HIV Vpr Targets PU.1 in Macrophages to Evade the Innate Immune Response and Promote Viral Spread

by

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Dedication

To Andrew and Mom

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enthusiasm and curiously for science and discovery is infectious and made the research so enjoyable. Thank you.

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Abstract

Human immunodeficiency virus (HIV-1) establishes a persistent infection despite the host immune response and treatment with antiretroviral therapy. HIV evades the immune response primarily due to the activity of several accessory proteins. One of these accessory proteins is Viral protein R (Vpr). The importance of Vpr for successful infection is reflected in its universal conservation amongst all primate lentiviruses, including HIV-1. Several studies have demonstrated Vpr is paradoxically unnecessary for viral spread between CD4+ T lymphocytes, the main cellular target of HIV. However, it remains necessary for maximal spread between macrophages and from macrophages to CD4+ T lymphocytes in both *in vitro* and *in vivo* systems. Vpr suppresses intrinsic and innate immune responses through mechanisms not well understood.

Efficient viral spread from infected macrophages to uninfected cells is achieved by virological synapse formation when HIV Env on the surface of the infected cell binds HIV receptors on the surface of the uninfected cell, forming a channel through which HIV can be directly passed. Therefore, efficient Env production is vital for maximal viral spread. Previous studies determined that Vpr counteracts a macrophage-specific restriction factor, which we demonstrate in Chapter 2 to be the macrophage mannose receptor (MR). MR augments virion entry by binding mannose residues on HIV Env, likely stabilizing the virion at the cell membrane, but restricts HIV infection upon egress. After infection, MR restricts viral spread by meditating the transfer of Env and Env containing virions to the

lysosome for degradation. Two HIV accessory proteins target MR. Nef interacts with the cytoplasmic tail of MR at the cell surface, facilitating lysosomal degradation of MR. And Vpr counteracts the detrimental effects of MR on Env by reducing transcription of the MR gene, *MRC1.*

In Chapter 3, we demonstrate that transcriptional suppression of *MRC1* is caused by Vpr-mediated degradation of the master myeloid transcription factor, PU.1. PU.1 acts alone and in coordination with other transcription factors to regulate the expression of MR and many additional antiviral factors. PU.1 regulated factors are intimately involved in pathogen sensing and immune signaling through Toll-like receptors, Type I interferons, interferon-stimulated genes, and other host restriction factors capable of targeting HIV Env for degradation such as IFITM3. The Vpr-mediated degradation of PU.1 requires Vpr's cellular co-factor, DCAF1 in the Cul4A-E3 ubiquitin ligase complex and results in degradation of PU.1 which can be prevented with pharmacological inhibition of the proteasome. Like Vpr itself, Vpr's ability to counteract PU.1 is highly conserved across several HIV-1, HIV-2, and SIV *vpr* alleles. The innate immune response is suppressed both in infected macrophages and uninfected bystander cells from *vpr*-expressing HIV cultures. Virion-associated Vpr can rapidly degrade PU.1 after virion fusion, which partially explains the bystander effect. Knockdown of PU.1 in infected macrophages with HIV lacking Vpr restored Env expression and viral spread. Altogether, Vpr counteracts PU.1 in macrophages, suppressing the immune response during HIV infection and promoting viral spread.

CHAPTER 1

Introduction[1]

After large numbers of young, heterosexual men mysteriously suffered from opportunistic infections and rare malignancies in 1981, the Centers for Disease Control (CDC) finally gave the new disease a name - acquired immunodeficiency syndrome (AIDS) (Centers for Disease Control (CDC), 1981). Within a couple of years, the causative agent for AIDS was identified as a newly discovered retrovirus that would eventually be named human immunodeficiency virus (HIV) (Barré-Sinoussi et al., 1983; Coffin et al., 1986; Gallo et al., 1984). The first HIV genomes were isolated from $CD4^+$ T lymphocytes in persons living with HIV (PLWH). HIV infection of T lymphocytes is extremely toxic to the cells, with a half-life of approximately two days post infection (unlike macrophages, which can withstand active HIV infection for weeks) (Ho et al., 1995). Therefore, over time, PLWH steadily lose their $CD4^+$ T cells, compromising their immune systems. Before the advent of antiretroviral therapy (ART) - and even now in the absence of ART - PLWH slowly lose their $CD4^+$ T lymphocytes due to the toxicity of the virus. Once T cell counts dip below 200 cells/mm^3 of blood, the person is said to have developed AIDS and is

[1] Sections of this chapter have been published in the following manuscript:
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unable to fight off opportunistic infections or rare malignancies such as Kaposi's sarcoma, eventually leading to death (Biggar et al., 2007; Greene, 2007; Hahn et al., 2000).

HIV transmission between individuals occurs when there is an exchange of body fluids. These fluids include blood, semen, and breast milk, which means HIV can be transmitted from mother to child during breastfeeding, between sexual partners during intercourse, through transfusions of contaminated blood, or by sharing needles for intravenous drug use (Bryson, 1996; Chaisson et al., 1987). Many hemophiliacs or people undergoing medical procedures acquired HIV from blood transfusions before a test for HIV-contaminated blood was available (Ragni et al., 1987). With time came the realization that it was not just homosexual people infected with HIV. In fact by the late 1990s, most people infected with HIV identified as heterosexual particularly in sub-Saharan Africa (Gray et al., 2001).

The future of the HIV epidemic

As of today, over 40 million people have died of HIV infection, and over a million people acquire HIV every year, according to the World Health Organization. New antivirals are constantly under development, and now pre-exposure prophylaxis (PrEP) will likely be the curative agent of AIDS. Initially in the 1980s it was thought there would be a vaccine within two years. That has yet to materialize. All putative AIDS vaccines to date have provided no protection or have increased the likelihood of infection (Haynes et al., 2023; Hiv, 2023; Kim et al., 2021). Excitement around new mRNA vaccines such as the ones successfully developed and deployed in record time for SARS-CoV-2 have reinvigorated hopes that an HIV vaccine is possible. However, the effectiveness of pre-exposure

prophylaxis (PrEP) cannot be overstated. It has reduced HIV transmission successfully in many countries, the drugs are less toxic than they used to be, and new drug combinations that do not need to be taken daily are on the market or soon to be released that would move PrEP injections to every 2-6 mo (LaPreze and Nowiki, 2022; Riddell et al., 2018). This is where the future of treatments should go.

Curative treatments for HIV have proven far more difficult to develop than initially anticipated. This is because of the retroviral nature of the virus and HIV latency. HIV causes a chronic viral infection that results in AIDS if left untreated. HIV primarily targets the host immune system by infecting $CD4^+$ T cells, which often leads to the death of the infected cell due to the cytotoxic nature of active infection. With the development of antiretroviral therapy (ART) to block new infections, it was hoped that with time, all the HIV infected T cells would be eradicated either through immune-mediated clearance or cytotoxic effects from the replicating virus. However, a discordance between the amount of HIV mRNA and proviral (genomic, integrated DNA) sequences was discovered that suggested the presence of more HIV DNA than mRNA (Psallidopoulos et al., 1989; Simmonds et al., 1990). This indicated HIV could survive in an integrated but latent form. The main cellular reservoir was identified as resting memory $CD4^+$ T cells (Chun et al., 1995; Finzi et al., 1997), though other reservoirs have since been discovered (Carter et al., 2010). Soon after, researchers calculated the mean half-life of latently-infected resting memory T cells to be nearly 44 months and that clearance of the latent reservoir could take 70 years or more (Finzi et al., 1999; Siliciano et al., 2003). These rare, latently infected cells can spontaneously reactivate. This means that if ART is interrupted, HIV springs back to a non-latent form from this rare HIV-infected population. Because we are

now only in the 5th decade of HIV infection and treatment, we do not know all the long-term consequences of extended HIV positivity, including whether the latent reservoir will ever disappear, whether people living with HIV long-term are prone to developing specific types of cancer or other yet-to-be-determined secondary health conditions. Although much research and money have been spent trying to understand HIV, there is still so much that we do not understand about HIV pathogenesis. Some of this will be discussed in this dissertation.

Evolutionary history of HIV

Genetic origins of HIV

Initially, HIV was thought to be the third member of the human T lymphocyte virus (HTLV) family due to the isolation of the HIV genome from a T lymphocyte and because initial tests showed some cross-reactivity between antibodies generated against HTLV-I to HIV (Barré-Sinoussi et al., 1983; Gallo et al., 1984). Indeed, HIV is truly a retrovirus just as both HTLV-1 and HTLV-2 are. However, HIV has since been shown to have a very different evolutionary origin story and is regarded as independent from HTLV. Through efforts from many groups, particularly that of Dr. Beatrice Hahn, we know that HIV arose through zoonotic transmission from non-human primates who themselves are infected with viruses similar to HIV called simian immunodeficiency virus (SIV) with a suffix to denote their species of origin as shown in **Figure 1.1** (Hahn et al., 2000; Sharp and Hahn, 2011).

HIV-1 came from chimpanzees (SIVcpz) and gorillas (SIVgor) (Huet et al., 1990). Sequence analysis of the SIVcpz genome suggests it is a mosaic composite of SIVrcm

from red-capped mangabeys and several other species (Sharp and Hahn, 2011). Because chimpanzees are known to eat other monkeys, it is likely they were exposed to several SIV genomes that recombined to form what we observe as SIVcpz (Goodall, 1986). HIV originating from gorillas (SIVgor) (Van Heuverswyn et al., 2006) was originally an infection from chimpanzees (Takehisa et al., 2009).

