indicated HIV protein in VL from experiments performed as in E. 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 SEM. ** $p < 0.002$ (two-tailed paired *t*-test).[9]

[9] This figure was created by Michael Mashiba and David Collins.

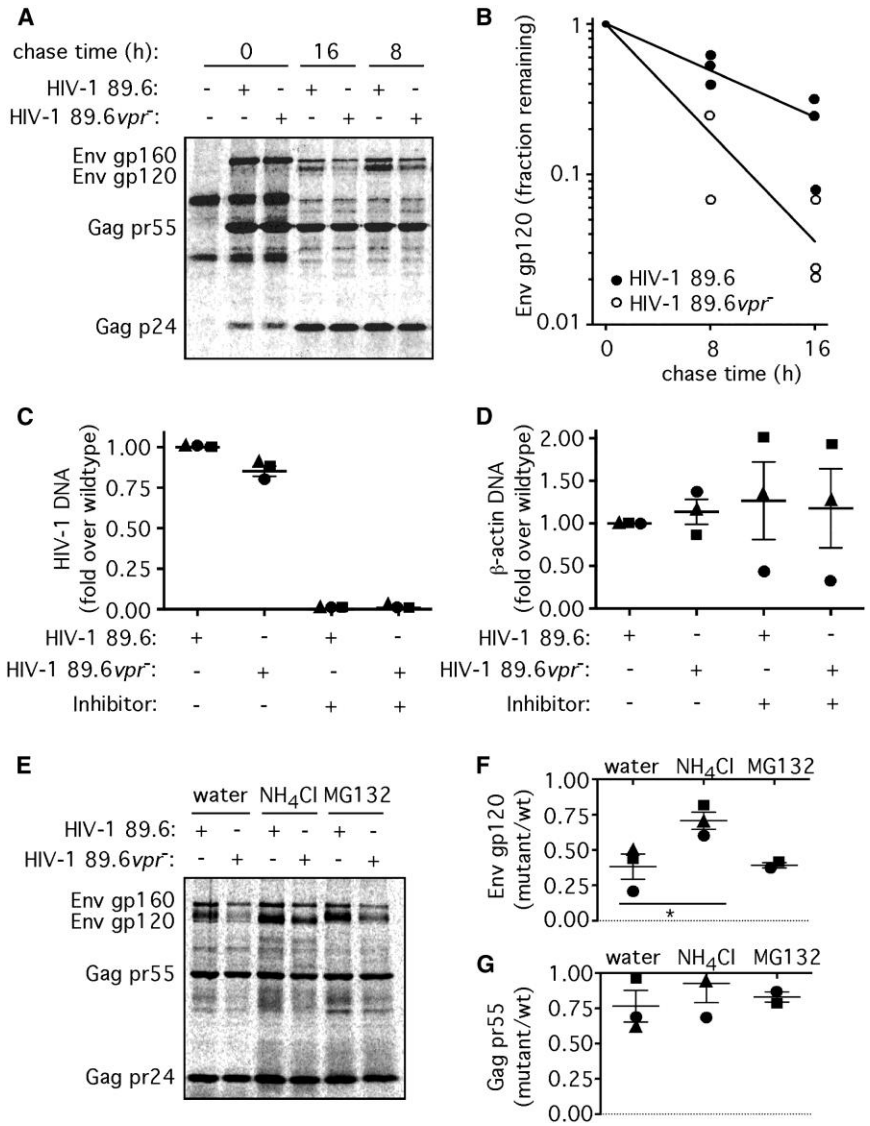

Figure 2.9: Vpr prevents lysosomal degradation of Env in primary human MDMs. (**A**) Radioimmunoprecipitation assay of HIV-1 proteins from primary human MDMs infected with 1 µg of wild-type 89.6, cultured for 10 days prior, and metabolically labeled with [^{35}S]Met/Cys for 1 hr. The labeled cells were chased for the indicated time periods, immunoprecipitated, and subjected to SDS-PAGE. (**B**) Summary of quantified data from the experiments performed as in (A). The fraction of gp120 remaining at 8 or 16 hours relative to T0 was calculated as described in Methods. Each dot represents a different donor from a separate experiment (n = 3). Best-fit curves were obtained by nonlinear regression analysis. (**C**,**D**) qPCR analysis of *gag* (C) and *β-actin* (D) DNA levels in cells analyzed in (B). Cells were treated with 10 µg/ml AMD3100 and 20 µM maraviroc or 2 µM raltegravir during infection where indicated. Each shape represents a different donor from a separate experiment (n=3). (**E**) Radioimmunoprecipitation assay of HIV-1 proteins from primary human MDMs infected and radiolabeled as described for (A). As indicated, labeled cells were chased for 8 hours with or without inhibitors of lysosomal and proteasomal degradation (20 µM NH_4Cl and 2.5 µM MG132, respectively). (**F,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 WCLs from MDMs infected by 89.6*vpr*$^{-}$ relative to wild-type 89.6. Each shape represents a different donor from a separate experiment (n = 3). Error bars represent SEM. $^{*}p < 0.05$, two-tailed paired t test.[10]

[10] This figure was created by Michael Mashiba.

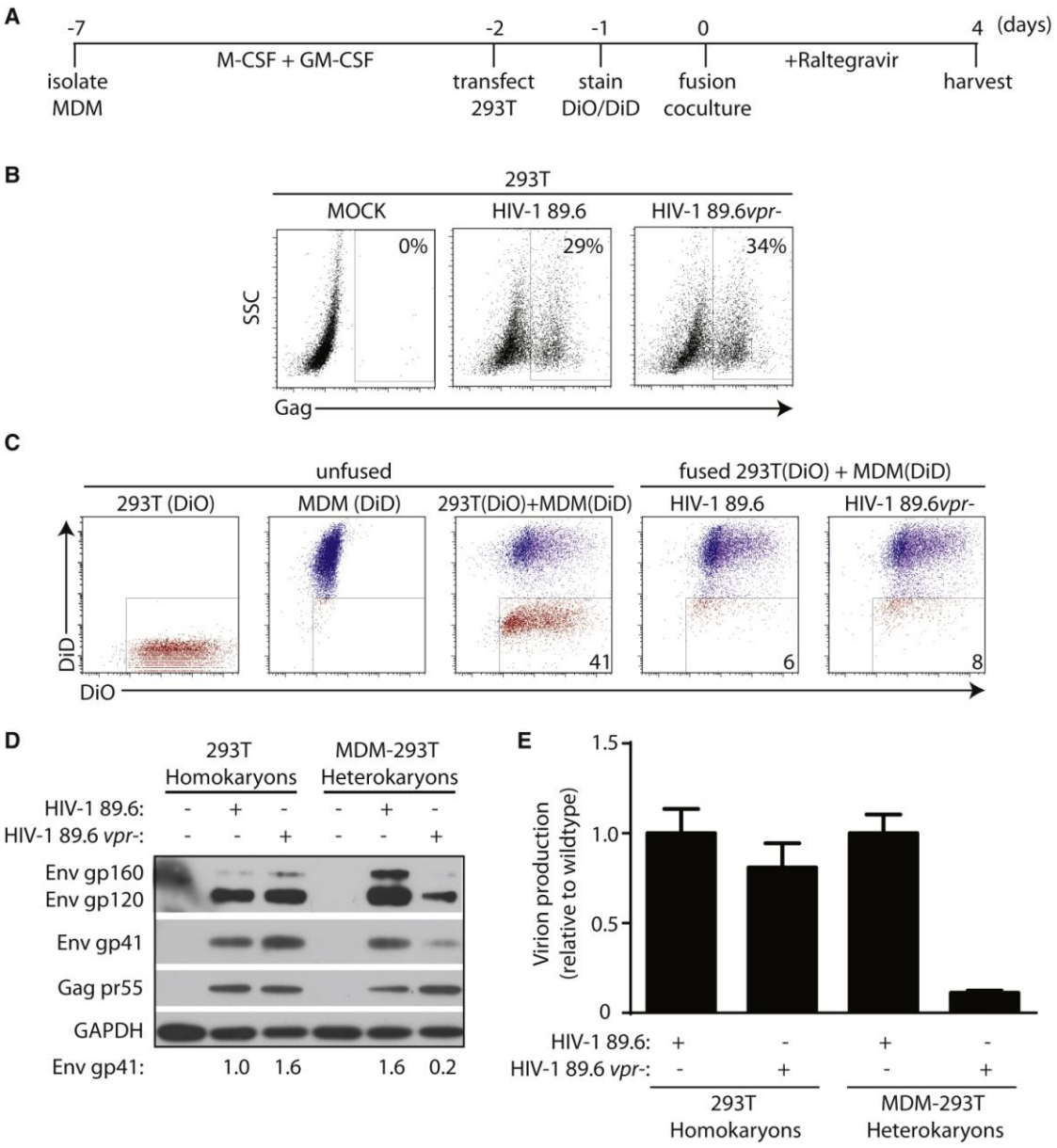

Figure 2.10: Vpr counteracts a dominant restriction of Env and virion production in MDMs. (**A**) Outline of experimental setup in which 293T cells transfected with HIV-1 and paramyxovirus fusion proteins were fused with uninfected MDMs for 4 days in the presence of raltegravir to prevent viral spread to unfused MDMs. (**B**) Flow cytometric analysis of intracellular Gag expression by 293T at 2 days post-transfection with the indicated HIV expression plasmid. (**C**) Flow cytometric analysis of 293T cells that were mock transfected (unfused) or transfected with paramyxovirus fusion proteins and the indicated HIV plasmids (fused), stained with DiO, and cultured as indicated with DiD-stained MDMs in the presence of raltegravir. Numbers represent frequency of residual unfused 293T cells. (**D**) Immunoblot of 293T cell homokaryons and MDM+293T heterokaryons. Env gp41 quantitation reflects densitometrically determined values relative to Gag pr55. (**E**) Virion production by 293T cell homokaryons and MDM-293T

heterokaryons measured and calculated as described in Figure 2.5 and normalized to wild-type. Error bars represent SEM.[11]

[11] This figure was created by David Collins.

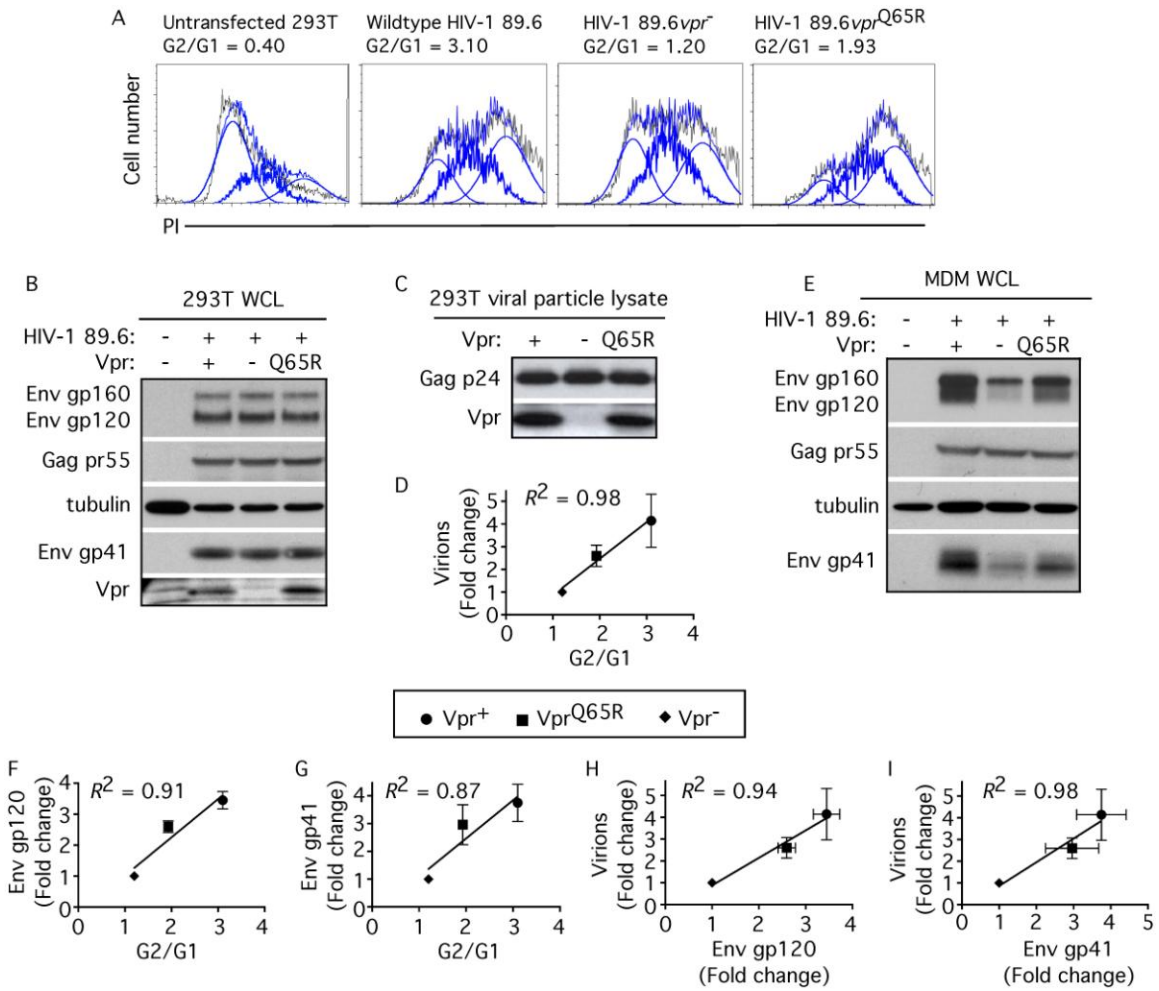

Figure 2.11: DCAF1-dependent functional activities correlate with Vpr–dependent virion production and Env expression. (**A**) Flow cytometric DNA content analysis of Gag^+ 293T cells transfected for 2d with the indicated HIV-1 genomic DNA plasmids. Black histograms represent raw data and blue histograms represent G1, S, G2 and summated model populations predicted by the Watson algorithm. (**B**,**C**,**E**) Immunoblot analysis of WCL, (B,E) or viral lysate (C) from the indicated cells transfected with the indicated genomic plasmid (B,C) or infected with the indicated virus (E). (**D**, **F**-**I**) 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 1 μg 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 SEM. Best-fit curves and R^2 values were obtained by linear regression analysis.[12]

[12] This figure was created by David Collins and Michael Mashiba.

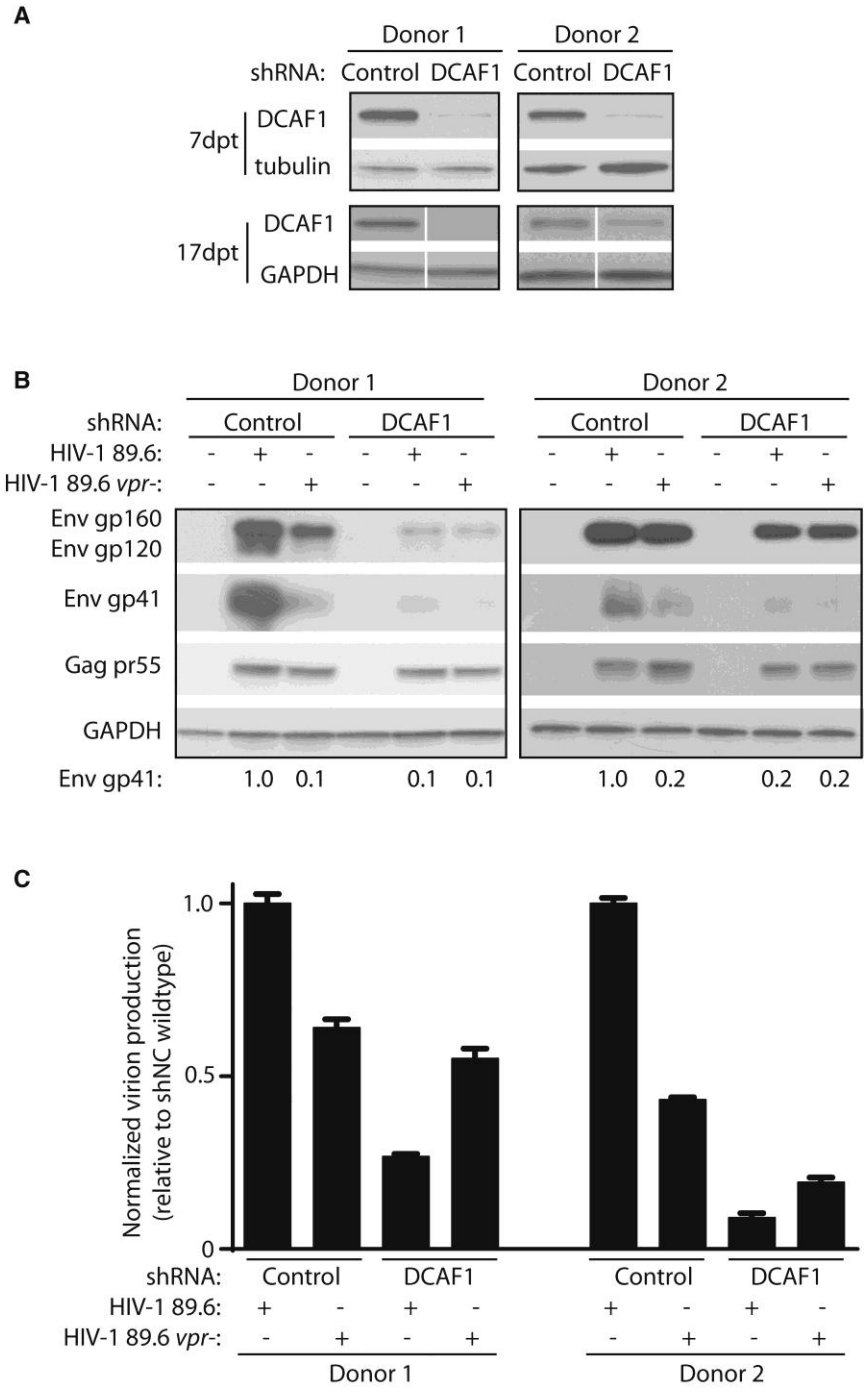

Figure 2.12: DCAF1 is required for Vpr to increase virion production and Env expression. (**A**) Immunoblot analysis of WCLs prepared from MDMs transduced with lentiviral vectors expressing shRNAs targeting DCAF1 or luciferase (Control) and incubated for 7 days (first 3 days with puromycin) (Pertel et al., 2011). dpt, days post-transduction. (**B**) Immunoblot analysis of WCLs from MDMs treated as described in (A) and then infected with 1 µg of wild-type 89.6 or 89.6*vpr*$^{-}$ for 10 days. Quantitation

reflects densitometric analysis of Env gp41 expression normalized for Gag pr55 expression. (**C**) Normalized virion production by MDMs treated and infected as described in (B). Error bars represent SEM.[13]

[13] This figure was created by David Collins.

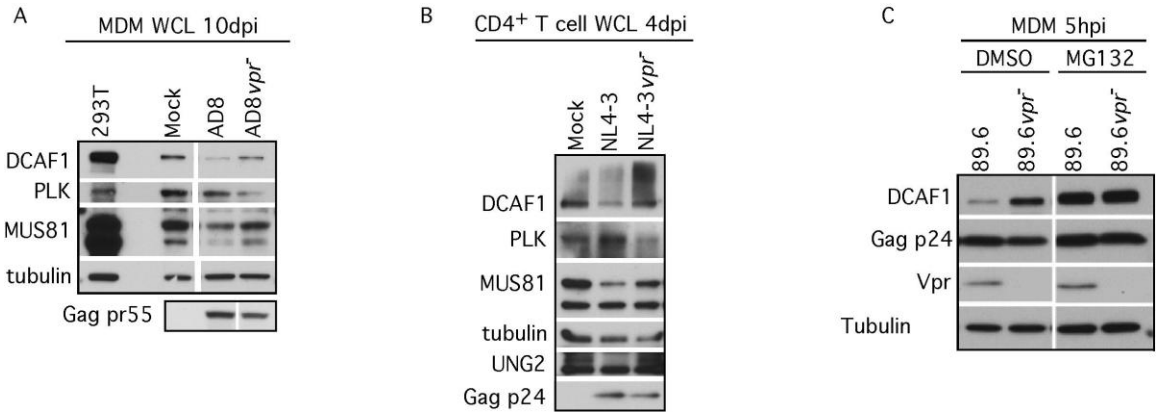

Figure 2.13: Vpr causes accumulation of PLK and turnover of MUS81, DCAF1 and UNG2 in MDM and primary cell targets of HIV-1. (**A**-**C**) Immunoblot analysis of WCL from cells infected with the indicated virus. Where indicated, cells were treated with 2.5μM MG132 or DMSO from -2 to 5hpi. AD8 infected MDM in (A) were infected by 1 μg of virus, CD4+ T cells in (B) were infected with 20μg of virus and MDM infected with 89.6 in (C) were infected with 100μg of virus.[14]

[14] This figure was created by Michael Mashiba.

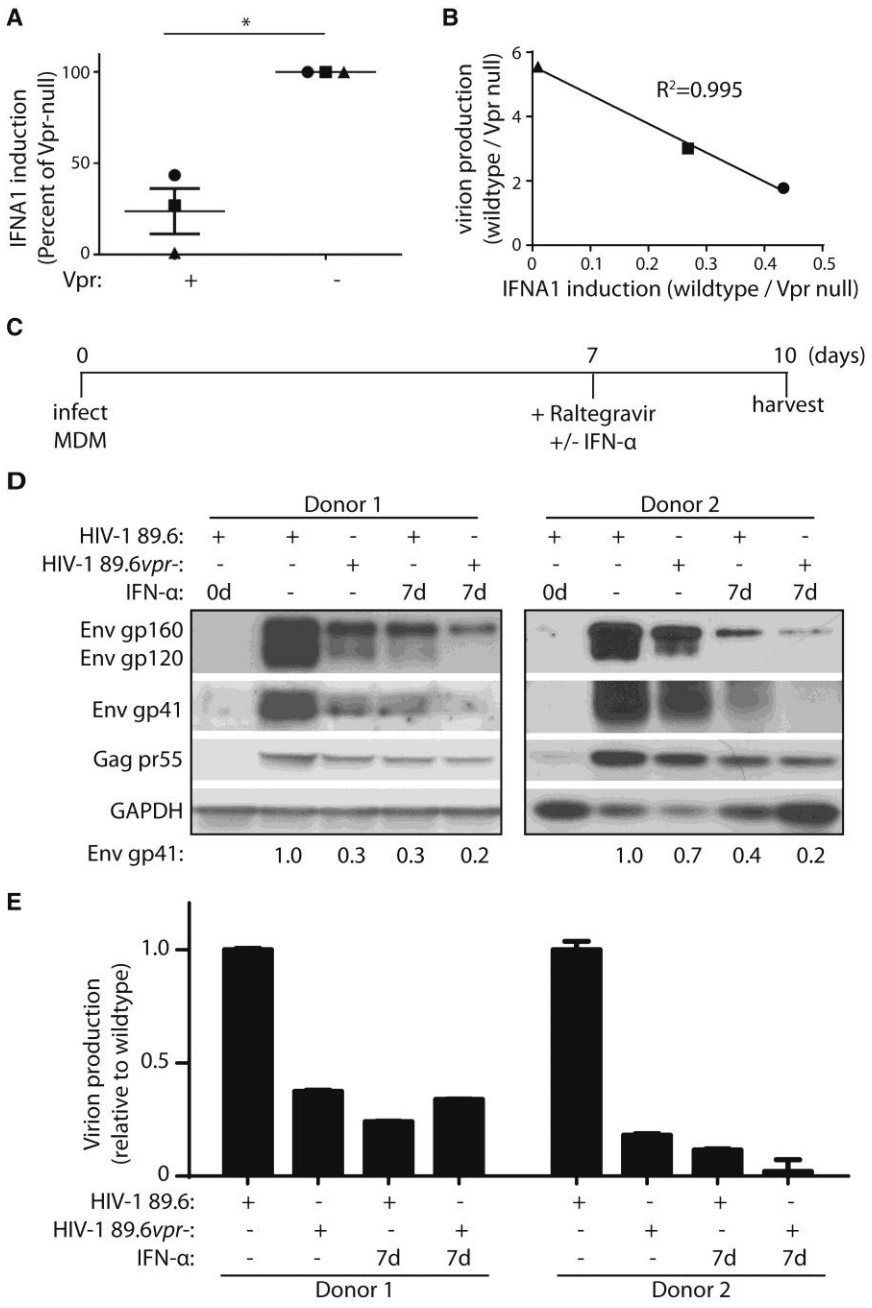

Figure 2.14: Vpr counteracts an antiviral interferon response in MDMs that restricts Env and virion production. (**A**) *IFNA1* gene induction in MDMs infected for 12 hours with 100 µg of wild-type 89.6 or 89.6*vpr*⁻ as measured by qRT-PCR, normalized to *ACTB*, and expressed as fold induction relative to mock and normalized to Vpr null (n = 3 donors). (**B**) Graphical analysis demonstrating an inverse correlation between *IFNA1* gene induction as measured in (A) and Vpr stimulation of virion production at 10 days post-infection with 1 µg of each virus. Best-fit curve and R^2 value were obtained by linear regression analysis (n = 3 donors). (**C**–**E**) (C) Outline of experimental setup for MDMs treated with 500 U/mL recombinant human IFNα and assessed for Env

expression (D) and virion production (E) in two independent experiments. Error bars represent SEM. *$p < 0.05$, two-tailed paired t test.[15]

[15] This figure was created by David Collins and Valeri Terry.

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Chapter 3

HIV-1 Vpr promotes infection of CD4$^+$ T lymphocytes: evidence for a macrophage-dependent stage of HIV-1 infection

Summary

Vpr is a conserved primate lentiviral protein that promotes infection of T lymphocytes in vivo by an unknown mechanism. Here we demonstrate that Vpr and its cellular co-factor, DCAF1, are necessary for efficient cell-to-cell spread of HIV-1 from macrophages to CD4$^+$ T lymphocytes when there is inadequate cell-free virus to support direct T lymphocyte infection. Remarkably, Vpr functioned to counteract a macrophage-specific intrinsic antiviral pathway that targeted Env-containing virions to LAMP1$^+$ lysosomal compartments. This restriction of Env also impaired virological synapses formed through interactions between HIV-1 Env on infected macrophages and CD4 on T lymphocytes. Treatment of infected macrophages with exogenous interferon-alpha induced virion degradation and blocked synapse formation, overcoming the effects of Vpr. These results provide a mechanism that explains the in vivo requirement for Vpr and suggests that a macrophage-dependent stage of HIV-1 infection drives the evolutionary conservation of Vpr.

Introduction

HIV-1 Vpr is conserved in all lentiviruses. However, decades of research have not revealed a functional explanation for its evolutionary conservation. $CD4^+$ T lymphocytes are the most abundant cellular target of HIV-1 in vivo and are widely regarded as the main drivers of viremia, persistence and progression to acquired immunodeficiency syndrome (Rockstroh et al.). While Vpr enables robust T lymphocyte infection and rapid disease progression in vivo (Hoch et al., 1995; Lang et al., 1993) and in ex vivo human lymphoid tissue (Rucker et al., 2004), Vpr is dispensable and may actually be detrimental to HIV-1 replication in T lymphocytes in vitro (Balliet et al., 1994; Planelles et al., 1995; Rogel et al., 1995). Recent work using transformed cell lines has defined a molecular mechanism by which Vpr limits immune detection of HIV-1 through modulation of host cellular ubiquitin ligase pathways and activation of a cellular nuclease (Laguette et al., 2014). Vpr modulates these pathways at least in part through its interaction with its cellular co-factor DCAF1 (also known as VprBP) (Belzile et al., 2010; Le Rouzic et al., 2007). Vpr utilizes this pathway to counteract a macrophage-specific restriction of HIV-1 Env glycoprotein expression (Mashiba et al., 2014). However, in T lymphocytes, there is no defect in Env expression in the absence of Vpr (Mashiba et al., 2014) and it remains unclear how Vpr enhances HIV-1 replication in $CD4^+$ T lymphocytes in vivo (Guenzel et al., 2014; Kirchhoff, 2010).

In this study, we describe cell culture conditions in which HIV-1 infection of primary T lymphocytes depended entirely on contact-dependent spread from macrophages; a mode of spread that evaded neutralization by some antibodies.

Under these conditions, Vpr enhanced the formation of virological synapses (VS) between infected macrophages and primary T lymphocytes. Mechanistic studies revealed that Vpr functioned to prevent an innate immune response that dramatically reduced HIV-1 Env expression, normal virion trafficking and VS formation in macrophage-T lymphocyte co-cultures. The addition of exogenous interferon-α (IFN) effectively counteracted the ability of Vpr to promote spread from macrophages to T lymphocytes. Our results highlight the importance of macrophages in HIV-1 pathogenesis and explain a requirement for Vpr in HIV-1 infection of T lymphocytes, providing a previously elusive explanation for Vpr's strong evolutionary conservation.

Results

Efficient infection of primary CD4⁺ T lymphocytes requires contact-dependent HIV-1 spread from infected macrophages.

To evaluate a role for Vpr in T lymphocyte infection that explained in vivo observations, we developed an assay to measure HIV-1 spread from primary macrophages to autologous CD4⁺ T lymphocytes. As outlined in **Figure 3.1A**, we inoculated primary monocyte-derived macrophages (MDM) with HIV-1 and allowed infection to establish for two days before co-cultivation with activated autologous CD4⁺ T lymphocytes for an additional two days to enable viral spread. MDM-T lymphocyte co-cultures produced an average of nine-fold more HIV-1 than infected MDM alone, suggesting that co-cultivation resulted in efficient spread between macrophages and T lymphocytes (**Figure 3.1B**).

To measure the frequency of infection in each cell type, we used flow cytometry to distinguish macrophages from T lymphocytes by expression of surface markers and measured infection by intracellular Gag staining (**Figure 3.2A**). Detection of Gag^+ cells was dependent on reverse transcriptase activity demonstrating that our assay measures productive HIV-1 replication (**Figure 3.2B**). Although HIV-1 infects and depletes $CD4^+$ T lymphocytes to cause acquired immunodeficiency syndrome in vivo, infection of primary $CD4^+$ T lymphocytes by cell-free virus was inefficient in vitro after two days of continuous culture (**Figures 3.1A**, **3.2A**) using an inoculum comparable to the amount of virus present in MDM-T lymphocyte co-cultures (data not shown). In comparison, co-cultivation of activated T lymphocytes with infected MDM increased T lymphocyte infection by thirty-fold (**Figure 3.1C**).