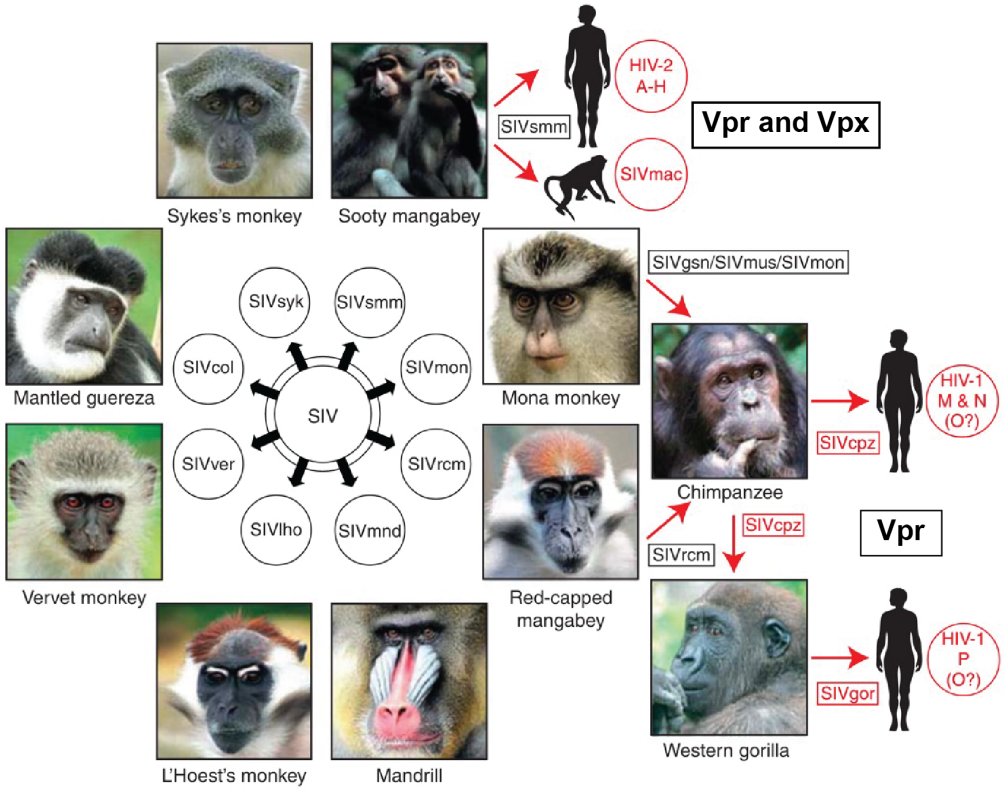

Figure 1.1. Evolutionary origins of HIV.[2] Old World monkeys are naturally infected with simian immunodeficiency viruses (SIVs). The suffix denotes their primate species of origin (e.g., SIVsmm from sooty mangabeys). Several of these SIVs have crossed the species barrier to great apes and humans, generating new pathogens. Known examples of cross-species transmissions and the resulting viruses are highlighted in red. Strains that crossed into humans are marked by whether the genomes contain *vpr* and/or *vpx*.

[2] This figure and corresponding figure legend were modified by Maria C. Virgilio from their original version from the following publication:
Sharp, P.M., Hahn, B.H., 2011. Origins of HIV and the AIDS Pandemic. Cold Spring Harb Perspect Med 1, a006841. https://doi.org/10.1101/cshperspect.a006841

SIV has jumped into humans many times in history, and some of these successful jumps have led to distinct lineages or “groups” of HIV-1. The current groups are M, N, O, and P, all of which arose from unique transmission events. Group M is the main group, responsible for the pandemic form of HIV-1. All other groups account for a very small percentage of HIV-1 infection. Group O is the next most prevalent group (De Leys et al., 1990; Gürtler et al., 1994), followed by Group N, which has only been identified in fewer than 20 people (Vallari et al., 2010). Group P is the most rare, with only two documented cases (Plantier et al., 2009; Vallari et al., 2011).

HIV-2 was discovered in 1986 with genomes isolated from two individuals from West Africa. This new retrovirus was able to infect CD4+ T lymphocytes and elements of the genome were similar to HIV, yet the virus was distinct (Clavel et al., 1986). Sequence analysis of the genome suggested HIV-2 was transferred from SIVsmm (SIV from sooty mangabey) (Chen et al., 1996; Gao et al., 1992). However, unlike HIV-1, HIV-2 infections rarely progress to AIDS (Rowland-Jones and Whittle, 2007). Because of the identification of this second HIV, the original HIV became known as HIV-1 (Guyader et al., 1987).

The HIV and SIV genomes

Retroviruses are a family of RNA viruses characterized by their ability to reverse transcribe their viral RNA genome into genomic DNA, a process believed to be impossible prior to the identification of an RNA-dependent DNA polymerase, commonly referred to as reverse transcriptase. (Baltimore, 1970; Poiesz et al., 1980; Temin and Mizutani, 1970). The retrovirus DNA genome then requires integration into the host DNA genome, where the retrovirus uses a virally encoded promoter to hijack the host’s transcriptional

machinery to turn the viral DNA (provirus) back into genomic RNA (gRNA) and viral mRNAs [reviewed in (Gouvarchin Ghaleh et al., 2020)].

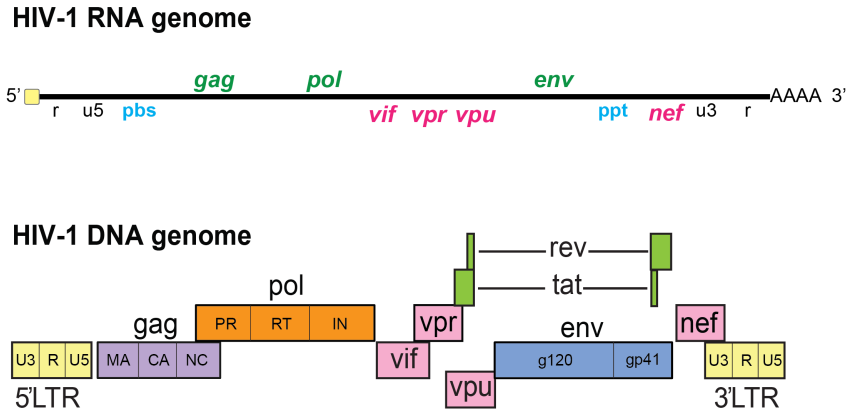

Figure 1.2. Essential components of the HIV-1 RNA and DNA genomes.[3] Major components of the HIV-1 genomic mRNA before reverse transcription (top), and DNA genome after reverse transcription (bottom). Viral gRNA resembles host mRNAs with a 5' cap and polyA tail. The LTR sequence is incomplete until the DNA/provirus form is produced. The major protein products encoded within the HIV-1 provirus are labeled. Each color represents one of the nine open reading frames and LTRs in the HIV-1 genome.

Like other lentiviruses, the HIV-1 viral RNA genome is extremely compact and fits all the necessary genetic information into a 9.3 kB genome, which is close to the maximal size genome packageable into a retrovirus particle (Gélinas and Temin, 1986; Ratner et al., 1985). The viral DNA genome is bookended with long terminal repeats (LTRs), which are identical sequences that can be divided into three elements: U3, R, and U5. The U3 region is derived from a sequence unique to the 3' end of the viral RNA, R (repeat) is from a sequence repeated at both ends of the viral RNA, and U5 is from the sequence unique to the 5' end of the RNA. The LTR sequences are only complete in the viral DNA genome

[3] This figure was made by Maria C. Virgilio and partially adapted from an earlier version by Mark Painter: Painter, M.M., Collins, K.L., 2019. HIV and Retroviruses, in: Schmidt, T.M. (Ed.), Encyclopedia of Microbiology (Fourth Edition). Academic Press, Oxford, pp. 613–628. https://doi.org/10.1016/B978-0-12-801238-3.66202-5

and through the process of reverse transcription, are identical copies of one another, hence referred to as terminal repeats. LTRs contain the regulatory information necessary for viral expression as a provirus, including enhancers, transcription factor binding sites, the TATA box, the major splice donor, and transcription start site (Coffin et al., 1997a; Nabel and Baltimore, 1987; Verdin et al., 1993).

All retroviral genomes contain the genetic information to produce the same basic structural proteins necessary to complete the viral lifecycle – Gag, Pro-Pol, and Env (Coffin et al., 1997b). A replication-competent provirus can be thought of as coding all the structural proteins and genomic features necessary for transcription, including cis regulatory sequences in the 5'UTR such as the major splice donor site (MSD) used in all the spliced viral RNAs, promoter, and transcriptional start site [reviewed in (Kim et al., 1998)]. Gag protein provides structure to the virus particle. Gag is proteolytically processed into several mature proteins: MA (matrix), CA (capsid), and NC (nucleocapsid). Viral entry requires *env* (envelope), which codes for both the surface (SU; gp120) and transmembrane protein (TM; gp41) of the virion. This complex is what interacts with the cellular receptor proteins, ultimately leading to fusion of the viral membrane with the cellular membrane. Pol is a polyprotein encoding several proteins with enzymatic activity used to complete the viral lifecycle. These include PR (protease), which proteolytically processes several HIV proteins for viral particle maturation after assembly and release, RT (reverse transcriptase), which has both DNA polymerase and RNase H activities, and IN (integrase) for viral cDNA integration into the host genome. Both *gag* and *pol* are in the same open reading frame; however *pol* translation relies on a single frameshift at the

junction between *gag* and *pol* at a slippery region near the *gag* stop codon (Jacks et al., 1988).

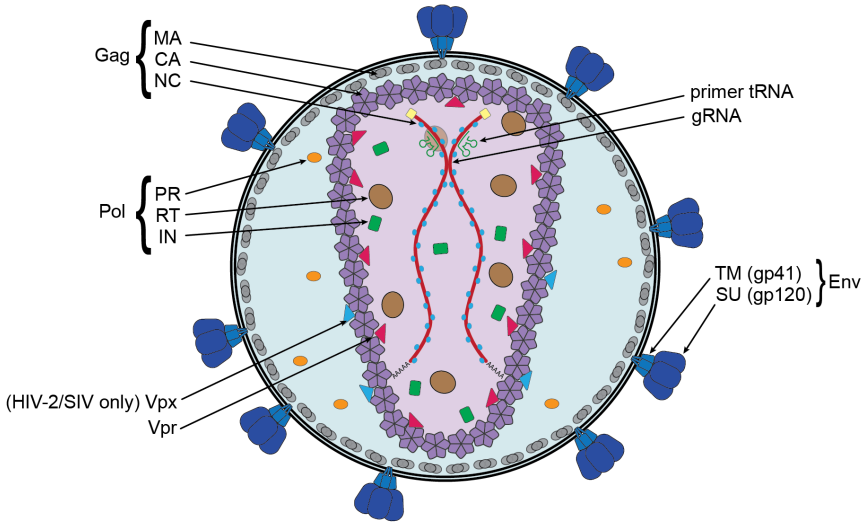

Figure 1.3. Model of a mature lentivirus virion.[4] Components of a mature lentiviral virion are depicted. Essential protein products are labeled based on their processed and precursor proteins. Vpr and Vpx are both depicted associated with capsid.

In addition to the three major structural proteins (Gag, Pol, and Env), HIV encodes two addition essential proteins, Tat and Rev, which are encoded by the only two spliced genes for HIV and SIV. The transcriptional *trans*-activator (Tat) protein is required for transcription elongation, enhancing HIV transcription significantly through binding to the TAR element in the LTR (Dayton et al., 1986; Fisher et al., 1986; Muesing et al., 1987; Rosen et al., 1985). Rev mediates the efficient transport of unspliced and singly spliced viral RNAs from the nucleus to the cytoplasm of infected cells. The RNA export activity helps to delineate the 'early' and 'late' genes. The small RNAs encoding Tat, Rev, and

[4] This figure was adapted by Maria C. Virgilio from an earlier version published by Mark Painter: Painter, M.M., Collins, K.L., 2019. HIV and Retroviruses, in: Schmidt, T.M. (Ed.), Encyclopedia of Microbiology (Fourth Edition). Academic Press, Oxford, pp. 613–628. https://doi.org/10.1016/B978-0-12-801238-3.66202-5

Nef are predominately expressed early in infection. Once enough Rev is produced, it mediates the export of viral RNAs to the cytoplasm, and the later stages of viral product production proceeds, leading to new virion assembly. Rev mediates the export of viral RNAs through recognition and binding to the Rev response element (RRE) found in the *env* ORF, which is spliced out of Rev-independent RNAs (Malim et al., 1990).

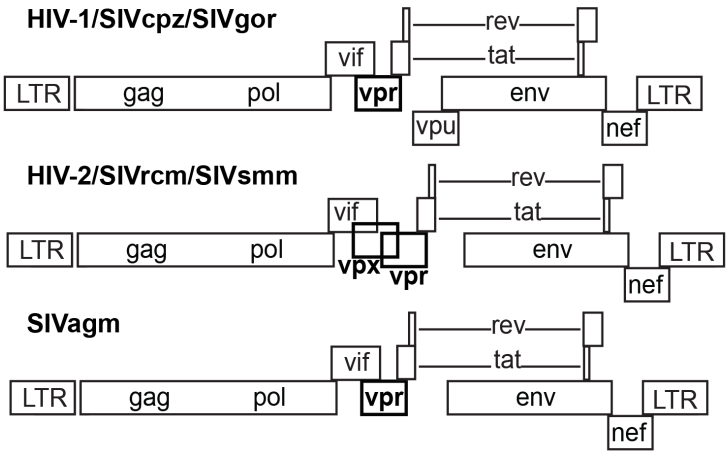

Figure 1.4. Accessory protein coding genes differ between HIV and SIV genomes.[5] Genomic maps for HIV-1, HIV-2 and select SIV genomes. HIV-1, SIVcpz, and SIZgor genomes contain *vpr* and *vpu* genes but not *vpx*. HIV-2, SIVrcm, and SIVsmm contain both *vpx* and *vpr*. SIVagm contains *vpr* but not *vpu* or *vpx*.

The remainder of the lentiviral genome contains a collection of genes not strictly necessary for viral assembly but are instead referred to as 'accessory proteins'. The HIV-1 accessory proteins are Vif, Vpu, Vpr, and Nef. HIV-2 and some SIV genomes code for another accessory protein called Vpx in addition to Vpr (**Figure 1.4**). These accessory proteins derive their title from the observation that they are not generally necessary for viral spread within *in vitro* systems lacking a complete immune response i.e., lacking cytotoxic T lymphocytes (CTLs) and antibodies. Lentiviral accessory proteins counteract

[5] This figure was created by Maria C. Virgilio

host defenses during infection and help the virus evade both innate and adaptive immune responses. Although the enhancement of viral spread for some of these accesry proteins is most easily measured *in vivo*, they can also be observed within *in vitro* settings.

The accessory proteins Nef, Vpu, and Vif

HIV and SIV Nefs are 25-34 kDa myristoylated proteins that associate with proteins at the cytosol-facing side of the plasma membrane and other organellar membranes. The name comes from a mistaken observations that Nef was a 'negative factor' of HIV, because Nef was thought to act on the regulatory region of HIV (LTR), contributing to HIV latency (Cheng-Mayer et al., 1989). With time, it was shown that Nef is important for pathogenicity, because strains lacking Nef are associated with long-term survival (Deacon et al., 1995; Dyer et al., 1997). The localization of Nef to the plasma membrane allows Nef to bind the cytoplasmic tails of host defense factors and traffic them to the lysosome for degradation (Collins and Collins, 2014).

Like other pathogens, HIV must successfully evade cytotoxic T lymphocyte (CTL) killing of HIV-infected cells. CTLs find their target cells through recognition of foreign antigens presented by major histocompatibility complex class I (MHC-I). HIV evades detection by CTLs through Nef-mediated mis-localization of MHC-I away from the cell surface (Collins et al., 1998; Schwartz et al., 1996). Nef accomplishes this by stabilizing an interaction between MHC-I and the clathrin adaptor protein-1 (AP-1), which normally regulates the clathrin-dependent trafficking of proteins between the trans-Golgi network and endosomes. The cytoplasmic tail of MHC-I contains a critical tyrosine residue (YXXA) that mediates an unstable interaction with the tyrosine-binding pocket in the μ1 subunit of

AP-1. The tyrosine in MHC-I can only weakly bind AP-1 in some cell types, and a complex of MHC-I and AP-1 is not normally detected in T lymphocytes. Nef stabilizes this weak interaction between MHC-I and AP-1, providing additional scaffolding contacts between the two proteins. The acidic cluster in Nef forms an electrostatic interaction with AP-1, and the polyproline (PxxP) repeats in Nef connect the MHC-I cytoplasmic tail to μ1 (Gall et al., 1998; Jia et al., 2012; Wonderlich et al., 2008). The stabilization of this complex allows AP-1 to direct MHC-I to the endolysosomal compartment, where it is degraded in lysosomes (Roeth et al., 2004).

Analogously to MHC-I and AP-1, Nef also targets the HIV receptor CD4 on the cell surface with help from AP-2 (clathrin adaptor protein complex 2). Removal of CD4 from the cell surface after infection enables HIV to avoid CD4-mediated inhibition of egress, which would otherwise happen by nascent virions binding to cell-surface CD4, anchoring virions to the producer cells and preventing spread (Lama et al., 1999; Ross et al., 1999). Nef mediates the interaction between CD4 and AP-2 by binding to a dileucine motif ([E/D]XXXL[L/I]) on the cytoplasmic tail of CD4, inducing clathrin-mediated endocytosis of CD4 and recruitment of CD4 to the lysosome (Aiken et al., 1994; Chaudhuri et al., 2007; Piguet et al., 1999). In line with these findings, there is some evidence that Nef also downmodulates both HIV co-receptors, CCR5 and CXCR4, though the mechanism is not defined (Michel et al., 2005; Venzke et al., 2006).

There are several lines of evidence that Nef enhances infection of both T cells and macrophages (Miller et al., 1994). One is the Nef-mediated removal of the macrophage mannose receptor (MR). Like MHC-I and CD4, MR has a short cytoplasmic tail that includes a SDXXXLΦ motif, resembling the dileucine motif ([E/D]XXXL[L/I]) on the

cytoplasmic tail of CD4. Addition of the cytoplasmic domain to a CD4-MR chimeric surface protein was enough to induce endocytosis (Vigerust et al., 2015, 2005). Unlike CD4, MR does not appear to be immediately degraded following endocytosis, suggesting a different endocytic journey (Stahl et al., 1980).