The capacity for MDM to efficiently infect autologous primary $CD4^+$ T lymphocytes depended on direct cell-to-cell contact because infection was not detected when the cells were separated by a virus-permeable transwell insert (**Figures 3.1D,E**). Direct cell-to-cell transmission of HIV-1 across virological synapses between infected and target cells has been previously described and is known to be highly resistant to antibody neutralization (Durham et al., 2012; Schiffner et al., 2013). Consistent with this mode of spread, we observed that MDM-dependent spread to autologous primary $CD4^+$ T lymphocytes was highly resistant to neutralizing antibodies against Env gp120 (b12), Env gp41 (Z13E1) and CD4 (SIM2) that were capable of neutralizing greater than 95% of free virus infection of MDM (**Figure 3.1F**). When added at the time of co-cultivation, only 2G12, which has

been previously demonstrated to block cell-to-cell spread (Duncan et al., 2014), was able to efficiently neutralize MDM-dependent T lymphocyte infection (**Figure 3.1F**).

Previous studies have demonstrated that uninfected dendritic cells and MDM can infect T lymphocytes through a "*trans"* mechanism in which virions bound to lectin receptors are transferred to T lymphocytes (**Figure 3.1G**) (McDonald, 2010; Peressin et al., 2014). This contrasts with *"cis"* infection that requires HIV-1 replication in MDM. To determine the mode of infection that was active in our system, we used the protocol described in **Figure 3.1A** but substituted an HIV-1 molecular clone that can infect T lymphocytes but not macrophages (NL4-3). Similar to HIV-1 89.6, NL4-3 did not infect primary T lymphocytes when cultured with free virus. Consistent with previous reports (O'Doherty et al., 2000), however, this virus infected a high percentage of T lymphocytes upon spinoculation (**Figure 3.1H**). As expected, NL4-3 did not infect MDM (**Figure 3.1H**) and MDM treated with NL4-3 as outlined in **Figure 3.1A** did not spread infection to primary $CD4^+$ T lymphocytes (**Figure 3.1H**). Thus, spread of infection from MDM to primary $CD4^+$ T lymphocytes required productive HIV-1 replication in MDM under the conditions of our assay. In summary, efficient infection of primary $CD4^+$ T lymphocytes required contact-dependent, neutralizing antibody-resistant, *cis*-mediated virus transfer from HIV-1 infected macrophages.

Vpr enables macrophage-dependent T lymphocyte infection.

The HIV-1 Vpr protein is necessary for optimal infection and spread in MDM cultures but can actually be detrimental to spread of infection in actively replicating

cells due to its inhibitory effects on cell cycle progression (Goh et al., 1998; Rogel et al., 1995; Stivahtis et al., 1997). Because $CD4^+$ T lymphocytes are the main target of HIV-1 in vivo, Vpr's role in HIV-1 infection and its evolutionary conservation across lentiviral species targeting a wide range of primates has remained enigmatic (Mashiba and Collins, 2013). We hypothesized that the mode of spread we describe here in which T lymphocyte infection is entirely dependent on infected MDM might reveal a crucial role for Vpr in enabling efficient T lymphocyte infection. To address this, we co-cultivated activated primary $CD4^+$ T lymphocytes with autologous MDM infected by HIV-1 89.6 containing or lacking Vpr (**Figure 3.3A**). Indeed, we observed a striking enhancement of infection by Vpr in our co-culture assay as measured by virion production (nine-fold, **Figure 3.3B**) and frequency of T lymphocyte infection (three-fold, **Figure 3.3C**).

Because Vpr stimulates HIV-1 spread among macrophages (**Figure 3.3C**) (Connor et al., 1995; Mashiba et al., 2014), it was possible that the stimulation of T lymphocyte infection we observed may result from an increase in the number of infected MDM that could amplify virus production. To address this, we measured spread of HIV-1 from infected MDM to T lymphocytes under conditions in which HIV-1 could only infect MDM for a single round and subsequent spreading infection could only occur in T lymphocytes. This was accomplished by using T-lymphotropic HIV-1 NL4-3 pseudotyped with macrophage-tropic YU2 Env (**Figure 3.3D**). This virus utilizes YU-2 Env protein to efficiently infect MDM for one round of viral replication. However, *de novo* virions produced by the infected macrophages express only NL4-3 Env and thus can only infect T lymphocytes. As previously

reported (Mashiba et al., 2014), this virus initially infected macrophages equally in the presence or absence of Vpr expression (**Figure 3.3E**). Remarkably, however, Vpr significantly enhanced spread of HIV-1 from infected MDM to T lymphocytes (four-fold, **Figure 3.3E**). In contrast, Vpr did not stimulate direct infection of primary T lymphocytes via spinoculation (**Figure 3.3E**), consistent with previous studies (Balliet et al., 1994). These data indicate that Vpr promotes the directional spread of HIV-1 from macrophages to T lymphocytes and that this activity of Vpr is conserved in diverse HIV-1 isolates.

Vpr-dependent HIV-1 spread from macrophages to T lymphocytes requires DCAF1.

Vpr interacts with the cellular protein DDB1-and-CUL4-associated factor 1 (DCAF1, also known as VprBP) to modulate ubiquitylation and proteasomal degradation pathways (Ahn et al., 2010; Belzile et al., 2010; Casey Klockow et al., 2013; Collins and Collins, 2014). Recent work has demonstrated that DCAF1 is an essential co-factor for Vpr to evade the induction of a type I interferon response, and thereby counteract macrophage restriction of Env and virion production (Laguette et al., 2014; Mashiba et al., 2014). To determine whether this pathway was required for spread of HIV-1 from infected MDM to primary T lymphocytes, we employed the Vpr Q65R mutant of 89.6 that is deficient at interacting with DCAF1 and relatively defective at inducing DCAF1-dependent cell cycle arrest (DeHart et al., 2007; Mashiba et al., 2014). We found that Vpr Q65R was proportionally defective at enhancing HIV-1 spread from MDM to $CD4^+$ T lymphocytes (**Figure 3.4A**). To more directly address the requirement of DCAF1 for Vpr-dependent spread, we silenced

DCAF1 in infected MDM and co-cultured these cells with autologous T lymphocytes (**Figure 3.4B**). Remarkably, we found that DCAF1 silencing abrogated the ability of Vpr to stimulate transmission of HIV-1 from MDM to $CD4^+$ T lymphocytes (**Figure 3.4C**). These data demonstrate that Vpr requires DCAF1 to promote MDM-to-T lymphocyte spread of HIV-1.

Vpr prevents lysosomal targeting of Env-containing virions in macrophages.

MDM infected by HIV-1 lacking Vpr mount an innate immune response that restricts Env expression by accelerating lysosomal degradation of Env, and Vpr prevents the induction of this pathway via a DCAF1-dependent mechanism (Mashiba et al., 2014). Because DCAF1 was also required for Vpr-dependent MDM-T lymphocyte spread of HIV-1 (**Figures 3.4A-C**), we assessed whether this pathway was active in MDM-T lymphocyte cocultures. We analyzed co-culture whole-cell lysates for steady-state Env expression by quantitative immunoblot in the presence or absence of Vpr (**Figure 3.4D**). Indeed, we observed a loss of Env gp160, gp120 and gp41 relative to the HIV Gag precursor pr55 in the absence of Vpr in cocultures (**Figure 3.4E**), similar to what was previously reported in HIV-1 infected MDM (Mashiba et al., 2014).

Because virions incorporate HIV-1 Env and because Env is required for Vpr-dependent changes in virion production (Mashiba et al., 2014), we hypothesized that in the absence of Vpr, Env-containing virions are targeted for lysosomal degradation in macrophages. To test this, we examined the localization of mature virions (Gag $MAp17^+$) with LAMP1, a marker of lysosomes. Because HIV-infected

cells form syncytia, infected MDM are frequently multinucleated, which we also observed (**Figure 3.5A**). Remarkably, in the absence of Vpr, mature virions (magenta puncta in **Figure 3.5A**, lower two panels) frequently co-localized with LAMP1. In comparison, expression of Vpr reduced co-localization of mature virions with lysosomal markers (**Figures 3.5A,B**). In addition, we observed more virions present in LAMP1$^+$ compartments when lysosomal acidification was blocked by NH_4Cl treatment, indicating that colocalization with LAMP1 represents bona fide lysosomal targeting that results in significant degradation (**Figure 3.5B**).

Vpr also prevented targeting of virions to lysosomes in MDM infected by YU-2 Env-pseudotyped HIV-1 NL4-3. Moreover, lysosomal targeting of virions was not observed without expression of Env from the integrated provirus (YU-2 Env-pseudotyped HIV-1 NL4-3*env*$^-$, **Figure 3.5C**). These studies reveal that in the absence of HIV-1 Vpr, MDM restrict HIV-1 by targeting Env-containing virions for lysosomal degradation. Furthermore, the capacity of Vpr to counteract this restriction is conserved among disparate HIV-1 clones.

Because restriction of Env expression and virion release by infected MDM is inducible by type I interferon (Mashiba et al., 2014), we treated MDM with exogenous IFNα to assess its effects on virion localization. Interestingly, IFNα stimulated lysosomal targeting of virions even in MDM expressing Vpr (**Figure 3.5B**), confirming the model that Vpr acts primarily by preventing the induction of an innate immune restriction pathway.

Vpr increases Env-dependent virological synapse formation between macrophages and T lymphocytes.

Infection of T lymphocytes in our culture system occurs by direct cell-to-cell spread, which requires formation of a transient VS between the infected cell and its target. Formation of VS requires interactions between HIV-1 Env on infected cells and CD4 on target cells (Jolly et al., 2004). Upon VS formation, high concentrations of mature virions localize to VS to mediate cell-to-cell spread (Dale et al., 2013). Because Vpr rescues Env and Env-containing virions from lysosomal degradation, we hypothesized that Vpr would also enable the formation of VS in the co-culture system. To determine whether Vpr affects VS formation between MDM and primary T lymphocytes, we used laser-scanning confocal microscopy to visualize areas of co-localization between surface CD4 on T lymphocytes and mature virions in MDM. We pre-stained T lymphocytes with an anti-CD4 antibody (DK4003) that does not disrupt the ability of CD4 to bind Env, and co-cultured these cells with infected MDM briefly to allow formation of cellular contacts. We then washed away unbound cells and stained with an antibody against Gag MAp17 to visualize mature virions, as previously described (Duncan et al., 2014; Giese and Marsh, 2014; Groot et al., 2008). Virological synapses were identified as regions of co-localization between CD4 (green puncta in **Figure 3.6A**) on T lymphocytes and mature Gag on MDM (red puncta in **Figure 3.6A**). We identified similar numbers of MDM infected with wild type and mutant virus, and infected MDM were frequently multi-nucleated syncytia (**Figure 3.6A**). However, we consistently observed significantly more VS per

infected MDM in the presence of Vpr (**Figures 3.6A,B**). These results explain why spread of HIV-1 from MDM to T lymphocytes is dramatically enhanced by Vpr.

As has been shown for other types of cell-to-cell spread (Jolly et al., 2004), we observed that VS between MDM and primary T lymphocytes did not form in the absence of de novo Env expression (YU2 Env-pseudotyped HIV-1 89.*6env*$^{-}$) (**Figure 3.6C**). Furthermore, consistent with a previous report (Massanella et al., 2009), VS formation was efficiently blocked by treating infected MDM with a high concentration (10 μg/ml) of the broadly-neutralizing anti-Env gp120 antibody b12 at the time of co-culture (**Figure 3.6C**). Thus, VS formation between HIV-1 infected MDM and primary T lymphocytes requires HIV-1 Env expression and is dramatically enhanced by expression of Vpr in MDM.

Vpr enhances Env expression by preventing the induction of a type I interferon-inducible restriction that degrades Env (Mashiba et al., 2014) and targets Env-containing virions for lysosomal degradation (**Figure 3.5**). Therefore we asked whether the addition of IFN-α to infected macrophages affected VS formation with T lymphocytes. Indeed, we observed that IFN-α significantly reduced the number of VS detected per infected MDM even when MDM expressed Vpr (**Figure 3.6C**). In sum, these results are consistent with a model in which Vpr increases HIV-1 infection of T lymphocytes by preventing the induction of a type I interferon-mediated restriction of Env-dependent VS formation in MDM.

Methods

Antibodies

Antibodies to CAp24 (KC57-FITC, Beckman Coulter), CD3 (OKT3-Pacific Blue, BioLegend) and CD14 (HCD14-APC, BioLegend) were used for flow cytometry. Antibodies to the following proteins were used for immunoblot analysis: DCAF1 (11612-1-AP Proteintech), GAPDH (Santa Cruz Biotech), Gag pr55 (HIV-Ig), Env gp160/120, Env gp41, and Vpr (AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog 288 from Dr. Michael Phelan (Hatch et al., 1992), 11557 from Dr. Michael Zwick (Zwick et al., 2001), 3951 from Dr. Jeffrey Kopp, and 3957 from NABI and NHLBI). Antibodies to the following proteins were used for microscopy: CD4 [DK4003 (Centre for AIDS Reagents, NIBSC, contributed by Dr. D Healey)], Gag MAp17 [4C9 (Centre for AIDS Reagents, NIBSC, contributed by Drs. R B Ferns and R S Tedder)] and LAMP1 [H4A3 (Abcam)]. Secondary antibodies were FITC-conjugated goat anti-mouse IgG (H+L) and AlexaFluor 647-conjugated goat anti-mouse IgG2a (BD Biosciences). Neutralizing antibodies 2G12, b12, SIM.2, and Z13E1 (AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog 1476 from Dr. Hermann Katinger (Buchacher et al., 1994), 2640 from Dr. Dennis Burton and Carlos Barbas (Burton et al., 1991), 723 from Dr. James E.K. Hildreth (McCallus et al., 1992)) were used at a 1:1000 dilution for neutralization studies at the time of coculture, and b12 was used at 1:100 to block VS formation.

Viral constructs

p89.6 and pNL4-3 were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalogs 3552 and 114 from Dr. Ronald G. Collman and Dr. Malcolm Martin, respectively (Adachi et al., 1986; Collman et al., 1992; Li et al., 1991). p89.6*vpr*$^-$, p89.*6env*$^-$, p89.6*vpr*Q65R, pNL4-3*env*$^-$, pNL4-3*vpr*$^-$, and pNL4-3*vpr*$^-$*env*$^-$ were constructed as previously described (Mashiba et al., 2014). pSIV3$^+$, psPAX2, pAPM-1221 (shNC) and pDCAF-APM.1-3 (shDCAF1) were obtained from Dr. Jeremy Luban (Pertel et al., 2011). pYU-2*env* was obtained from Joseph Sodroski (Sullivan et al., 1995).

Virus preparation

Virus stocks were obtained by transfection of 293T cells with virus expression plasmids using polyethylenimine, as described (Mashiba et al., 2014; McNamara et al., 2012). Pseudotyped virus was produced by co-transfecting 293T cells with provirus and Env expression plasmid, as described (Mashiba et al., 2014). 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 (Mashiba et al., 2014).

Cell isolation, HIV-1 infection and MDM-T lymphocyte coculture

Leukocytes isolated from anonymous donors by apheresis were obtained from New York Blood Center Component Laboratory. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll density gradient separation, as described (Norman et al., 2011). CD14$^+$ monocytes and CD4$^+$ T lymphocytes were

isolated as previously described (Mashiba et al., 2014). Briefly, 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% Certified endotoxin-low fetal bovine serum (Gibco, Invitrogen)], penicillin (10 Units/ml), streptomycin (10 μg/ml), L-glutamine (292 μg/ml), carrier-free M-CSF (50 ng/ml, R&D Systems) and GM-CSF (50 ng/ml R&D Systems) for seven days. MDM were incubated with 5 μg HIV-1 for six hours and cultured in fresh medium for two to four days. $CD4^+$ T lymphocytes were isolated by CD8 negative selection (DynaBeads, Life Technologies), cultured in R10 for several days and activated with 5 μg/ml phytohaemagglutinin (PHA-L, Calbiochem) overnight before addition of 500 IU/ml recombinant human IL-2 (R&D Systems). T lymphocytes were infected with 5 μg or 50 μg HIV-1 by spinoculation at 2500 RPM for 2-3h with 8 μg/ml polybrene (Sigma) 72h following PHA stimulation, as described (Norman et al., 2011), or incubated with virus for two days, where indicated. For coculture experiments, HIV-1-infected MDM were cocultured with autologous $CD4^+$ T lymphocytes 72 hours after PHA activation for two days. Infected T lymphocyte monocultures or cocultures were maintained in R10 and IL-2 until analyzed.