HIV Vpu (viral protein u) counteracts the interferon-induced BST-2 (bone marrow stromal antigen 2) also known as tetherin (Neil et al., 2008; Van Damme et al., 2008). Vpu also counteracts CD4. Both BST-2 and CD4 are cell surface proteins capable of binding HIV Env, preventing virion release. Guanylate binding proteins (GBPs) are an interferon-inducible subfamily of guanosine triphosphates (GTPases) with well described anti-pathogen activity. One of these family members, guanylate binding protein 5 (GBP5), impairs the infectivity of HIV in macrophages by interfering with HIV Env likely by trafficking Env from the Golgi to the lysosome, which is partially counteracted by Vpu (Krapp et al., 2016). Like Vif and Vpr, Vpu uses a Cullin1/F-box E3 ubiquitin ligase complex to enact poly-ubiquitination and subsequent lysosomal degradation of target proteins (Harris et al., 2012; Margottin et al., 1998). Furthermore, HIV-1 Vpu can recruit newly synthesized CD4 for ubiquitination and degradation in addition to the surface protein (Binette et al., 2007).

Although HIV and SIV have very similar genomes, they are not the same with respect to their accessory proteins. SIVsmm/mac (sooty mangabey/macaque), SIVagm (African green monkey), and HIV-2 do not have a *vpu* gene whereas HIV-1 does. This is important when considering the important function of accessory proteins to evade immune system detection. Whereas HIV-1 and some SIVs use Vpu to remove tethrin/BST-2 from the cell surface (Iwabu et al., 2009; Rong and Perelson, 2009), most

SIVs use Nef instead of Vpu because they lack a *vpu* ORF *(Jia et al., 2009; Zhang et al., 2009)*. The adaptation to remove BST-2/tetherin in the absence of a particular accessory protein highlights the importance of counteracting certain host restriction factors for both SIV and HIV.

Vif (viral infectivity factor) is a primate lentiviral accessory protein best known for antagonizing APOBEC3G (apolipoprotein mRNA editing enzyme 3G). Like other members of the APOBEC3 family of deaminases with broad antiviral activity (Malim and Emerman, 2008), APOBEC3G attacks single-stranded DNA, converting cytidine to uridine, causing hypermutation of HIV-1 cDNA during reverse transcription (Sheehy et al., 2002). APOBEC3G can be packaged into virions and is therefore present during reverse transcription when the virus particle infects the next cell. There is some evidence that both Vif and Vpr are capable of counteracting APOBEC3G, emphasizing the significant antiviral activity of the restriction factor (Norman et al., 2011). Mutations induced by APOBEC3G activate the DNA damage response in infected cells, leading to the upregulating of natural killer cell activating-ligands on the cell surface and death of the infected cell (Norman et al., 2011). Using a mechanism common to HIV accessory proteins, Vif acts as an adaptor protein between APOBEC3G and EloBC within the Cullin-5 E3 ubiquitin ligase complex, where APOBEC3G is poly-ubiquitinated and marked for degradation via the proteasome (Yu et al., 2003).

The role of Vpr and Vpx in HIV and SIV pathogenesis

Viral protein R

HIV *vpr* produces a 96-amino acid 14 kDa accessory protein (Wong-Staal et al., 1987). Many biochemical functions have been attributed to Vpr. The earliest observation was cytotoxicity in T cells (Cohen et al., 1990b). Many mechanisms of HIV pathogenesis have subsequently been attributed to Vpr, including evasion of the immune response; however, Vpr remains an enigmatic protein with functions that are only partially defined.

Early studies of HIV-1 Vpr suggested it could transactivate the LTR and bind viral genomic RNA; however subsequent studies have shown these claims to be highly unlikely (Mashiba et al., 2014; Wang and Su, 2019). Vpr is the only HIV accessory protein that can be packaged into virus particles (**Figure 1.3**), suggesting it may play a role in early infection before *de novo* viral protein synthesis occurs (Cohen et al., 1990a). This is supported by studies demonstrating virus-like particles (VLPs) loaded with Vpr molecules in producer cells and delivered to new cells by trans-complementation still showed a Vpr phenotype (Connor et al., 1995). Incorporation of Vpr in HIV particles is in nearly a 1:1 molar ratio with Gag and is mediated by a specific interaction between the carboxy terminus of Vpr with Gag p6 (Paxton et al., 1993).

Multiple groups have definitively shown Vpr to be important for spread *in vivo*. Several lines of evidence support this finding. HIV-1-infected people make antibodies to Vpr, confirming it is expressed *in vivo* (Wong-Staal et al., 1987). Monkeys infected with SIV with a mutation in the *vpr* ATG show progression and increased viral burden upon reversion of the ATG codon (Lang et al., 1993). Moreover, there are several instances of long-term non-progressors (people whose HIV infection does not progress to AIDS)

where the founder viruses lacked a functional *vpr* (Ali et al., 2018; Beaumont et al., 2001; Wang et al., 1996). Perhaps most convincingly, *vpr* is highly conserved among lentiviruses and is found in all HIV and SIV isolates, strongly supporting a critical role for lentiviral pathogenesis (Tristem et al., 1992).

Despite the evolutionary conservation of *vpr* and its fundamental role in HIV pathogenesis, the mechanism underlying Vpr function has remained elusive. This is because several studies have shown that *vpr* and *vpx* are dispensable for viral replication in $CD4^+$ T cell lines, because deletion of the *vpr* ORF only minimally decreased viral replication (Adachi et al., 1991; Akari et al., 1992; Cohen et al., 1990b; Dedera et al., 1989; Ogawa et al., 1989). In fact, Vpr can even hinder replication in dividing cells and is selected against in serially passaged cell lines (Balliet et al., 1994; Planelles et al., 1996, 1995; Rogel et al., 1995). These are curious observations given that the primary cellular target of HIV is CD4 T lymphocytes, but the significance becomes clearer in light of more mechanistic studies on the activity of Vpr on different cellular targets of HIV.

Vpr is an adaptor protein

Vpr behaves as an adaptor protein, counteracting host defenses as part of an E3 ubiquitin ligase complex (Collins and Collins, 2014). These E3 ligases facilitate the transfer of ubiquitin from E2 ubiquitin-conjugating enzymes to specific target proteins under normal cellular conditions. Ubiquitination is a post-translational protein modification that regulates protein degradation and trafficking (Cooper, 2000). Vpr behaves as a substrate adaptor between host proteins. Through immunoprecipitation studies, Vpr's binding partner was identified and creatively named VprBP for Vpr binding protein, though

it was later renamed DCAF1 (damaged DNA binding protein 1-cullin 4-associated factor 1) (Belzile et al., 2007; Zhao et al., 1994). Through DCAF1, Vpr interacts with damaged DNA binding protein 1 (DDB1) as part of the Cul4A E3 ubiquitin ligase complex where host proteins recruited by Vpr are polyubiquitylated and then degraded via the proteasome (**Figure 1.5**). Vpr recruits many host proteins to DCAF1 for degradation with varying consequences on the HIV infected cells. Through DCAF1, Vpr induces cell cycle arrest in diving cells, promotes viral replication in non-dividing cells, and counteracts the innate immune response.

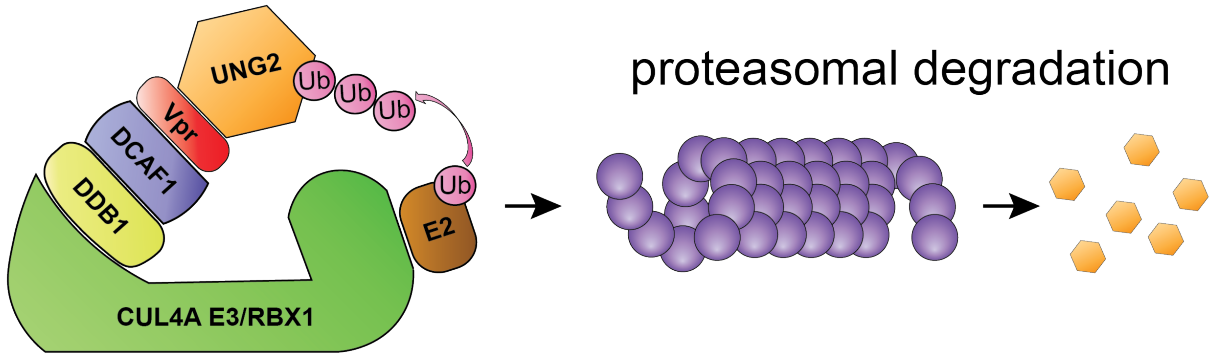

Figure 1.5. HIV-1 Vpr recruits host proteins to a ubiquitin ligase complex.[6] Graphical depiction of the molecular complex formed between HIV-1 Vpr and cellular proteins. Vpr interacts with DCAF1/DDB1/Rbx1/Cullin4A E3 ubiquitin ligase complex with UNG2. UNG2 is polyubiquitylated and degraded by the proteasome.

Vpr mediates cell cycle arrest in dividing cells

The best characterized function of Vpr is that it manages to arrest cell cycle at the G2/M phase (Jowett et al., 1995). As an adaptor protein between host restriction factors and DCAF1, it is no surprise that the arrest at the G2/M phase requires DCAF1 (Belzile

[6] This figure was adapted by Maria C. Virgilio from an earlier version by David Collins:
Collins, D.R., Collins, K.L., 2014. HIV-1 accessory proteins adapt cellular adaptors to facilitate immune evasion. PLoS pathogens 10, e1003851. https://doi.org/10.1371/journal.ppat.1003851

et al., 2007; Hrecka et al., 2007). In normal cell cycle progression, the transition from G2 to M is driven by the activation of the p34cdc2/cyclin B complex, which requires de-phosphorylation. Vpr somehow prevents this dephosphorylation of p34cdc2/cyclin B (He et al., 1995). Reversal of the G2-M block can be achieved through introduction of the phosphatase inhibitor okadaic acid (Re et al., 1995). Vpr is not alone in targeting this particular cellular process. Simian virus 40 (SV40), polyoma virus, HTLV, and adenovirus all express proteins targeting the G2/M checkpoint, suggesting a strong benefit to viral replication by disrupting transitions to mitosis (Zhao and Elder, 2005).

How exactly Vpr mediates cell cycle arrest is still incompletely understood. Some progress was made when Vpr was shown to mediate the degradation of MUS81-EME1 endonucleases, which causes premature activation of the SLX4 endonuclease complex (SLX4com), leading to cell cycle arrest at the G2/M stage of dividing cells (Laguette et al., 2014). SLX4 is a multidomain scaffold protein interacting with several nucleases, including Mus81-EME1. The complex is involved in DNA repair and resolution of Holliday junctions during homologous recombination and upregulated during stalled DNA replication forks (Fekairi et al., 2009). Specificity was confirmed by silencing of SLX4 subunits, which reduced Vpr-induced G2/M arrest. However, how well conserved the Vpr-SLX4-DCAF1 complex formation and induction of cell cycle arrest is still up for debate (Fregoso and Emerman, 2016). Still, the more intriguing finding from Laguette, et al. led to their proposal that cell cycle arrest is an unintended consequence of Vpr-mediated degradation of SLX4 complex proteins (Laguette et al., 2014). Instead, they propose SLX4com plays a role in induction of the type I interferon response; and by reducing the abundance of the complex, Vpr helps HIV evade immune sensing.

The uracil glycosidases UNG2 and SMUG1 are part of the DNA base-excision repair pathway and were the first substrates identified for Vpr-mediated degradation via DCAF1 (Schröfelbauer et al., 2005). However, they do not seem to contribute to G2 cell cycle arrest (Selig et al., 1997), and the benefit to HIV is highly contested. Some studies have suggested Vpr recruits UNG2 to virions to suppress mutations in the viral cDNA during reverse transcription and enhance viral spread in macrophages (Chen et al., 2004), or that UNG2 is a restriction factor counteracted by Vpr because it increases the uracilated viral cDNA (Weil et al., 2013), or that it has no effect at all on viral infection of many cell types (Kaiser and Emerman, 2006).

Most recently, the helicase-like transcription factor (HLTF), which is a DNA-binding protein involved in DNA repair, was identified as a target of Vpr in both macrophages and T cells (Hrecka et al., 2016; Lahouassa et al., 2016). HLTF was shown to moderately enhance HIV replication in CD4 T cells but the benefit of HLTF was only measurable in competition assays (Yan et al., 2019). As with other cellular targets of Vpr, HLTF poly-ubiquitination and degradation via the proteasome is DCAF1-dependent (Lahouassa et al., 2016; Yan et al., 2019).

The role of Vpr in cell cycle arrest and subsequent toxicity in dividing cells such as $CD4^+$ T cells is widely documented, if still incompletely understood. As mentioned above, *vpr* is unnecessary for viral replication in CD4+ T cell lines and is toxic for dividing cells due to the cell cycle arrest phenotype (Adachi et al., 1991; Akari et al., 1992; Balliet et al., 1994; Cohen et al., 1990a; Dedera et al., 1989; Ogawa et al., 1989; Planelles et al., 1996, 1995; Rogel et al., 1995). What is equally well documented is the necessity of Vpr for maximal spread of HIV in non-dividing cells, particularly macrophages.

Vpr aids in innate immune system evasion and counteracts host restriction factors targeting Env

Very early in HIV investigations, a clear benefit for HIV spread in macrophages was observed (Balliet et al., 1994; Connor et al., 1995; Eckstein et al., 2001; Hattori et al., 1990; Mashiba et al., 2014; Westervelt et al., 1992). As terminally differentiated cells, macrophages are more resistant to the cytotoxic effects observed in $CD4^+$ T cells, as described below. Importantly, unlike other retroviruses, productive HIV infection does not require breakdown of the nuclear envelope during cell division (Humphries and Temin, 1974; Lewis and Emerman, 1994). Assays measuring HIV spread have demonstrated that HIV spreads efficiently in macrophages but only when *vpr* is intact (**Figure 1.6**). Likewise, *vpr* mutant viruses have no apparent effect on spread in T cells alone, but do affect spread in tissues containing both macrophages and T cells (**Figure 1.6**) (Eckstein et al., 2001; Hattori et al., 1990).

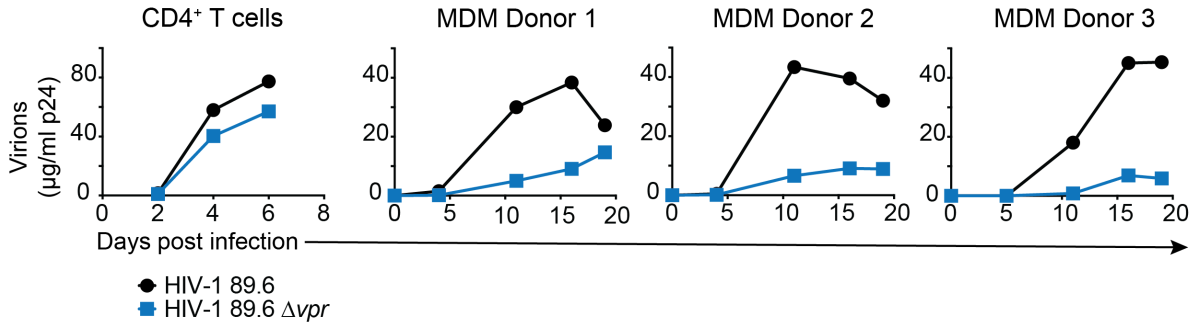

Figure 1.6. Vpr increases virion production in HIV-infected MDMs but not $CD4^+$ T cells.[7] Virion production by primary human $CD4^+$ T cells or monocyte-derived macrophages (MDM) infected with 1µg of the indicated virus for 6d or 18d, respectively.

[7] This figure and corresponding figure legend were modified by Maria C. Virgilio from their original version from the following publication:

As antigen-presenting cells, macrophages are important members of the immune community and release interferons and cytokines to initiate innate and adaptive immune responses to pathogens. In addition to cell cycle arrest, the other major role of Vpr supported by the literature is to dampen in innate immune response to HIV infection in macrophages. This involves degradation of SLX4 complex protein, reducing expression of type I interferons *IFNA*, *IFNB*, and *MXA* (Laguette et al., 2014). IRF3 is an interferon response factor triggered by Toll-like receptor (TLR) signaling that mediates the upregulation of interferon stimulated genes, particularly *ISG15,* and type I interferons, all of which have strong anti-HIV activity (Okumura et al., 2006). Both Vpr and Vif target IRF3 for degradation in macrophages in a process that is reversible by pharmacological inhibition of the proteasome with MG132 (Okumura et al., 2008). Vpr also targets an upstream member of the IRF3-IFN induction pathway, the TANK-binding kinase, which prevents activation of IRF3 (Harman et al., 2015). The targeting of multiple cellular proteins involved in the upregulation of the interferon response highlights the importance of inhibiting IRF transcription factors, preventing upregulation of their target genes during viral pathogenesis.

Intimately connected with the interferon response induced by HIV infection and counteracted by Vpr are several cellular restriction factors that target HIV Env for degradation. Disruption of Env trafficking not only prevents new virions from budding off the infected cell for cell-free virus spread, but it also prevents cell-to-cell viral spread by

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Mashiba, M., Collins, D.R., Terry, V.H., Collins, K.L., 2014. Vpr overcomes macrophage-specific restriction of HIV-1 Env expression and virion production. Cell Host and Microbe 16, 722–735. https://doi.org/10.1016/j.chom.2014.10.014

preventing syncytia formation and virological synapse formation (Collins et al., 2015; Sattentau, 2008). TET2 is a tet methylcytosine dioxygenase enzyme that demethylates the genome (Wang et al., 2018) and is monoubiquitylated by the DCAF1-Cul4A E3 ligase, promoting binding to chromatin (Nakagawa et al., 2015). Vpr targets TET2 for polyubiquitylation and degradation using the DCAF1-Cul4A UB ligase complex in HIV-infected macrophages (Lv et al., 2018). The degradation of TET2 enhances HIV replication and maintains expression of the pro-inflammatory cytokine IL-6, which is known to enhance HIV infection (Nakajima et al., 1989). Env processing and incorporation into new virions is significantly enhanced by the presence of Vpr in HIV-infected macrophages; however several host restriction factors target Env and are thought to prevent spread. Some of these antiviral factors targeting Env are interferon-induced transmembrane proteins (IFITMs). Of the three commonly described IFITMs, both IFITM2 and IFITM3 mediate the degradation of HIV Env and reduce spread (Yu et al., 2015). Interestingly, the expression of IFITM3 is TET2-dependent in macrophages. During HIV infection, IFITM3 expression is reduced in a Vpr and DCAF1 dependent manner. The dependence of IFITM3 expression on TET2 was confirmed when reduced *IFITM3* expression was observed with TET2 knockdown (Wang and Su, 2019).