Flow cytometry

Surface staining for CD3 and CD14 was performed before fixation and intracellular staining for Gag CAp24 as described previously (Carter et al., 2010; Mashiba et al., 2014). Flow cytometric data was acquired using a FACSCanto

instrument with FACSDiva collection software (BD) or a FACScan (BD, Cytek) with FlowJo software (TreeStar) and analyzed using FlowJo. Cell cycle analysis of 293T cells was performed previously (Mashiba et al., 2014).

Immunoblot

MDM or MDM-T lymphocyte cocultures were lysed in Blue Loading Buffer (Cell Signaling), sonicated with a Misonix sonicator (Qsonica, LLC.) and clarified by centrifugation at 13000 RPM. Lysates were analyzed by SDS-PAGE immunoblot and protein levels were quantified using Adobe Photoshop as described (Mashiba et al., 2014; Norman et al., 2011).

CAp24 ELISA

CAp24 ELISA was performed as previously described and quantitation of mass is based upon commercial standards (ViroGen) (Mashiba et al., 2014).

RNAi

Short hairpin RNA-mediated knockdown of DCAF1 was performed as previously described (Mashiba et al., 2014; 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 R10 with M-CSF (50 ng/ml) and GM-CSF (50 ng/ml) plus 20 μg VSV-G-pseudotyped lentivirus containing a shRNA cassette targeting luciferase (Control) or DCAF1. Following an overnight incubation, the cells were

cultured for 3 days in fresh medium before addition of 10 μg/ml puromycin for 3 additional days prior to HIV-1 infection.

Laser-scanning confocal microscopy (LSCM)

LSCM of MDM or MDM-T lymphocyte VS was performed as described previously (Duncan et al., 2014; Groot et al., 2008), with modifications. Briefly, MDM were differentiated on Nunc Lab-Tek 4-well chambered borosilicate cover glass (Thermo Fisher). For VS visualization, autologous, PHA/IL-2-activated $CD4^+$ T lymphocytes were pre-stained for surface CD4 for one hour with primary antibody plus 30 minutes with secondary antibody and cocultured for four hours at room temperature with MDM before gentle washing with warm RPMI. For experiments using exogenous IFN, infected MDM were treated with 500U/mL recombinant IFNα (Calbiochem) two days before harvest. For LAMP1 staining, infected MDM were treated with 20 μM ammonium chloride for the final eight hours to prevent lysosomal acidification. Cells were fixed in 4% paraformaldehyde for one hour at room temperature and permeabilized with 0.1% saponin (Sigma) in 10% pooled human AB and goat sera for F_C-receptor blocking for one hour at room temperature, and endogenous biotin was blocked using endogenous biotin-blocking kit (Life Technologies) before staining for Gag p18 and/or LAMP1 for one hour primary and 30 minutes secondary using the antibodies listed above. Actin cytoskeleton was visualized by Phalloidin-TRITC (Sigma) and nuclei were stained using DAPI (Fisher Scientific). Cells were preserved in ProLong Gold anti-fade (Life Technologies) and visualized on a Leica SPX5 inverted confocal microscope at the University of

Michigan Microscopy and Image-Analysis Laboratory. Images of optical sections of approximately 1 μm depth were captured at 20X dry or 100X oil-immersion objective magnification. Images were processed using ImageJ (NIH) and co-localization was quantitated by automated spots analysis using Imaris (BitPlane). Each Gag MAp17^{+} puncta with signal 2-fold or greater above background based on a raltegravir-inhibited infected MDM control was identified in an automated manner, and fluorescence intensity in each channel was quantitated for each Gag^{+} spot. Co-localization was defined as the number of Gag^{+} spots that were also positive for LAMP1 or CD4 (VS) two-fold or greater above isotype staining controls, per Gag^{+} cell imaged.

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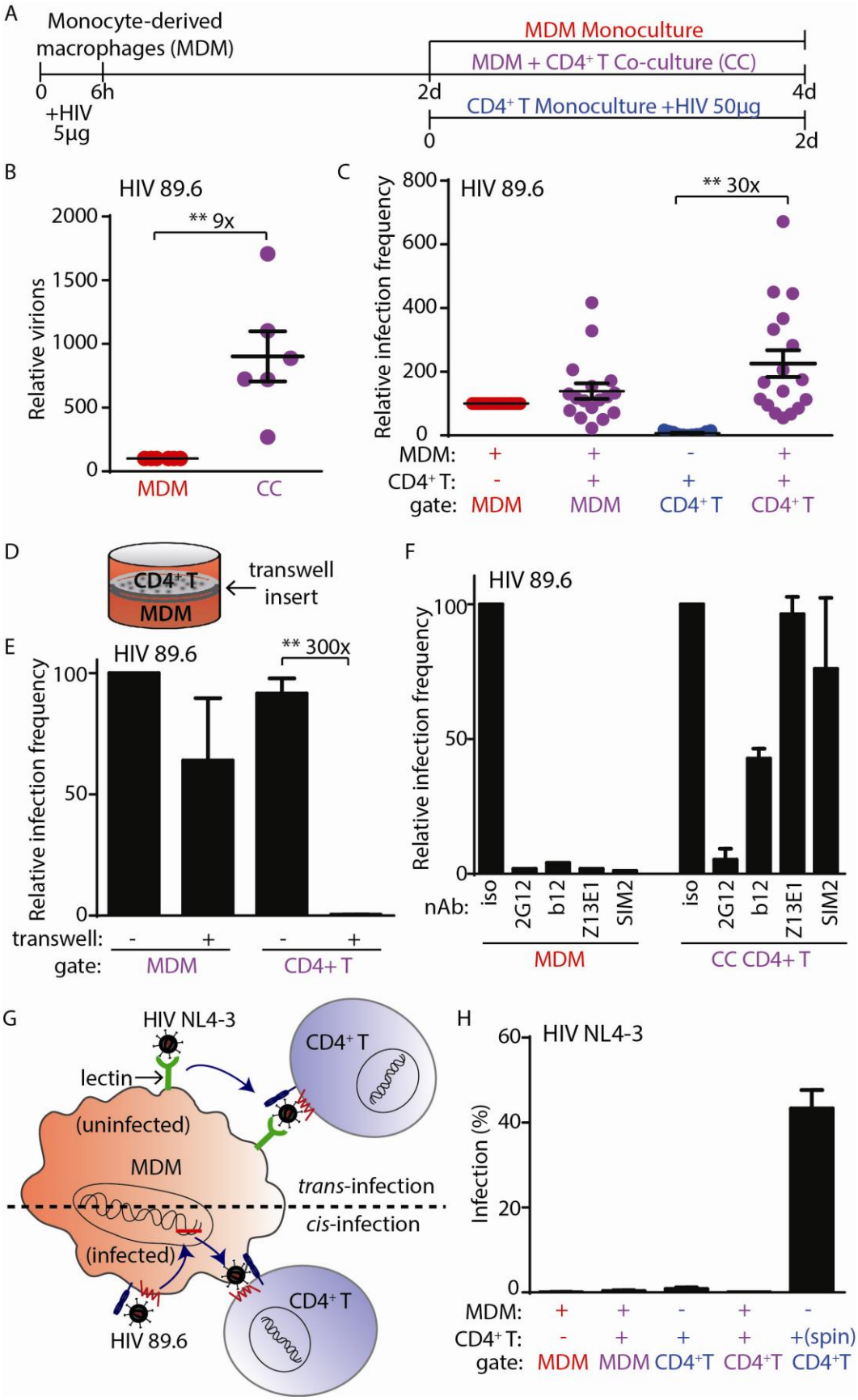
A
Monocyte-derived macrophages (MDM)
MDM Monoculture
MDM + CD4+ T Co-culture (CC)
CD4+ T Monoculture +HIV 50µg
0
6h
+HIV 5µg
2d
4d
0
2d
B
HIV 89.6
** 9x
Relative virions
2000
1500
1000
500
0
MDM
CC
C
HIV 89.6
** 30x
Relative infection frequency
800
600
400
200
0
MDM: + + - +
CD4+ T: - + + +
gate: MDM MDM CD4+ T CD4+ T
D
CD4+ T
MDM
transwell insert
E
HIV 89.6
** 300x
Relative infection frequency
100
50
0
transwell: - + - +
gate: MDM CD4+ T
F
HIV 89.6
Relative infection frequency
100
50
0
nAb: iso 2G12 b12 Z13E1 SIM2 iso 2G12 b12 Z13E1 SIM2
MDM
CC CD4+ T
G
HIV NL4-3
lectin
CD4+ T
(uninfected)
MDM
trans-infection
cis-infection
(infected)
CD4+ T
HIV 89.6
H
HIV NL4-3
Infection (%)
60
40
20
0
MDM: + + - + -
CD4+ T: - + + + +(spin)
gate: MDM MDM CD4+T CD4+T CD4+T

Figure 3.1: Efficient HIV infection of T lymphocytes requires contact with infected macrophages. (**A**) Graphical outline of experimental setup depicting HIV-1 infection of macrophages and co-cultivation with autologous, PHA-activated $CD4^+$ T lymphocytes as detailed in Methods. (**B**) Summary graph of relative quantity of virions released into culture supernatant as measured by Gag CAp24 ELISA (n=6 donors). (**C**) Summary graph of relative infected cell frequency in the indicated cultures as measured by flow cytometry (n=11 donors for $CD4^+$ T or 17 donors for MDM and CC). (**D**) Diagrammatic representation of virus-permeant transwell. (**E**) Summary graph of relative infected cell frequency in cocultures prepared as shown in A in the presence or absence of transwell inserts (n=4 donors). (**F**) Summary graph of relative infected cell frequency in the indicated cultures prepared as shown in A. Neutralizing antibodies to HIV Env gp120 (2G12, b12), gp41 (Z13E1) or CD4 (SIM.2) were added at the time of initial infection (MDM) or at the time of $CD4^+$ T addition and cocultivation (CC). (**G**) Diagram illustrating *trans*- and *cis*-infection of T lymphocytes. (**H**) Summary graph of infected cell frequency in the indicated cell type after addition of HIV-1 NL4-3 as described in A. For "spin" condition, PHA activated $CD4^+$ primary T lymphocytes were centrifuged at 2500 RPM with 50µg HIV-1 NL4-3 in polybrene (n=4 donors). Error bars represent SEM. **p<0.01, student's paired t-test.[1]

[1] This figure was created by David Collins.

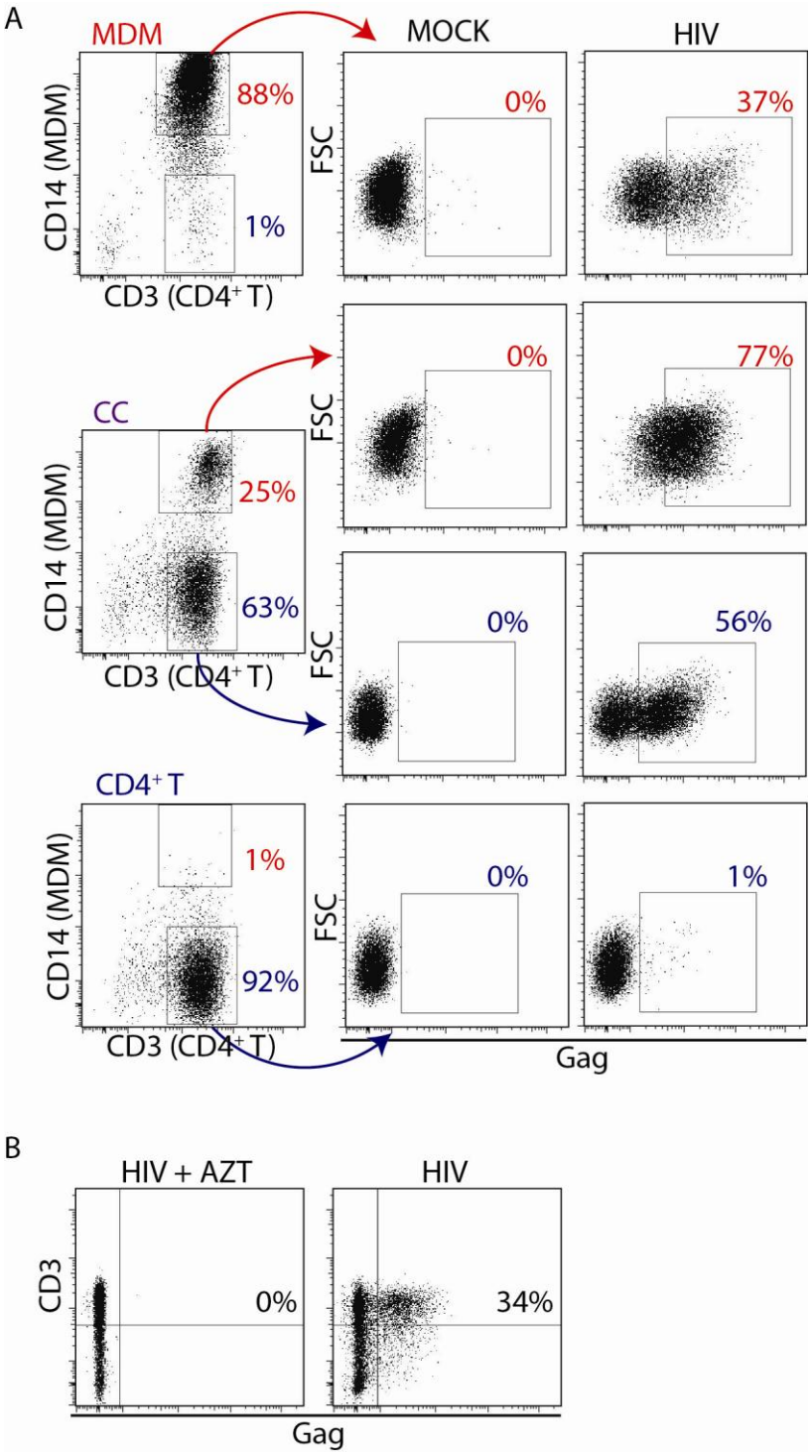

Figure 3.2: Flow cytometric analysis of HIV infection in macrophage-T lymphocyte cocultures. (**A**) Representative flow cytometric dot plots illustrating segregation of $CD14^+$ MDM from $CD3^+$ T lymphocytes in cocultures and subsequent assessment of HIV-1 infection by intracellular Gag CAp24 stain after treatment of the indicated cultures treated as shown in Figure 3.1A. (**B**) Representative flow cytometric dot plots of

intracellular Gag CAp24 staining (x-axes) vs. surface CD3 expression (y-axes) of MDM-$CD4^+$ T lymphocyte cocultures infected as shown in Figure 3.1A in the presence or absence of the reverse transcriptase inhibitor zidovudine (AZT).[2]

[2] This figure was created by David Collins.