Previous work from our lab suggests the benefit of Vpr to achieve optimal spread in macrophages likely involves counteracting many restriction factors targeting Env and the benefits are additive with spread. Mashiba et al. found no effect of Vpr on virions produced from macrophages on the first round of infection (Mashiba et al., 2014). Instead, Vpr boosted infectivity with subsequent rounds of infection, particularly at low multiplicity of infection. Maximal virion production was only achieved when both Vpr and Env were

expressed within the same cells. Macrophages infected with HIV lacking Vpr produced dramatically reduced amounts of Env due to increased lysosomal degradation of Env, which was reversible with ammonium chloride treatment (Collins et al., 2015; Mashiba et al., 2014). Incredibly, heterokaryon studies confirmed the macrophage-specific restriction of Env. HIV-infected HEK 293T cells, which are of non-hematopoietic origin cell line, display no HIV restriction. However, when restriction factors are provided in *trans* through fusion with macrophages, the restriction returns (Mashiba et al., 2014). Using both a Vpr mutant (Vpr^{Q65R}) defective at DCAF1 interactions or DCAF1 knockdown recovers the restriction on Env. Vpr-*null* infected macrophages produced more *IFN* RNA after the initial infection, and exogenous IFNα drastically reduced *env* expression and virion production. Importantly, the restriction on Env counteracted by Vpr severely impaired virological synapses from forming between HIV Env on infected macrophages with CD4 on autologous T lymphocytes, limiting cell-to-cell spread (Collins et al., 2015). The Vpr-enhanced spread via virological synapses between macrophages and T cells required cell-to-cell contact that were refractory to neutralizing antibodies (Collins et al., 2015). Finally, virion synapses between MDMs and autologous T cells increased Gag p24 in culture, solving the paradoxical observation that Vpr is required for maximal infection of T cells in vivo, but numerous studies have shown Vpr only marginally impacts infection of pure T cell cultures in vitro (Collins et al., 2015; Mashiba et al., 2014). Altogether, the findings from our lab and others strongly support the finding that Vpr is important for efficient infection of macrophages and spread by counteracting known and unidentified host restriction factors targeting Env and by suppressing the innate immune response.

Vpr and Vpx are related but distinct accessory proteins

HIV-1 fails to transduce dendritic cells and monocytes due to a restriction at the point of viral cDNA synthesis in these cells (Kaushik et al., 2009; Nègre et al., 2000). Conversely, HIV-2 and related SIVsmm/SIVmac transduce myeloid cells efficiently due to the expression of Vpx, an accessory protein not produced by HIV-1 (Tristem et al., 1992; Yu et al., 1991). Myeloid cells, particularly dendritic cells, are not permissive to HIV infection, due to the naturally high levels of SAMHD1 (sterile alpha motif and HD domain-containing protein-1). SAMHD1 restricts viral infection by reducing the concentration of intracellular deoxynucleoside triphosphates (dNTPs) to levels too low for successful synthesis of viral DNA by reverse transcriptase (Lahouassa et al., 2012). Silencing SAMHD1 in non-permissive cells such as monocyte-derived dendritic cells increased their susceptibility to infection (Laguette et al., 2011). Like Vpr, Vpx acts as an adaptor between SAMHD1 and DCAF1 in the Cul4A-DCAF1 E3 ubiquitin ligase complex, leading to proteasomal degradation of SAMHD1 (Hrecka et al., 2011).

In addition to an association with DCAF1, Vpr and Vpx share many genetic and functional similarities. This is unsurprising given that Vpr and Vpx likely share a common genetic ancestor (Tristem et al., 1992). Early HIV-2 sequencing confirmed some sequence homology between HIV-1 and HIV-2, but with several differences. Using the ROD isolate of HIV-2, a region unique to HIV-2 and SIVsmm/SIVmac isolates compared to HIV-1 was identified and called ORF X or viral protein X (Vpx) (Guyader et al., 1987). Vpr and Vpx are the only accessory proteins capable of being packaged into virus particles (Yu et al., 1990, 1988). However, how they are packaged differs. Unlike Vpr, which is found associated with Gag inside the viral capsid, Vpx is instead packaged on

the outside (Horton et al., 1994; Yu et al., 1993). Importantly, both Vpr and Vpx are essential accessory proteins supporting HIV replication and spread in macrophages.

HIV lifecycle

Entry

In the bloodstream, HIV targets cells of interest through recognition of its primary cellular receptor, CD4, in addition to one of two co-receptors: CXCR4 or CCR5, by the HIV-encoded envelope (Env) protein (**Figure 1.7**). It has been suggested that all transmitted founder viruses—the initial viruses that establish infection—are CCR5-tropic [reviewed in (Alkhatib, 2009; Berger et al., 1999)], but eventually CXCR4-tropic (X4) viruses predominate in the most infected individuals (Connor et al., 1997; Fouchier et al., 1996; Koot et al., 1999; Scarlatti et al., 1997). Exceptions to CCR5-tropic (R5) founder viruses are well established and often found in CCR5−/− individuals (Biti et al., 1997; Dean et al., 1996). Homozygous loss of CCR5 is usually due to a naturally occurring 32 base pair deletion in CCR5 (CCR5Δ32), and homozygous individuals are highly resistant to HIV infection (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). Both X4-tropic and dual-tropic viruses (which have Env that can recognize both CCR5 and CXCR4) have been found in CCR5Δ32 homozygous individuals (Gorry et al., 2002; Naif et al., 2002). Although CXCR4 is more widely expressed in the hematopoietic compartment, both CCR5 and CXCR4 are expressed on $CD4^+$ T cells and hematopoietic stem and progenitor cells (HSPCs) (Carter et al., 2011, 2010; Nixon et al., 2013; Sebastian et al., 2017). CCR5 is highly expressed on macrophages and activated T cells, but very little is found on quiescent T cells (Bonecchi et al., 1998; Han et al., 2004; Pierson et al., 2000; Rabin et

al., 1999). For HSPCs, the more primitive stem cells express less CCR5 than progenitors, however proviral sequences that express X4- and R5-tropic Env have been identified in both stem cell and progenitor populations (Carter et al., 2011, 2010; Sebastian et al., 2017).

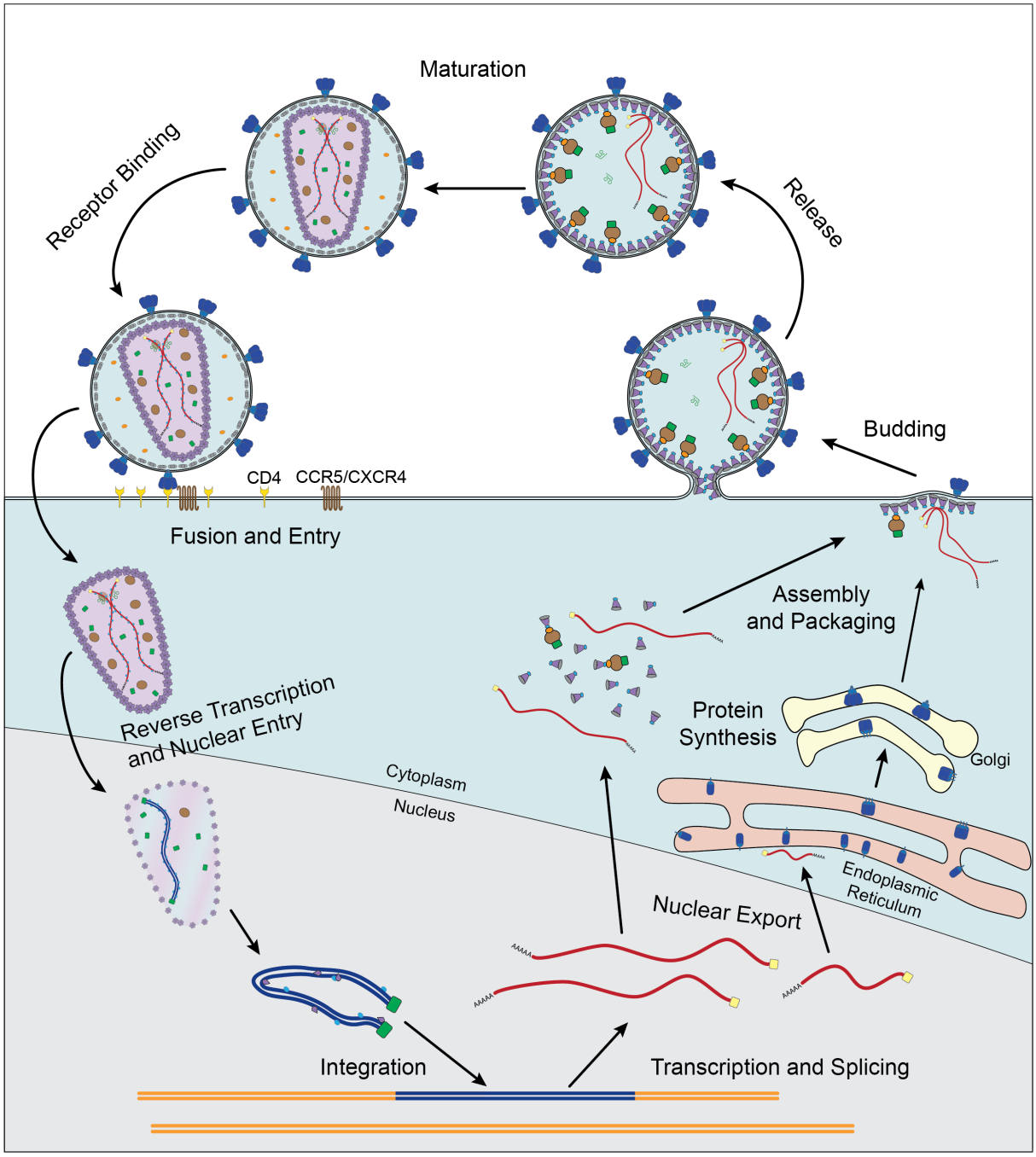

Figure 1.7. Diagram of the major steps within the HIV-1 lifecycle.[8] Virions bind receptors at the cell surface, fuse, and enter. The genomic RNA undergoes reverse transcription. The cDNA genome permanently integrates into the host chromatin, where it is transcribed, and translated or exported out of the nucleus All components for new virions assemble at the cell surface for release and final maturation.