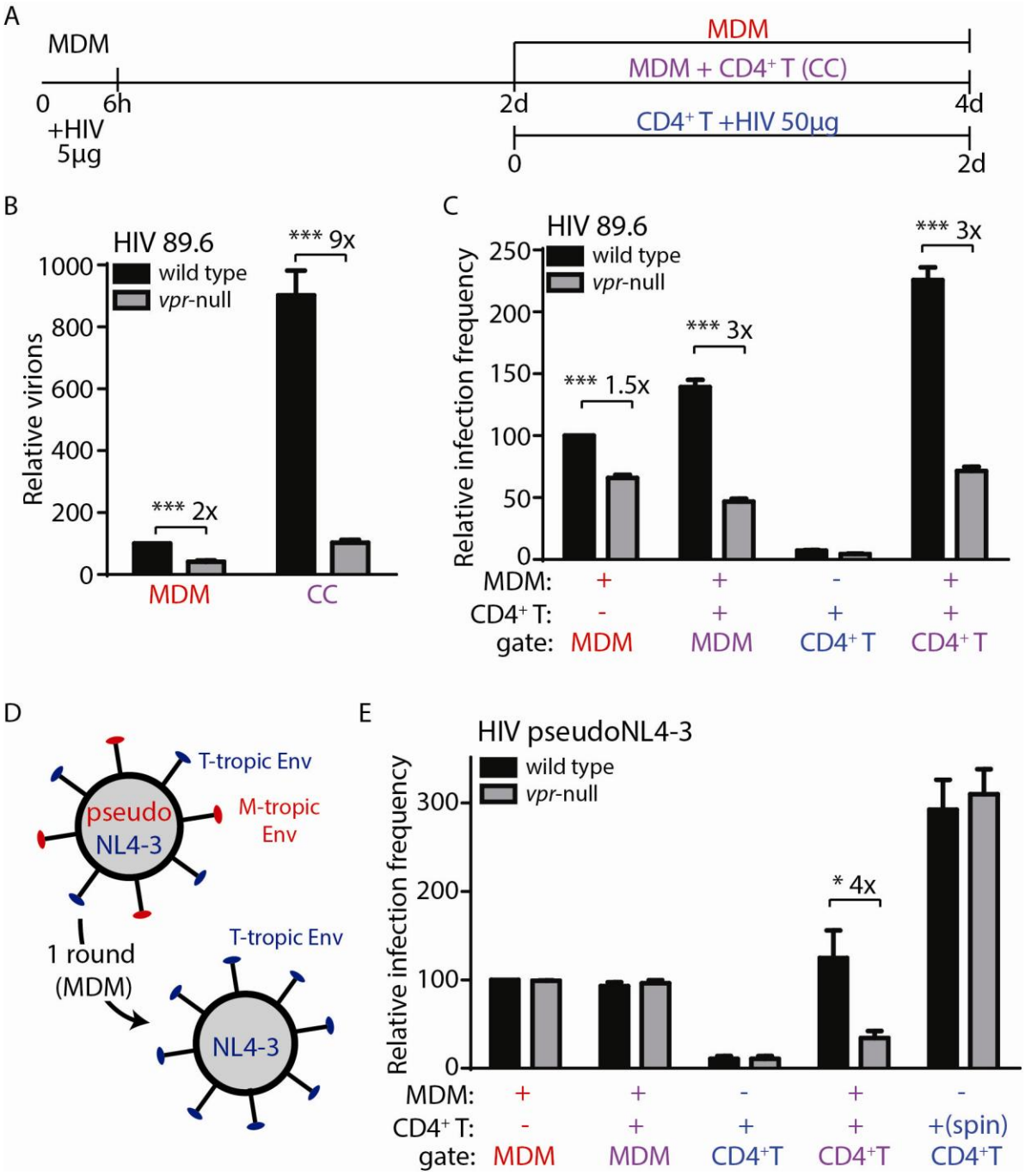

Figure 3.3: Vpr enhances macrophage-dependent infection of CD4$^+$ T lymphocytes. (**A**) Graphical outline of experimental setup as in Figure 3.1A. (**B**) Summary graph of relative quantity of virions released into the supernatant of the indicated cultures after inoculation with type (black bars) or *vpr*-null (gray bars) HIV-1 89.6 (n=6 donors). (**C**) Summary graph of relative infected cell frequency in the indicated cultures (n=11 donors for CD4$^+$ T or 17 donors for MDM and CC). (**D**) Diagram illustrating HIV-1 NL4-3 pseudotyped with YU-2 Env (Buchacher et al.) to infect macrophages for a single round and subsequently spread to T lymphocytes using NL4-3 Env (blue). (**E**) Summary graph

of relative infected cell frequency in the indicated cell type after addition of HIV-1 YU-2 pseudo-NL4-3 as described in A. For "spin" condition, PHA activated $CD4^+$ primary T lymphocytes were centrifuged for 2500 RPM with 50µg HIV-1 NL4-3 in polybrene (n=3 donors). Data normalized relative to wild-type MDM. Error bars represent SEM. *p<0.05, ***p<0.001, student's paired t-test.[3]

[3] This figure was created by David Collins.

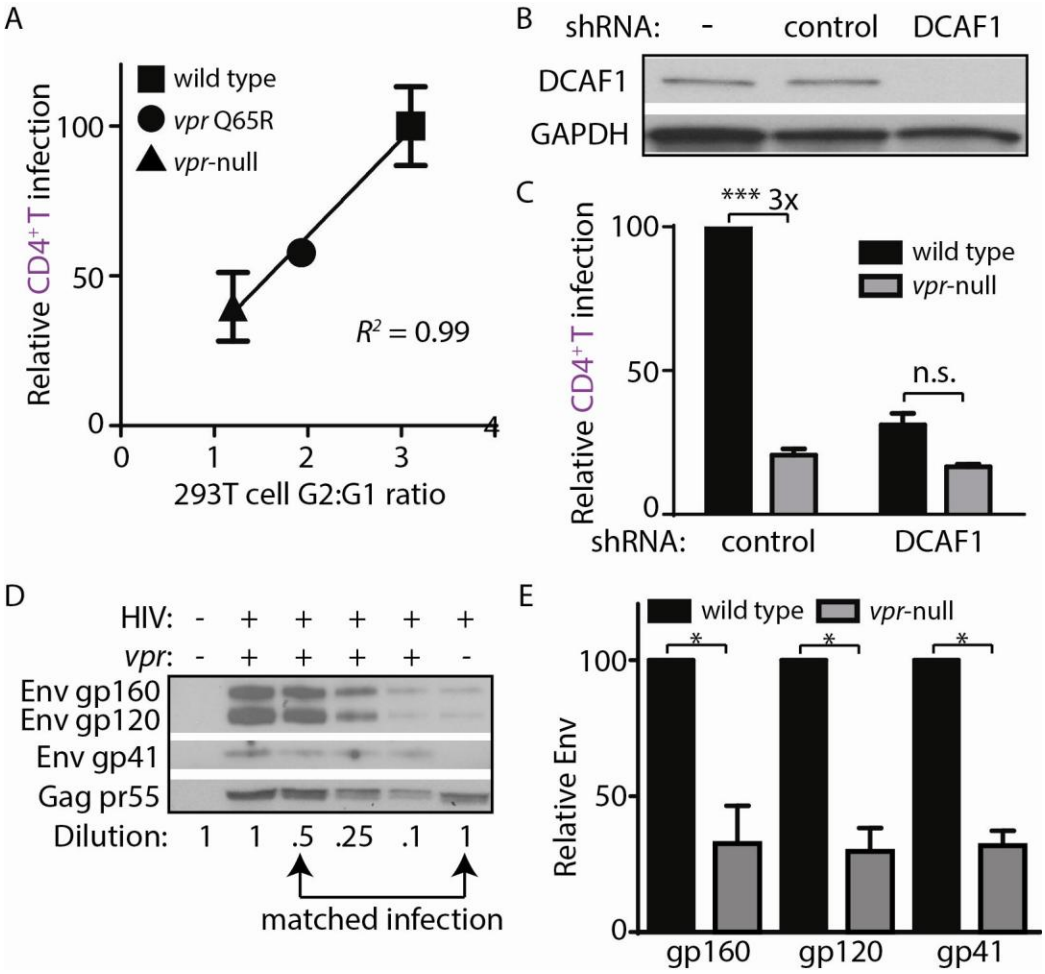

Figure 3.4: DCAF1 is required for Vpr-dependent HIV-1 spread from macrophages to CD4$^+$ T lymphocytes. (**A**) Scatter-plot of Vpr-dependent cell cycle arrest of 293T cells (x-axis) versus Vpr-dependent increase in cocultured T lymphocyte infection (y-axis). Best-fit curve from linear regression analysis, R^2=0.99 (n=4 donors). (**B**) Immunoblot of DCAF1 and GAPDH in MDM seven days after transduction with lentivirus encoding shRNA targeting luciferase ("control") or DCAF1. (**C**) Summary graph showing infection frequency of T lymphocytes in co-culture (Figures 3.1A and 3.3A) by MDM treated with the indicated shRNA (n=3 donors). (**D**) Immunoblot of HIV-1 89.6 Env and Gag in MDM-T lymphocyte coculture whole-cell lysates diluted as indicated. Arrows denote lysates with comparable levels of Gag pr55 in the presence and absence of Vpr. (**E**) Summary graph of Env levels quantified by densitometry and normalized to Gag pr55 levels (n=4 donors). Error bars represent SEM. *p<0.05, ***p<0.001, "n.s."p>0.05, student's paired t-test.[4]

[4] This figure was created by David Collins.

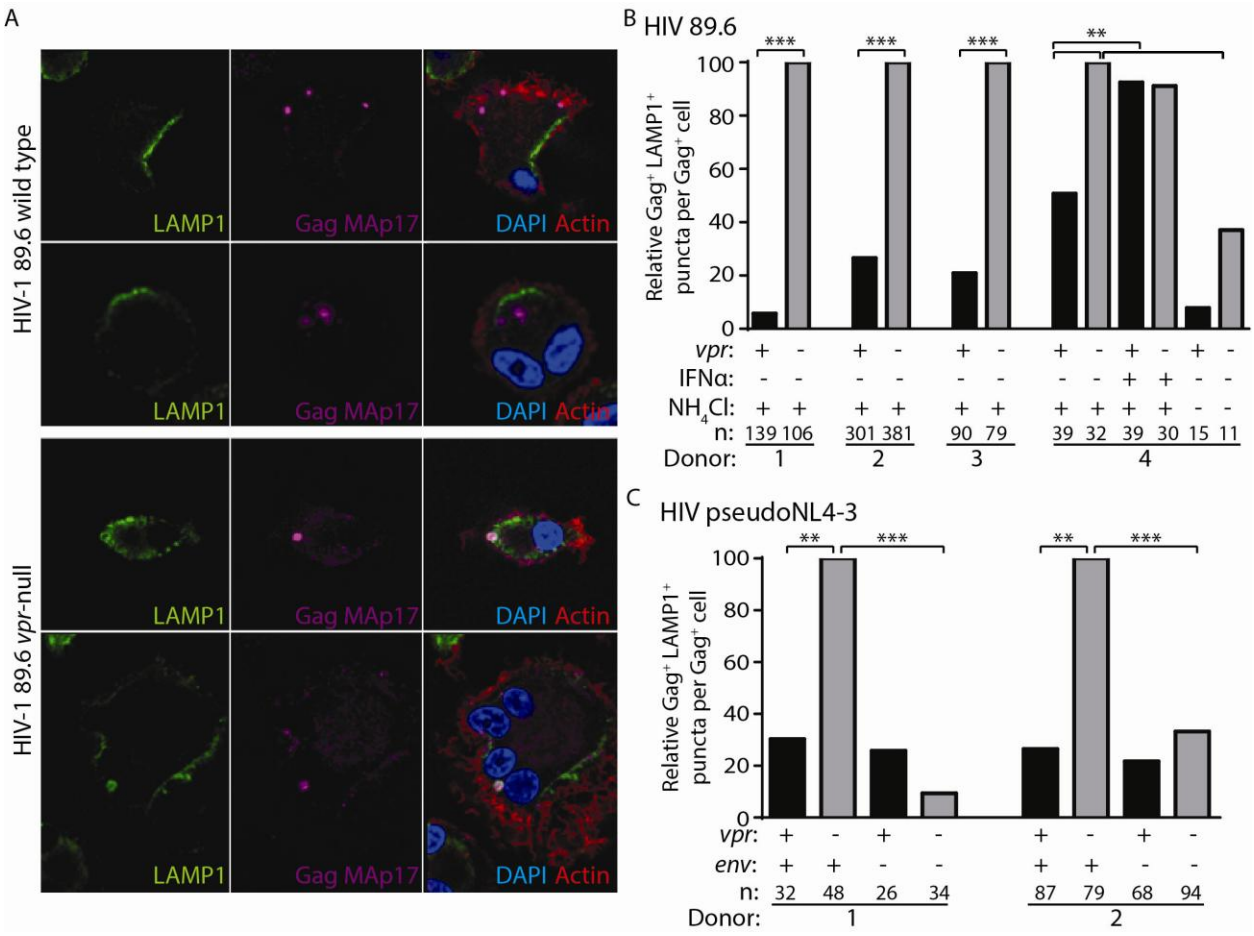

Figure 3.5: Vpr prevents Env-dependent targeting of HIV virions to lysosomes in macrophages. (**A**) Representative confocal micrographs depicting subcellular localization of lysosome marker LAMP1 (green) and HIV-1 Gag MAp17 (magenta) in MDM infected by wild type or Vpr-null HIV-1 89.6 for ten days. Merged images (right panels) include phalloidin staining of actin cytoskeleton (Buchacher et al.) and DAPI staining of nuclei (blue). (**B**) Summary graph of LAMP1$^+$ Gag MAp17$^+$ co-localized puncta per Gag$^+$ cell across 'n' number of MDM from four donors infected as shown in Figure 3.1A expressed as percent colocalization relative to *vpr*-null. MDM from donor 4 were treated with IFNα for the final two days of infection. Lysosomal acidification was blocked with NH_4Cl for the final 8 hours where indicated. (**C**) Summary graph as in B of MDM from two donors infected with wild-type, *vpr*-null, *env*-null, or *vpr*- and *env*-null NL4-3 pseudotyped with YU-2 Env and treated with NH_4Cl for the final 8 hours. **p<0.01, ***p<0.001, Fisher's exact test.[5]

[5] This figure was created by David Collins.