Reverse transcription

As a retrovirus, one of the defining features of HIV infection is reverse transcription post-entry into the host cell. Reverse transcription converts the viral single-stranded RNA genome into a double-stranded DNA genome via the virally encoded reverse transcriptase (RT) [reviewed in (Hu and Hughes, 2012)]. Reverse transcription begins shortly after the viral capsid enters the cytoplasm. Reverse transcription initiates from the 3' terminal 18 nucleotides of a host $tRNA_{Lys3}$ that are complementary to the 18-nucleotide viral primer binding site (PBS) near the 5' end of the genome (Jiang et al., 1993; Ratner et al., 1985). Priming for RT from a host-derived tRNA is common to all retroviruses and endogenous retroviruses (Taylor, 1977). Viral reverse transcriptase primes from the 3'-OH of the tRNA and synthesizes complementary DNA (cDNA) through the U5 and R sequences of the minus-strand strong-stop DNA. The RNase H activity of RT degrades the genomic RNA complementary to the newly synthesized viral DNA, exposing the single strand. Through a process facilitated by NC, the minus strand DNA translocates to the 3' end of the viral RNA genome where the R region from the DNA anneals to the complementary *r* sequence on the gRNA. As two copies of the viral RNA genome are packaged into each viral particle, the minus-strand translocation can proceed to either

[8] This figure was adapted by Maria C. Virgilio from an earlier version by Mark Painter:
Painter, M.M., Collins, K.L., 2019. HIV and Retroviruses, in: Schmidt, T.M. (Ed.), Encyclopedia of Microbiology (Fourth Edition). Academic Press, Oxford, pp. 613–628. https://doi.org/10.1016/B978-0-12-801238-3.66202-5

copy. Synthesis of the minus-strand continues along the gRNA from the strong-stop DNA generating one full strand of viral DNA. The RNase H activity of RT degrades the remaining gRNA of the second strand, leaving behind small RNase H-resistant pieces of the RNA genome, that are poly-purine tracts (ppt) that act as primers for reverse strand DNA synthesis. The tRNA primer is then removed by the RNase H activity of RT, exposing both PBS single-stranded sites, which anneal to form an intermediate circle, and each strand serves as a template for the other. Eventually the elongation of the minus strand displaces the plus strand, and synthesis of a double stranded cDNA genome is completed. The end result is a dsDNA viral genome with two duplicate copies of the LTR on the termini of the genome. This dsDNA is then transported into the nucleus where it uses a mix of host- and virus-encoded proteins to permanently insert itself into the human chromosome [reviewed in (Lesbats et al., 2016)].

The timing of reverse transcription remains a deeply contentious topic of debate. Historically, reverse transcription is thought to occur as the viral capsid carrying the two copies of the viral RNA genome traverses the cytoplasm and is complete by the time the capsid reached the nuclear pore (NP). Being greater in diameter than an NP, the capsid is thought to disassemble either enroute to the nucleus or just before it arrives, releasing the DNA genome into the nucleus through the NP [reviewed in (Bukrinsky, 2004)]. However, several recent studies contradict the current dogma using several lines of evidence. First, fluorescently tagged capsid and cDNA genomes were both found inside the nucleus of HIV-infected cells suggested at least some capsid is able to transmit through the NP (Hulme et al., 2015). Second, the diameter of capsid at the widest point is approximately 60nm (Briggs et al., 2003) and somewhat greater than that of a nuclear

pore diameter at 40-60nm (Hoelz et al., 2011; von Appen et al., 2015), however both the nuclear pore and the capsid core demonstrate flexibility (Beck and Baumeister, 2016; Mattei et al., 2016). Furthermore, electron micrographs taken of a complete viral capsid passing through the nuclear pore demonstrate the capsid can indeed traverse the NP and arrive inside the nucleus whole (Zila et al., 2021). Others have also provided evidence that an intact viral core disassembles only once inside the nucleus by showing GFP-tagged CA loses florescence only once inside the nucleus (Burdick et al., 2020). This same group also included evidence that integration occurs within 1.5μM of the NP. Third, blocking entry of capsid to the nucleus interferes with reverse transcription suggesting reverse transcription completes once inside the nucleus and not before (Dharan et al., 2020). The recent papers addressing the timing and specifics of reverse transcription and capsid uncoating used complementary but distinct experimental approaches to demonstrate that reverse transcription does not complete in the cytoplasm and the viral capsid core does not disassemble until the viral genome is deposited inside the nucleus and is complete only shortly before integration (**Figure 1.7**) (Burdick et al., 2024, 2020; Dharan et al., 2020; Müller et al., 2021). The updated model supports the hypothesis that an intact viral capsid is necessary to protect viral genome intermediates from innate immune sensing pathways that could respond to double stranded RNA or RNA-DNA intermediates in the cytoplasm.

Integration and insertion site preference

Retroviruses exhibit differences in their integration site preferences (Serrao et al., 2015; Wu et al., 2005). HIV preferentially integrates into introns of actively expressed

genes (Einkauf et al., 2019; Han et al., 2004; Ho et al., 2013; Ikeda et al., 2007; Lewinski et al., 2006; Schröder et al., 2002). Unlike many other retroviruses, HIV can infect non-dividing cells, and the cDNA genome is able to traverse the nuclear membrane through interaction between viral and host proteins (Anderson and Maldarelli, 2018; Burdick et al., 2017; Cherepanov et al., 2003; Di Nunzio et al., 2012; Fernandez and Machado, 2019; Lelek et al., 2015). The nuclear pore complex (NPC), associated nucleoporin proteins (Nups), and the cleavage and polyadenylation specificity factor 6 (CPSF6) facilitate HIV integration into euchromatic regions of the host genome through interactions between the viral capsid and DNA genome-protein integration complex (Burdick et al., 2020; Chin et al., 2015; Lelek et al., 2015). This results in a strong preference for integration into chromatic regions near the nuclear pores (Burdick et al., 2020). Integrations near the nuclear envelope are common to lentiviruses except for the gammaretrovirus M-MLV (Moloney murine leukemia virus) (Marini et al., 2015).

Once HIV has integrated into a host chromosome, it persists for the life of the cell. If the HIV genome is replication-competent and if the infected cell is long-lived, there is potential for the provirus to become activated and contribute to viremia at any point. Resting $CD4^+$ T cells have a long half-life (Finzi et al., 1999), and HIV DNA levels remain stable in patients on ART after many years. Resting $CD4^+$ T cells can remain quiescent, or they can homeostatically proliferate without reactivation of the latent virus. Hence, any prolonged interruption of therapy creates potential for viral rebound.

Viral gene expression

Understanding of transcriptional control mechanisms of HIV is well established in T cells. For example, HIV preferentially integrates into the introns of actively transcribed genes, presumably to ensure access to transcriptional machinery (Lewinski et al., 2006; Schröder et al., 2002). In HIV-infected T cells, two nucleosomes are invariably present in the HIV 5' LTR of integrated provirus, with nucleosome-1 (Nuc-1) positioned at the transcriptional start site (TSS), blocking the release of promoter-proximal transcription complexes in latency (Rafati et al., 2011; Verdin et al., 1993). In addition to nucleosome positioning, recruitment of histone lysine methyltransferases (HKMT) and deacetylases (HDAC) (Nguyen et al., 2017; Tyagi et al., 2010) to the 5' LTR are associated with the establishment of heterochromatin and HIV latency from cell line and primary T cell models of latency (Tyagi et al., 2010).

The HIV LTR contains many important regulatory sequences. These include sites for binding of transcription factors such as NF-κB and NFAT, the promoter, and the trans-activation response element (TAR) element, which is required for activation of the HIV promoter (Dingwall et al., 1989). NF-κB is an important transcriptional regulator in hematopoietic cells and is sequestered to the cytoplasm of resting cells by the inhibitor of κB (IκB). With T cell activation, IκB releases NF-κB for translocation to the nucleus (Li et al., 1999). Once in the nucleus, NF-κB binds the HIV LTR, displacing silencing factors and recruiting HDACs (Nabel and Baltimore, 1987; Van Lint et al., 1996). Although NF-κB activation is sufficient to reactivate latent virus in Jurkat cell models of HIV latency, T_{RM} (resting memory) cells have limiting amounts of NF-κB in the nucleus (Tyagi et al., 2010), and it is insufficient in primary T_{RM} cell models to initiate transcription, requiring co-

activation with positive elongation factor b (P-TEFb) (Tyagi et al., 2010). P-TEFb is a complex made up of cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CycT1). It is present only at low levels in resting T cells, and it exists mostly sequestered by the 7SK small nuclear RNA and hexamethylene bisacetamide (HMBA)-induced protein (HEXIM1), part of the 7SK small nuclear ribonuclear protein (7SK snRNP) (Nguyen et al., 2001). T cell activation results in the rapid phosphorylation of preexisting CDK9 and the dissociation of P-TEFb from 7SK snRNP (Kim et al., 2011). HIV Tat assists in this process by competitively displacing HEXIM1 to free P-TEFb from the inhibitory complex (Barboric et al., 2007; Contreras et al., 2007; Sedore et al., 2007). Activated P-TEFb is then recruited to nascent HIV transcripts, where it facilitates transcription elongation and reactivation of latent virus (Kim et al., 2011; Tyagi et al., 2010).