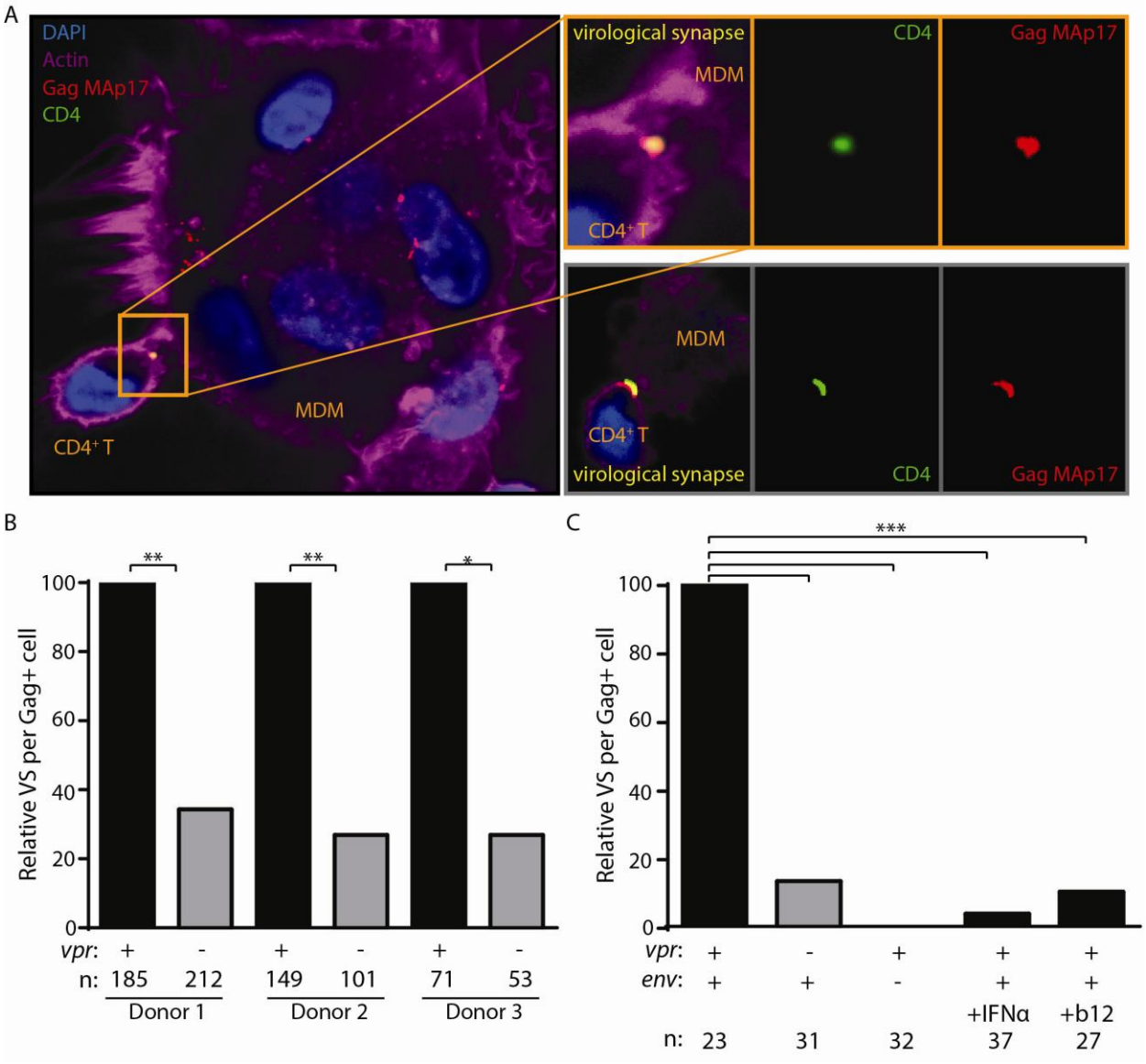

Figure 3.6: Vpr promotes Env-dependent virological synapse formation between macrophages and CD4$^+$ T lymphocytes. (**A**) Representative confocal micrographs of MDM infected as shown in Figures 3.1A and 3.3A and briefly cocultured with CD4$^+$ T lymphocytes pre-stained for surface CD4. Co-localization between HIV-1 Gag MAp17 (Buchacher et al.) in MDM and surface CD4 (green) on T lymphocytes at 100X magnification is indicated as virological synapses (VS). Merged images include phalloidin staining of actin (magenta) and DAPI staining of nuclei (blue). Inset depicts magnified VS from same image (top) or from a different representative image (bottom). (**B**) Summary graph of VS observed at 20X magnification per 'n' number of Gag$^+$ MDM from three donors infected by wild type or *vpr*-null HIV-1 89.6. (**C**) Summary graph of VS as in B of MDM infected with YU-2 Env-pseudotyped *env*-null 89.6 (third column), wild type 89.6-infected MDM treated with anti-Env gp120 neutralizing antibody b12 during co-cultivation with CD4$^+$ T lymphocytes (fourth column) or treated for two days

prior to coculture with interferon-α (IFNα, final column). *p<0.05 **p<0.01 ***p<0.001, Fisher's exact test.[6]

[6] This figure was created by David Collins.

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Chapter 4

Discussion[1]

Summary and discussion of results

Vpr is a highly conserved HIV-1 accessory protein and virulence factor associated with high viral loads and progression to AIDS (Lang et al., 1993; Somasundaran et al., 2002). In vitro, Vpr promotes HIV-1 infection of restricted primary cells such as macrophages (Le Rouzic and Benichou, 2005). However, the mechanism by which Vpr facilitates infection is poorly understood. The work presented in this dissertation describes an immunoevasive function of Vpr that facilitates HIV-1 spread among macrophages and from macrophages to $CD4^+$ T lymphocytes.

In agreement with other studies, we observed that Vpr enhances viral infection and spread in macrophages (Chen et al., 2004; Connor et al., 1995; Eckstein et al., 2001). In Chapter 2, we demonstrate that Vpr enhanced spread by increasing the number of virions released per infected cell when HIV-1 Env was also expressed. In addition, all Vprs tested (89.6, NL4-3, and AD8) dramatically increased the

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expression of HIV-1 Env, a necessary component of HIV-1 virions that binds to the CD4 receptor and coreceptors to facilitate viral fusion to target cells (Freed and Martin, 1995). Indeed, we observed a greater effect of Vpr when HIV-1 was allowed to spread through the MDM culture.

The results presented in Chapter 2 demonstrate that macrophages express a cell type-specific restriction factor that targets newly made Env protein and Env-containing virions. This determination is strongly supported by several key observations: (1) Vpr enhanced virion production only when Env was expressed (null mutations in 89.6 and NL4-3 *env* eliminated the ability of Vpr to stimulate virion production); (2) Vpr stimulated Env expression and Env-containing virion production in restricted cells (macrophages), but not in permissive cell lines (293T); (3) permissive cells acquired the capacity to restrict Env expression and release of Env-containing viral particles when fused to restricted cells (primary macrophages), indicating that macrophages harbor a dominant restriction factor; and (4) Vpr counteracts the restriction in macrophages and in 293T-macrophage heterokaryons. In sum, these data demonstrate that in the absence of Vpr, a dominant factor restricts the expression of Env protein and the release of Env-containing virions in macrophages. Moreover, Vpr functions to counteract this restriction.

While our data clearly demonstrate that restriction of Env occurs via accelerated lysosomal degradation in MDMs lacking Vpr, additional studies are needed to understand how Vpr evades this restriction. Prior studies have shown that cellular proteins that bind Env can also reduce release of Env-containing virions (Lama et al., 1999; Ross et al., 1999). Thus, Vpr could enhance virion release by

counteracting an MDM-specific factor that binds Env on budding virions, reducing virion release. Based on pulse-chase analysis of cell-associated p24 summarized herein, Vpr did not detectably affect virion assembly, budding, retention, and degradation up to 16 hours post-synthesis. However, if MDMs both retain and degrade virions in the absence of Vpr, virion retention could mask virion degradation at early time points.

We also found that virions released by MDMs lacking Vpr had, on average, 2- to 3-fold less Env, confirming our observations that Env is targeted by an MDM-specific factor that is counteracted by Vpr. Interestingly, CD4 expression also interferes with Env incorporation into virions produced by transfected 293 cells that lack Nef. Nef expression counteracts this interference and promotes Env incorporation into virions (Lama et al., 1999). The interference mediated by CD4 appears to be analogous to the MDM-specific restriction we describe herein, which is counteracted by Vpr. However, when normalized for virion content, the effect of Vpr on Env incorporation into virions did not appear to enhance infectivity on a per-virion basis (data not shown). Instead, Vpr enhances spread by enhancing virion release and/or direct cell-to-cell spread of HIV-1.

Previously identified Vpr-dependent phenotypes, including cell-cycle arrest observed in permissive cell-line systems, depend on an interaction with the Cul4A-DDB1-DCAF1 ubiquitin ligase (Ahn et al., 2010; Belzile et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Wen et al., 2007), which activates the SLX4-endonuclease complex. Vpr- and DCAF1-dependent activation of the SLX4 endonuclease complex may suppress accumulation of unprocessed HIV-1 DNA

intermediates that otherwise induce *IFNα* and *IFNβ* (Laguette et al., 2014). Thus, the macrophage-specific restriction we describe may result from cellular detection of viral infection and activation of an innate immune response. Several lines of evidence presented herein support this model: (1) the Vpr-dependent SLX4 pathway that suppresses innate immune detection of viral infection was active in primary macrophages; (2) DCAF1, a cellular cofactor required for Vpr to activate the SLX4 complex was also required for Vpr-dependent stabilization of Env and efficient release of Env-containing virions in macrophages; (3) Vpr significantly reduced *IFNα* induction in macrophages from three donors at early time points; (4) the magnitude of *IFNα* induction observed in donor macrophages correlated with the magnitude of the Vpr-dependent virion production phenotype detected in macrophages from the same donor at later time points; and (5) addition of IFNα dramatically reduced Env expression and release of virions even in infected macrophage cultures that expressed Vpr. Thus, these data support a model in which Vpr functions to prevent innate immune recognition of infection, *IFN* induction, and the activation of downstream pathways that disrupt Env protein expression and virion release. Whether SLX4 is directly required for *IFN* modulation and whether low amounts of type I IFN induced at the time of initial infection are sufficient for the macrophage restriction will require further experimentation.

Importantly, our results provide strong and convincing evidence that the Vpr-DCAF1 complex is linked to a virological endpoint in a restricted cell type. The observation that Vpr affects a late process in the viral life cycle was initially surprising, because the efficient incorporation of Vpr into virions suggests it acts on

a step in the viral life cycle preceding integration (Lu et al., 1995). Indeed, we report here that Vpr causes accelerated turnover of DCAF1 via a proteasomal mechanism as early as 5 hpi and prior to the establishment of productive infection. Thus, Vpr packaged into virions is sufficient to initiate DCAF1-dependent pathways. Interestingly, the proteasome inhibitor, MG132, blocked Vpr-induced early turnover of DCAF1, but did not affect macrophage restriction of Env expression that occurred later, after infection was established. This observation suggests that at least a component of Vpr's capacity to rescue Env expression resulted from an indirect, downstream effect of DCAF1 and Vpr. Whether viral sensing that occurs in the absence of Vpr results in the upregulation of a cellular factor that binds and disrupts Env and Env-containing virions is an interesting possibility that remains to be investigated.

In addition to DCAF1, Vpr also accelerates the turnover of cellular cofactors MUS81, the endonuclease contained within the SLX4 complex that is activated by Vpr to possibly degrade HIV-1 replication intermediates and limit induction of the innate immune response (Laguette et al., 2014), and UNG2, a uracil glycosylase (Priet et al., 2003; Wen et al., 2012). Interestingly, UNG2 may reduce the accumulation of uracilated DNA intermediates in HIV-1-infected primary $CD4^+$ T cells expressing Vpr (Norman et al., 2011). Whether UNG2 and the SLX4 complex work cooperatively to clear HIV-1 intermediates and prevent immune activation in HIV-1-infected primary cell systems is an interesting hypothesis that remains to be investigated.

In summary, Chapter 2 demonstrates that Vpr increases spread of HIV-1 in MDMs by counteracting an MDM-specific restriction of Env expression that leads to lysosomal degradation of Env and impaired release of Env-containing virions. Notably, this pathway relies upon the expression of the Vpr cofactor DCAF1, and this interaction is necessary for optimal infection of MDMs, a restricted primary cell type. Macrophages represent an important conduit for HIV-1 infection of $CD4^+$ T cells and are infected during the acute phase of HIV-1 infection (Hladik et al., 2007). Thus, these studies provide important insights into how HIV-1 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.

In Chapter 3, we show that under conditions in which efficient $CD4^+$ T lymphocyte infection required contact-dependent VS formation with infected macrophages, Vpr promoted VS-mediated transmission of HIV-1 to T lymphocytes. Moreover, we provide evidence that Vpr promoted infection by preventing the induction of an innate immune restriction of HIV-1 Env expression and in macrophages and Env-dependent virological synapse formation between macrophages and $CD4^+$ T cells.

Although $CD4^+$ T lymphocytes are the most abundant HIV-1-infected cell type in vivo and are responsible for much of its pathogenesis, T lymphocytes were relatively refractory to infection by cell-free HIV-1 in vitro. In Chapter 3, we demonstrate conditions under which HIV-1 infection of activated primary $CD4^+$ T lymphocytes required spread from infected macrophages. This allowed us to recapitulate the in vivo requirement for Vpr and provide a mechanism that explains

its evolutionary conservation, suggesting that similar conditions exist in vivo during the course of infection. As reported by others (Duncan et al., 2014; Groot et al., 2008), we demonstrate that HIV-1-infected macrophages efficiently spread HIV-1 to T lymphocytes across Env-dependent VS, and that this mode of spread is resistant to neutralization by some antibodies. Furthermore, we show that productive infection of macrophages was required for spread to T lymphocytes; passive *trans*-infection of T lymphocytes by uninfected macrophages was not observed under the conditions of our assay. These results reveal a critical role for macrophage infection in maximal HIV-1 infection of T lymphocytes.

We report that in the absence of Vpr, virions containing Env were targeted to macrophage lysosomes and fewer virions were localized to Env-dependent VS between macrophages and T lymphocytes. Indeed, our results illustrate that Vpr from multiple HIV-1 isolates promoted efficient macrophage-dependent T lymphocyte infection by this mechanism. This conserved function of Vpr provides a mechanistic explanation for its evolutionary conservation.

Finally, we provide confirmatory evidence that Vpr prevents the activation of an innate immune restriction of HIV-1 in macrophages. Vpr activates the SLX4 endonuclease complex through its adaptor protein, DCAF1, allowing HIV-1 to evade a type I interferon response (Laguette et al., 2014). This pathway is active in macrophages and may explain how Vpr prevents macrophage-specific restriction of Env (Mashiba et al., 2014). Consistent with this, we demonstrated that treatment of infected macrophages with exogenous IFN increased lysosomal targeting of virions and impaired VS formation with T lymphocytes. Restriction of HIV-1 by IFN is of

particular interest in light of recent evidence that IFN treatment may shrink the HIV-1 reservoir (Azzoni et al., 2013; Sun et al., 2014). Further elucidation of this pathway, including the mechanism by which HIV-1 is detected and the identity of the IFN-stimulated macrophage restriction factor are important areas for future investigation.

In sum, Chapter 3 describes a novel role for Vpr in promoting VS-mediated HIV-1 infection of T lymphocytes by evading innate immune restriction of Env in macrophages. These results underscore the importance of macrophages in HIV-1 pathogenesis and antiviral immunity, and provide a compelling explanation for the evolutionary conservation of Vpr.

Working models, limitations, and future directions

Taken together, the results presented in this dissertation support a model in which Vpr functions to prevent a type I interferon-inducible restriction of Env in macrophages that otherwise targets Env and Env^+ virions to lysosomes and prevents Env-dependent cell-to-cell spread of HIV-1. This model is summarized in **Figure 4.1**. These results bring about several important mechanistic questions, all of which can be explained by several non-exclusive alternative models. The common theme among the possible models is that Vpr counteracts an as yet unidentified restriction factor, which targets Env for lysosomal degradation in macrophages. This section will discuss various models that may explain how Vpr counteracts the restriction, how the restriction factor targets Env, and the role of macrophages in HIV-1 pathogenesis. In addition, this section includes discussion of

noteworthy limitations in the interpretation of the data presented in this dissertation as well as future directions that will facilitate stronger conclusions.

Mechanisms of Vpr-mediated counteraction of restriction

One remaining mechanistic question is how Vpr counteracts the putative cellular antiviral factor that restricts Env in macrophages. One possibility is that Vpr directly interacts with the restriction factor (RF) to induce DCAF1/CUL4 E3 ubiquitin ligase-mediated polyubiquitination and proteasomal degradation of the RF. In support of this model, Vpr uses this pathway to degrade other cellular factors, including UNG2 and MUS81 (Ahn et al., 2010; Laguette et al., 2014). Moreover, our results show that Vpr is required to overcome Env restriction in MDM. However, it is unclear whether Vpr directly interacts with the RF. Vpr and DCAF1 are primarily localized to the nucleus (Belzile et al., 2010), whereas Env is localized primarily to the ER/Golgi and plasma membrane (Stein and Engleman, 1990). Some targets of Vpr activity are nuclear, including UNG2, SMUG1, and MUS81, whereas others are both nuclear and cytosolic, such as Dicer (Casey Klockow et al., 2013; White et al., 2014). Thus, it is possible that Vpr may act outside the nucleus under certain conditions to induce proteasomal degradation of the RF. When the RF is identified by strategies that will be discussed below, it may be possible to track any interactions between Vpr and the RF by confocal immunofluorescence microscopy and/or live-cell microscopy to determine whether, how and where Vpr directly interacts with the RF.

In addition to the possibility that Vpr directly interacts with the RF, it is possible that Vpr indirectly counteracts or prevents the activity of the RF. Indeed, such an indirect model is supported by several lines of evidence. Vpr is packaged within the virion and is thus believed to act early in the viral replication cycle (Hrimech et al., 1999). However, we show that Vpr affects late events in viral replication, which may result from downstream effects of an essential early Vpr activity. If *de novo* expressed Vpr was sufficient to facilitate optimal viral spread, its packaging into the virion would likely not be an evolutionarily conserved feature. Moreover, recent evidence suggests that Vpr in the virion likely acts upon MUS81 and the SLX4 endonuclease complex early after infection to prevent innate immune detection of HIV-1 replication intermediates (Laguette et al., 2014). Indeed, our results are consistent with this model, as Vpr expression led to reduced *IFNA1* gene expression. That the magnitude of this effect at twelve hours post-infection correlated with the magnitude of Vpr-mediated enhancement of virion production in MDM after ten days of spread supports the notion that very early Vpr activities may influence long-term viral outcomes. In further support of this hypothesis, MDM treated with exogenous interferon-α induced restriction of Env expression, virion production and VS formation even in the presence of Vpr, suggesting that Vpr enhances Env expression, virion production and HIV-1 spread by counteracting interferon induction. However, although the restriction observed in MDM infected by Vpr-null HIV-1 is apparently inducible by exogenous IFN, it remains to be proven whether this restriction requires secretion of endogenous IFN.

While a model in which Vpr indirectly affects restriction of HIV-1 in MDM via modulation of an interferon response is best supported by our evidence, it remains unclear how interferon may activate the RF. It is possible that Vpr impairs transcription, translation, post-translational modifications, activation, or trafficking of the RF in MDM. Finally, it remains possible that interferon does not directly mediate the restriction. While we have shown that Vpr evades interferon induction, and that exogenous interferon can cause a similar restriction to that which is observed in the absence of Vpr, it remains to be conclusively demonstrated that endogenous interferon produced in the absence of Vpr actually leads to restriction. In preliminary data not shown in this dissertation, we were unable to measure interferon-α in culture supernatants from infected macrophages with or without Vpr. Moreover, effective blockade of the type I interferon receptor at the cell surface did not abrogate the effects of Vpr on Env expression and virion production. These data suggest that if interferon mediates the restriction in the absence of Vpr, it does so through an intracellular signaling mechanism. It is also possible that the type I interferon expression observed in the absence of Vpr is a surrogate marker for another antiviral response that leads to restriction of Env and virion production through an interferon-independent pathway. It is possible that high levels of exogenous interferon-α may induce such a pathway indirectly. Identification of the RF will facilitate future studies that will seek to delineate the mechanism by which Vpr counteracts it, directly or indirectly.

Taken together, the data shown in this dissertation best support an indirect mode of Vpr activity in overcoming macrophage restriction. However, in addition to

the identity of the restriction factor remaining unknown, significant limitations remain to be addressed. One such limitation is that the role of the SLX4 endonuclease complex in Vpr-mediated Env stabilization and virion release has not been directly evaluated. RNA interference-mediated knockdown of SLX4 in primary macrophages would allow evaluation of its necessity in mediating Vpr's effects on Env, virion production, and macrophage-to-T lymphocyte spread. Additionally, silencing of other Vpr molecular targets, including UNG2, may reveal importance for Vpr-mediated immune evasion. Experiments to address these limitations will inform and strengthen conclusions regarding the mechanism of Vpr-mediated counteraction of HIV-1 restriction in macrophages.

Mechanisms of Env restriction in MDM

Another open mechanistic question is how Env is restricted by the putative restriction factor suggested by the work presented herein. One possibility is that the RF binds to Env directly and redirects Env and Env^+ virions to lysosomes for degradation. Such a model is consistent with all evidence presented in this dissertation, and provides important clues that may assist in identification of the RF. Because the restriction we observe appears to be active in MDM but not in other cell types, Env-binding proteins that are expressed in macrophages but not in $CD4^+$ T lymphocytes are attractive candidates for RF identification. One such candidate, mannose receptor (MRC1), is discussed and partially evaluated in **Appendix**. Other candidate RFs may be identified by an unbiased screen approach, in which Env-binding cellular factors are co-immunoprecipitated with Env from infected MDM

differentially in the absence or presence of Vpr. Such proteins could be identified by tandem mass-spectrometry analysis. Once identified, such putative RFs can be confirmed by RNAi silencing in MDM. If Vpr expression does not rescue Env expression to levels above that in the absence of Vpr upon silencing of any putative RF, it would suggest that the silenced protein is the RF necessary for the observed phenotypes. Moreover, if expression of a putative RF in a permissive cell type, such as 293T cells, causes restriction of Env expression, it would suggest that the expressed protein is the RF and is sufficient to cause the observed phenotypes. In addition to the possibility that the RF is a macrophage-specific Env-binding protein, it is also possible that there are multiple RFs that act cooperatively or redundantly, which would make identification of the RF more complicated. It is furthermore possible that the RF is not macrophage-specific, but is rather differentially localized or trafficked in macrophages compared to non-restricted cell types.

Another possibility is that Env is indirectly restricted by the RF. For example, the RF could be a cellular factor that causes global disruption of Env trafficking, resulting in lysosomal accumulation. HIV-1 assembles at lipid raft microdomains at the plasma membrane (Ono and Freed, 2001) and at virus-containing compartments (VCC) which are specialized invaginations of the plasma membrane in macrophages (Tan and Sattentau, 2013). The exact molecular determinants of this Env localization are poorly understood, but it is possible that the RF induced in the absence of Vpr may disrupt these sites of Env localization to cause the observed restriction without directly interacting with Env. If this is the case, identification of the RF will be complicated. Important clues can be obtained by studying which

regions of Env are required for restriction in the absence of Vpr. For example, mutations within the Env cytoplasmic tail alter Env localization to sites of viral assembly (Muranyi et al., 2013). Using such mutants to study effects of Vpr on Env restriction in MDM is complicated by the inefficient spread of these viruses. However, because 293T-MDM heterokaryons exhibit Env restriction, pseudotyped viruses may be generated in 293T cells before fusion with MDM to study Env restriction in a non-spreading infection system. Determining which Env regions are required for restriction will enable biochemical analysis of protein-protein interactions between those regions of Env and cellular factors present in MDM. Such interactions may provide essential clues about how Env is degraded by the RF by either a direct or indirect mechanism.

Once the mechanism by which Env is restricted in macrophages is better understood, it will be of interest to test whether the same restriction mechanism can target other enveloped viruses. One possible experiment is to express HIV and envelope glycoproteins from other pathogenic viruses, including influenza HA, ebolavirus GP, SARS coronavirus S, and potentially others. As a first pass, this can be achieved by co-transfection of HIV-1 *env*- expressing or lacking Vpr and viral glycoprotein expression plasmids in 293T cells before fusion with primary MDM to form restricted heterokaryons, and glycoprotein stability can be assessed by immunoblot. Many restriction factors act broadly on a variety of viral pathogens, setting a precedent for studying the breadth of the novel restriction described in this dissertation.

Roles of macrophages in HIV-1 pathogenesis

Our work supports the notion that macrophage infection is critically important for HIV-1 infection and pathogenesis. This conclusion stems from convergent lines of evidence, including: (1) $CD4^+$ T lymphocyte infection by cell-free infection is highly inefficient compared to macrophage-dependent HIV-1 infection of $CD4^+$ T lymphocytes, (2) macrophage-to-T lymphocyte spread is enhanced by Vpr in vitro, recapitulating in vivo requirement for Vpr and providing a compelling explanation for Vpr's strong evolutionary conservation. However, one limitation of the present evidence is that the PBMC-derived MDM and PHA-activated $CD4^+$ T lymphocytes used in these studies may not accurately reflect physiologic conditions. To further address the role of Vpr in macrophage-to-T lymphocyte spread in more relevant settings, and thus strengthen any conclusions about the role of macrophages in vivo, it would be pertinent to assess macrophage-to-T lymphocyte spread in human lymphoid aggregate culture (HLAC) models infected ex vivo with HIV-1 expressing or lacking Vpr. Additionally, activation conditions for PBMC-derived $CD4^+$ T lymphocytes may be altered to better reflect physiologic conditions. Indeed, we had difficulty infecting PHA-activated T lymphocytes with CCR5-tropic HIV-1 in several donors (data not shown). Other groups have shown that strong T lymphocyte activation stimuli may reduce CCR5 expression and/or increase expression of CCR5-binding factors that compete with HIV-1 for binding (Creson et al., 1999; Schweighardt et al., 2004). Despite the implications of this work for understanding the role of infected macrophages in facilitating viral spread, much

remains to be learned about the specific contributions of these cells to HIV-1 pathogenesis.

A once-prominent model for HIV-1 transmission included a suggested role for macrophages at the infection site in initiating and establishing infection in an individual. This model stems from strong evidence that nearly all macrophage-tropic HIV-1 clones are CCR5-tropic, that CCR5-tropic viruses predominate early after infection, and that individuals homozygous for a CCR5 deletion are not susceptible to HIV-1 infection (reviewed in(Margolis and Shattock, 2006)). Indeed, vaginal macrophages are susceptible to HIV-1 infection (Shen et al., 2009), providing further support for a model in which macrophages may be among the first cells infected by HIV-1 at sites of sexual transmission. Moreover, HIV-1-infected macrophages modulate chemotaxis of T lymphocytes, potentially increasing cell-to-cell spread in vivo (Swingler et al., 1999). Current evidence suggests that most transmitted/founder HIV-1 clones are poorly macrophage-tropic, though all are able to utilize CCR5 for entry (Ochsenbauer et al., 2012). The importance of macrophage infection has been recently called into question by a report that SIV DNA isolated from mucosal macrophages can be attributed to phagocytosis of infected $CD4^+$ T lymphocytes(Calantone et al., 2014). However, another report demonstrates that such phagocytosis also leads to productive macrophage infection, even by transmitted/founder HIV-1 clones (Baxter et al., 2014). More work is required to better understand whether macrophages are sometimes or always critically important during HIV-1 transmission. Future work to assess whether macrophages within cervicovaginal or anal tissue specimens obtained from acutely HIV-1-infected

individuals harbor replicating virus would help to clarify the plausibility of this model.

In addition to a putative role in viral transmission, evidence suggests that macrophages are critical mediators of increased HIV-1 spread during and after viremia. Indeed, macrophage-to-T lymphocyte spread is highly efficient, is resistant to treatment with some antiretroviral inhibitors, and is resistant to antibody neutralization (Duncan et al., 2013; Duncan et al., 2014; Groot et al., 2008). Thus, macrophages may be critical for both HIV-1 spread and immune evasion, collectively allowing HIV-1 to establish a larger pool of persistently-infected cells and impairing current cure efforts. Work shown in this dissertation also supports an important role for macrophages in viral spread. Future directions to expand upon this work include work to demonstrate that macrophage-to-T lymphocyte spread contributes to HIV-1 spread and pathogenesis in ex vivo tissue models and/or in vivo animal models. In addition, it would be of interest to compare the efficiency of macrophage-to-T lymphocyte spread to the efficiency of T lymphocyte-to-T lymphocyte spread.

HIV-1-infected macrophages may also play a role in HIV-1 persistence. Infected macrophages have been shown to contribute to neuropathogenesis of HIV-1, attributed to their ability to cross the blood-brain barrier and resist treatment with antiretroviral inhibitors (reviewed in (Burdo et al., 2013)). Moreover, many tissue macrophages are long-lived in vivo and can support both latent and active viral replication and archive virions within VCC during treatment (reviewed in (Watters et al., 2013)). Continued investigation of the contributions of macrophages

to HIV-1 persistence and neuropathology are an essential component of designing improved treatments for infected patients.

Finally, macrophages may play a critical role in progression to AIDS. Indeed, our group and others have observed that HIV-1-infected macrophages can efficiently form syncytia with neighboring $CD4^+$ cells, including macrophages and T lymphocytes. In vivo, this may contribute significantly to $CD4^+$ T cell depletion. Early studies describe a strong association between syncytium-inducing HIV-1 envelopes and disease progression, although progression can still occur in the absence of syncytium-inducing envelopes (Koot et al., 1993; Richman and Bozzette, 1994). This notion should be re-evaluated using modern technologies, including HLAC models of $CD4^+$ T lymphocyte depletion. In addition, it would be of interest to test whether syncytium-inducing phenotypes correlate with progression in a large cohort of HIV-1 infected individuals, comparing progressors to nonprogressors and elite controllers. Interestingly, by increasing VS formation and macrophage-to-T lymphocyte spread, Vpr may enhance syncytia formation as an additional mechanism that may explain its reported effects on disease progression in animal models. This mechanism would provide an evolutionary advantage to Vpr-expressing HIV-1 variants by enabling the depletion of HIV-1-specific $CD4^+$ T lymphocytes, which are critical mediators of effective cellular antiviral immunity. Such immune evasion would eventually overcome selection within an individual for CTL escape mutants, which have significant fitness costs to viral replication (Leslie et al., 2004). This provides a possible explanation for Vpr's evolutionary conservation that warrants continued investigation.

Conclusions

In summary, the work presented in this dissertation expands our basic understanding of the role of Vpr in promoting spread of HIV-1. The work provides key mechanistic details that support a model in which Vpr and its cellular cofactor DCAF1 act to prevent the induction of a type I interferon-inducible, macrophage-specific restriction that targets HIV-1 Env and Env^+ virions to lysosomes for degradation and impairs formation of Env-dependent virological synapses. This information represents a critical advance in our understanding of Vpr function and explains its previously puzzling role in enhancing infection of $CD4^+$ T lymphocytes and contributing to pathogenesis in vivo. This work also supports a critical role for macrophages in HIV-1 infection and initiates several important new questions, both mechanistic and conceptual. Finally, this work lays a framework for identifying a novel HIV-1 restriction pathway, which may provide molecular targets for the design of new treatment strategies that may enhance natural immunity to HIV-1 in infected patients.

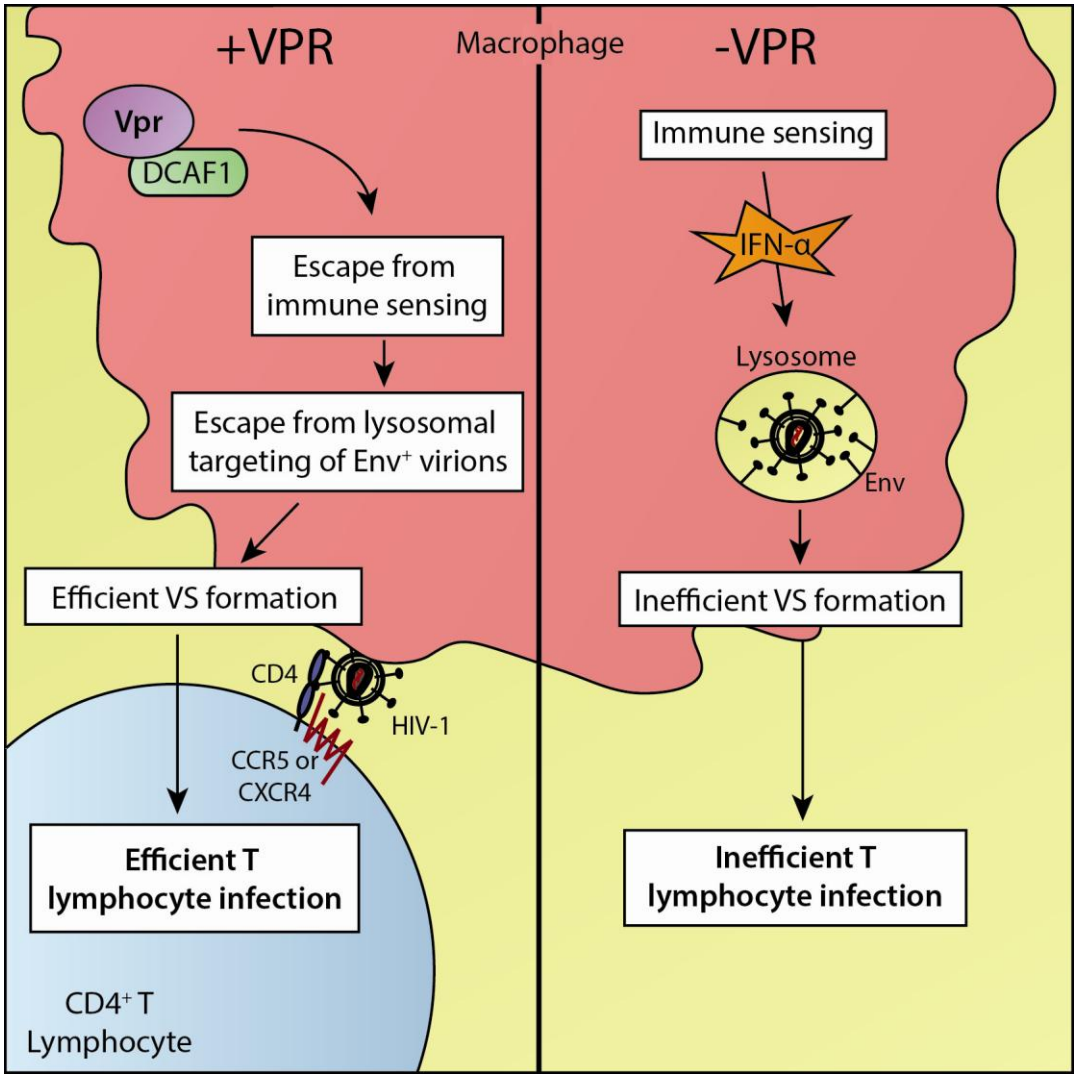

Figure 4.1. Working model of Vpr function. Graphical representation of a macrophage (magenta) infected by HIV-1 containing (left) or lacking (right) Vpr expression. Vpr interacts with DCAF1 to escape immune sensing, enabling efficient virological synapse (VS) formation and HIV-1 spread to CD4+ T lymphocytes (cyan). In the absence of Vpr, interferon-alpha (IFN-α) is induced upon infection; Env and Env-containing virions are targeted to lysosomes for degradation, preventing VS formation and impairing HIV-1 infection of CD4+ T lymphocytes. [2]

[2] This figure was created by David Collins.

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Appendix

MRC1 as a putative HIV-1 restriction factor counteracted by Vpr

Introduction

In this dissertation we provide evidence that Vpr counteracts a macrophage-specific restriction factor that targets Env and Env$^+$ virions to lysosomes for degradation and impairs virological synapse-mediated spread from macrophages to T lymphocytes. Based on our data, we speculate that this factor is likely to interact directly with HIV-1 Env and to be specifically expressed in macrophages. This appendix provides preliminary evidence for one such candidate restriction factor, mannose receptor (MRC1), as a potential Env-binding, macrophage-specific HIV-1 restriction factor counteracted by Vpr.

MRC1 is a C-type lectin receptor (CLR) that is primarily expressed by macrophages, and its carbohydrate recognition domain interacts with high-mannose residues on microbial proteins, including HIV-1 Env gp120 (Curtis et al., 1992; Taylor et al., 2005). MRC1 has been implicated in immune recognition of a variety of pathogens, including bacteria, fungi, and enveloped DNA and RNA viruses, and mediates internalization of such pathogens by macrophages (Fraser et al., 1998; Milone and Fitzgerald-Bocarsly, 1998). Additionally, surface-expressed MRC1 has

been shown to mediate CD4-independent HIV-1 entry into astrocytes (Liu et al., 2004), nonproductive HIV-1 entry into brain macrophages (Trujillo et al., 2007), and HIV-1 *trans*-infection of $CD4^+$ T lymphocytes (Nguyen and Hildreth, 2003). In this appendix, we provide evidence that HIV-1 Vpr leads to MRC1 downmodulation in a post-transcriptional, DCAF1-dependent manner.

Results

Vpr downmodulates MRC1

We hypothesized that Vpr may reduce expression levels of the cellular factor responsible for restriction of Env in macrophages. To examine whether MRC1, a candidate restriction factor, is affected by Vpr expression in infected macrophages, we infected MDM for seven to ten days with HIV-1 89.6 expressing or lacking Vpr, and performed immunoblot analyses on whole-cell lysates from several donors to analyze relative MRC1 protein expression. As reported in Chapter 2, Vpr expression correlated with higher Env expression. Surprisingly, Vpr expression also correlated with lower MRC1 protein levels at both time points (**Figure A1A**). Over eleven donors analyzed, seven displayed a significant Vpr-dependent reduction of MRC1 levels (average four-fold, **Figure A1B**). This effect suggests that Vpr may counteract MRC1 expression in infected MDM to overcome a putative restriction of Env and Env^+ virions.

Vpr-mediated MRC1 downmodulation is post-transcriptional and DCAF1-dependent

We next sought to begin to address the mechanism by which Vpr expression leads to MRC1 downmodulation in MDM. Our working model, discussed throughout this dissertation, predicts that Vpr may counteract restriction factor expression indirectly through DCAF1-dependent modulation of SLX4-mediated immune recognition. Because Vpr expression prevents *IFNA1* gene induction, we hypothesized that HIV-1 immune recognition in the absence of Vpr may lead to higher MRC1 transcription. We assessed *MRC1* gene expression by quantitative RT-PCR in three donors. At ten days post-infection we did not observe a significant effect of Vpr on *MRC1* expression levels (**Figure A1C**). These data suggest that Vpr acts post-transcriptionally to modulate MRC1 expression. We also sought to determine whether DCAF1 is required for Vpr-mediated MRC1 downmodulation. Using RNA interference to silence DCAF1 as in Chapter 2, we assessed Vpr-dependent MRC1 downmodulation in HIV-1-infected MDM from two donors in the presence or absence of Vpr. We observed a striking reversal of Vpr-dependent MRC1 downmodulation upon DCAF1 silencing in both donors (**Figure A1D**). These data suggest that DCAF1 is essential for Vpr-mediated MRC1 downmodulation.

Discussion and future directions

The preliminary data shown in this appendix supports the hypothesis that MRC1 may be an Env-binding, macrophage-specific restriction factor counteracted

by Vpr. The most important future direction will be to demonstrate that MRC1 is capable of restricting Env expression, virion production, and macrophage-to-T lymphocyte HIV-1 spread. To examine whether MRC1 is necessary for the observed restriction phenotypes, we plan to silence *MRC1* expression using RNA interference. To examine whether MRC1 is sufficient for the observed restriction phenotypes, we plan to transfect MRC1 into non-restricted cell lines and assess its effects on HIV-1 Env expression and virion release in both co-transfection and entry-mediated infection contexts. If MRC1 is capable of inducing the restriction phenotypes counteracted by Vpr, it will be of interest to investigate MRC1 colocalization with HIV-1 virions and $LAMP1^+$ lysosomes in the presence or absence of Env in HIV-1-infected MDM by confocal microscopy. It will also be important to determine whether type I interferon can induce the restriction, and if so, at what stage MRC1-mediated restriction is induced. We hypothesize that interferon treatment may modulate *MRC1* gene expression, translation, post-translational modifications, localization, and/or function. It is also possible that Vpr affects MRC1 through an interferon-independent mechanism. MRC1 co-immunoprecipitation with Env and/or Vpr will provide confirmatory evidence as to whether Env and/or Vpr interact directly with MRC1. Moreover, pulldown of MRC1 may also enable proteomic identification of important molecular complexes involved in the restriction pathway. In addition to these experiments, mannan, a high-mannose sugar that outcompetes Env gp120 for MRC1 binding, may be a useful tool in assessing a putative role for MRC1 in Env restriction in MDM.

It is also important to consider the possibility that MRC1 does not contribute to HIV-1 restriction in macrophages. Knockdown analysis may demonstrate that MRC1 is dispensable for Env restriction. In this case, it is possible that MRC1 is one of multiple redundant restriction factors counteracted by Vpr, or that MRC1 is not involved in the restriction. Overexpression in cell lines may clarify these possibilities by determining whether MRC1 is sufficient to induce restriction in permissive cells. If MRC1 is not required or involved in the restriction, new candidates can be identified in an unbiased proteomic screen. This screen would involve immunoprecipitation of Env from infected MDM expressing or lacking Vpr and mass spectrometry-based identification of proteins that interact differentially with Env in the presence or absence of Vpr. Hits from this screen can similarly be validated by RNA interference-mediated silencing in MDM and by overexpression in permissive cell lines.

Identification of the restriction factor that is counteracted by Vpr in macrophages and mediates restriction of HIV-1 spread will be useful for several reasons. It will provide a potential new therapeutic target for development of new treatments, and will also enable continued investigation into the molecular mechanisms of Vpr activity, macrophage restriction, and the importance of macrophages in HIV-1 pathogenesis. Our preliminary data implicate MRC1 as a strong candidate restriction factor, and continued examination of this hypothesis will yield stronger conclusions and open new avenues for investigation.

Methods

Immunoblotting and RNA interference

Antibodies to the following proteins were used for immunoblot analysis: tubulin and GAPDH (Sigma), HIV-Ig, gp120 and gp41 (AIDS Reagent Program, Division of AIDS, NIAID, NIH), MRC1 from Dr. Philip Stahl (Liu et al., 2004; Wileman et al., 1984). MDM were prepared, infected, lysed, immunoblotted, and densitometrically quantitated as described in Chapter 2. RNA interference to silence *DCAF1* expression was performed as in Chapter 2.

Quantitative RT-PCR

qRT-PCR was performed as described in Chapter 2, using QIAgen RNeasy kit extraction of RNA, oligo-dT + random nonamer cDNA priming, ABI Taqman Gene Expression Assay (Hs00267207_m1), FAM-MGB probe assay for MRC1 normalized to TaqMan Gene Expression Assay for beta-actin.

Author contributions

Dr. Kathleen Collins first hypothesized mannose receptor as a putative restriction factor counteracted by Vpr. Michael Mashiba initiated this line of investigation and contributed data for Figures A1A and A1B. Valeri Terry assisted with quantitative RT-PCR assays for Figure A1C. David Collins performed experiments for A1B,C, and D and wrote this appendix.

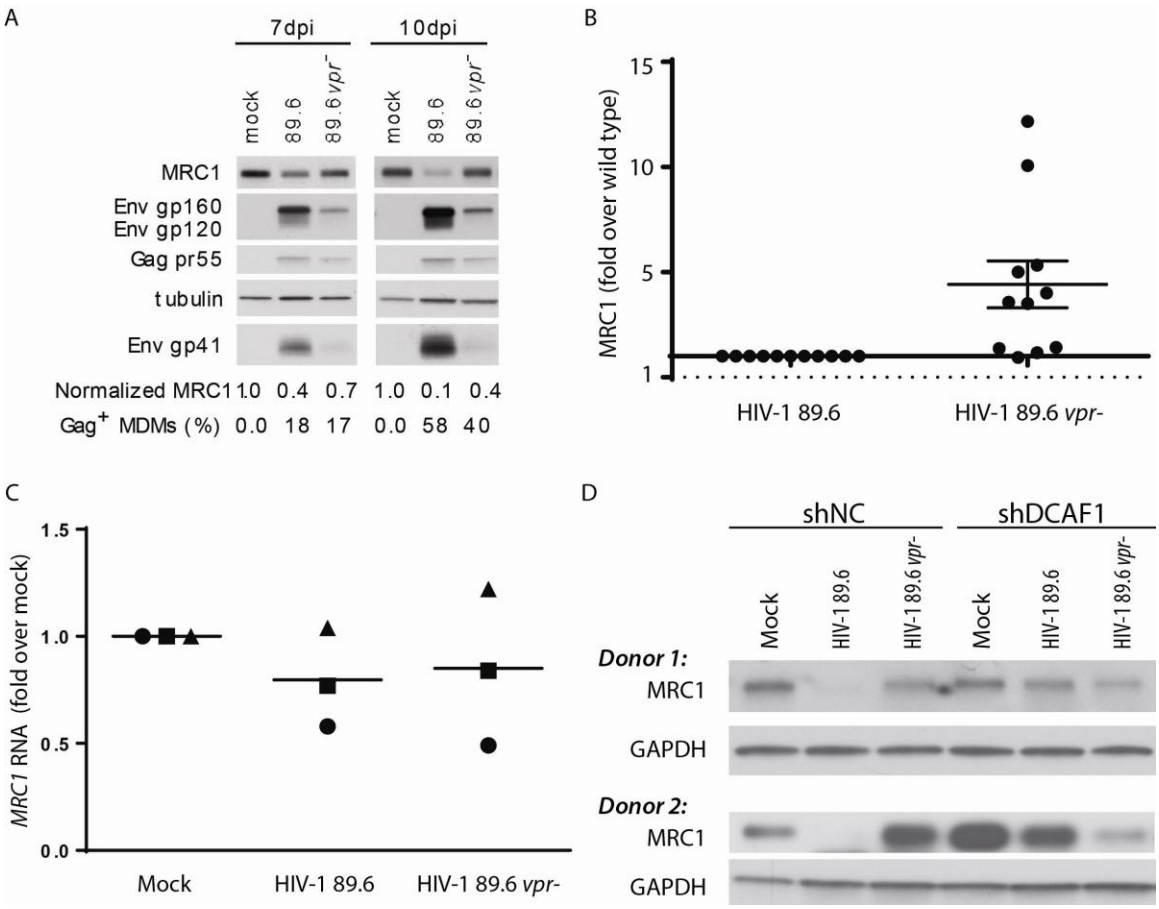

Figure A1: Vpr and DCAF1 alter MRC1 expression post-transcriptionally. (**A**) Immunoblot of MDM infected by HIV-1 89.6 expressing or lacking Vpr at seven and ten days post-infection (dpi), and densitometric quantitation of relative MRC1 expression. Percent Gag+ MDM for each condition as measured by intracellular Gag CAp24 staining and flow cytometry. (**B**) Summary graph of relative MRC1 expression measured by densitometric analysis of MDM lysates from eleven donors at ten dpi by immunoblot. Each data point represents a separate donor, and data are normalized to wild type MRC1 levels. Error bars represent standard error of the mean. (**C**) Summary graph of relative *MRC1* gene expression levels in MDM at ten dpi as measured by qRT-PCR as described in Methods, normalized to Mock-infected MDM. (**D**) Immunoblot analysis of MRC1 and GAPDH expression at ten dpi in MDM from two donors transduced with lentiviruses expressing the indicated shRNA and treated with the indicated infection conditions.[1]

[1]This figure includes data from Michael Mashiba, David Collins and Valeri Terry.

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