HIV latency

The potential for HIV latency was discovered several decades ago in patients receiving ART. First predictions of time to cure with continued therapy were approximately 2–3 years (Perelson et al., 1997) based on initial results showing a rapid decrease in plasma virus after initiation of ART (Ho et al., 1995) and a short half-life of actively infected CD4+ T cells (Perelson et al., 1997). These initial estimates proved wrong due to the presence of cells with a much longer half-life. Further in vitro studies showing that non-virus producing T cells could be stimulated in culture to produce virus provided definitive evidence for the existence of a longer-lived latent reservoir. The major reservoir was thought to be long-lived memory T cells (Chun et al., 2000, 1997; Finzi et al., 1999, 1997; Perelson et al., 1997). There is now evidence that other long-lived cells including naïve T

cells, stem cell memory T cells (Buzon et al., 2014; Hiener et al., 2017) and hematopoietic stem and progenitor cells (HSPCs), also contribute to the reservoir (Carter et al., 2010; McNamara et al., 2012). Virus may rebound upon interruption of ART through cellular stimulation and differentiation from cells within the latent reservoir. However, T cells and HSPCs have very different proliferative potentials and differentiation capabilities that contribute to viral latency and maintenance of the viral reservoir. The extent to which other long-lived cells such as tissue resident macrophages contribute to the reservoir is unknown and pose a barrier to achieving a cure.

Env synthesis and trafficking

Env is a large, structurally complex protein synthesized as a 160 kDa polyprotein that is translated directly into the lumen of the rough ER, where it is glycosylated at the many asparagine residues (Hunter and Swanstrom, 1990; Leonard et al., 1990). The enzyme complex oligosaccharyltransferase attaches glycan trees to each of the asparagine residues. Within eukaryotic cells, these glycan trees, which are composed of three glucoses, nine mannoses, and two N-acetylglucosamines, are trimmed to remove the glucoses post-translation but before reaching the Golgi (Doores et al., 2010). This modification leaves mannoses as the terminal residue, which is trimmed by mannosidases and is replaced with complex glycans on host proteins as they transit through the Golgi (Kornfeld and Kornfeld, 1985; Stanley et al., 2022). Proteins that terminate with mannose as opposed to the complex glycans are recognized as foreign and identified as a PAMP (pathogen associated molecular pattern) by the host. HIV Env glycan trees do not undergo the final trimming of mannose due to the high number of

glycosylation sites and because Env oligomerizes into trimers in the cis-Golgi, preventing mannosidases from accessing the mannose residues (Checkley et al., 2011; Doores et al., 2010; Schawaller et al., 1989). These Env trimers then pass through the trans-Golgi, where the 160 kDa polyprotein is cleaved by the host protease furin into two smaller proteins, gp120 and gp41 (Checkley et al., 2011), after which Env is trafficked to the cell surface. These two subunits of Env are important for viral entry (McCune et al., 1988). Gp120 binds CD4 at the cell surface, which induces a conformational change, exposing the co-receptor binding site. The binding of CCR5 or CXCR4 induces another conformational change that exposes a fusion peptide, a hydrophobic region on gp41 that inserts into the membrane of the target cell and promotes fusion (Bosch et al., 1989). Because Env is the only viral protein on the outside of the viral membrane, and processing of Env leaves remaining mannose residues that appear non-eukaryotic, Env is a common target of the innate immune response.

Env incorporation into virus particles and Viral assembly

MA regulates the intracellular trafficking and binding of Gag to the plasma membrane in coordination with plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP2) (Ono et al., 2004). MA also directs the incorporation of Env into virions, and CA helps coordinate Gag-Gag interactions, which ultimately form the viral core after virion release and maturation (Saad et al., 2006). Interestingly, Gag alone is sufficient to produce virus-like particles that bud from the plasma membrane (Göttlinger, 2001). NC mediates the packaging of viral genomic RNA and supports Gag multimerization (Cimarelli et al., 2000). The p6 domain of Gag stimulates the release of viral particle from

the plasma membrane by recruiting endosomal sorting complexes required for transport (ESCRT), which normally functions in cellular budding events (Adamson and Freed, 2007; Bieniasz, 2009).

Although most of the virus assembly takes place at the plasma membrane (Finzi et al., 2007; Welsch et al., 2007), in some cases new HIV particles assemble in internal compartments resembling late endosomes, primarily in HIV-infected macrophages (Gousset et al., 2008). These internal virus-containing compartments are specialized plasma membrane invaginations that can hold many virus particles that are rapidly released to the surface at cell-to-cell contact sites, forming virological synapses (Balasubramaniam and Freed, 2011).

Viral spread

Infection is initiated by either a small number of virions or a single virion [reviewed in (Joseph et al., 2015)]. Once infection is established, there are two main methods of viral transfer: cell-free and cell-to-cell transmission [reviewed in (Sattentau, 2008)]. With cell free virus, HIV spreads from host to host and can easily be transmitted through blood or saliva. Thereafter, cell-free virions can circulate in the bloodstream and rapidly disseminate. As important as cell-free viral infection is, there are also disadvantages. These include the need to rely on diffusion for movement to find cells that meet entry requirements, to bind multiple receptors for entry upon finding receptive cells, especially if those receptors are rare, and finally, to avoid anti-viral defense mechanisms such as humoral immunity, complement, cytokines, antibodies, and cellular defenses like

macrophages, activated lymphocytes, and affinity matured antibodies (Dutartre et al., 2016).

Cell-to-cell transmission involves contact between at least one infected cell and another uninfected cell. It is a much slower process than cell-free spread, but the overwhelming majority of new infections are from cell-to-cell spread and not cell-free virus (Dimitrov et al., 1993; Sato et al., 1992). As an antigen-presenting cell, infected macrophages efficiently transfer HIV to nearby T cells through direct interaction (Collins et al., 2015; Duncan et al., 2014; Groot et al., 2008). A host factor involved in cell aggregation, activated leukocyte cell adhesion molecule (ALCAM), mediates cell-to-cell transmission of HIV to T cells (Park et al., 2017). HIV proteins also contribute to cell-mediated transfer. HIV Env is expressed and trafficked to the cell surface during active infection, where it can interact with HIV receptors on uninfected cells, sometimes fusing cells together into syncytia (Jolly et al., 2004). In addition to syncytial formation, cell-to-cell spread can also be achieved through virological synapse formation. Synapse formation relies on actin polarization and congregation of several host and viral proteins at the plasma membrane including CD4, the co-receptors CXCR4 (X4) or CCR5 (R5), adhesion molecules, tetraspanins, with HIV Gag and Env (Balasubramaniam and Freed, 2011; Jolly et al., 2004). Cell-to-cell spread through virological synapses is most often observed between an HIV-infected antigen-presenting cell, such as a macrophage or dendritic cell, and a CD4+ T cells (McDonald et al., 2003; Monks et al., 1998). Cell-to-cell spread minimizes the exposure of virus to the host immune system and lessens selective pressures (Schiffner et al., 2013).

HIV targets cells of the hematopoietic compartment

Hematopoietic Stem and Progenitor Cells (HSPCs)

HSPCs have enormous proliferative potential, are extremely long-lived, and differentiate into all the hematopoietic lineages through hematopoiesis. Hematopoiesis is the process of building and maintaining the entire hematopoietic compartment through many stages of differentiation, starting from the most primitive hematopoietic stem cells (HSCs). Differentiation initially proceeds from the long- and short-term HSCs, which divide and differentiate into progenitors that eventually terminally differentiate into all the cells that make up the hematopoietic system, including dendritic cells, macrophages, erythrocytes, megakaryocytes, B and T cells. With each step through the differentiation process, stemness is lost along with proliferative potential [reviewed in (Jacobsen and Nerlov, 2019; Zhang et al., 2018)]. Although most differentiated cells in the hematopoietic compartment are tissue-resident, HSPCs remain primarily quiescent in the bone marrow, except during times of stress or need [reviewed in (Nakamura-Ishizu et al., 2014)].

HSPCs express CD4 at low levels, are capable of being infected with both X4 and R5-tropic HIV in vitro, and proviral sequences have been identified in HSPCs from patients (Carter et al., 2010; McNamara et al., 2012; Sebastian et al., 2017; Zaikos et al., 2018). Identical proviral sequences, including the same insertion site in the patient genome, were found in multiple compartments of the hematopoietic lineage, including HSPCs, PBMCs (peripheral blood mononuclear cells), and BMMCs (bone marrow mononuclear cells). A subset of these clonal sequences was matched to expressed cell-free virus (Zaikos et al., 2018). Some cells with clonal proviral sequences matching provirus from HSPCs were not $CD4^+$. The virus detected was defective, indicating that

natural infection could not have occurred. These infected lineages could only have arisen through differentiation into multiple lineages from a single, more primitive cell (Sebastian et al., 2017). This demonstrates the potential of a single infected HSPC to be infected, proliferate, and differentiate into several lineages while carrying a provirus (**Figure 1.8**).

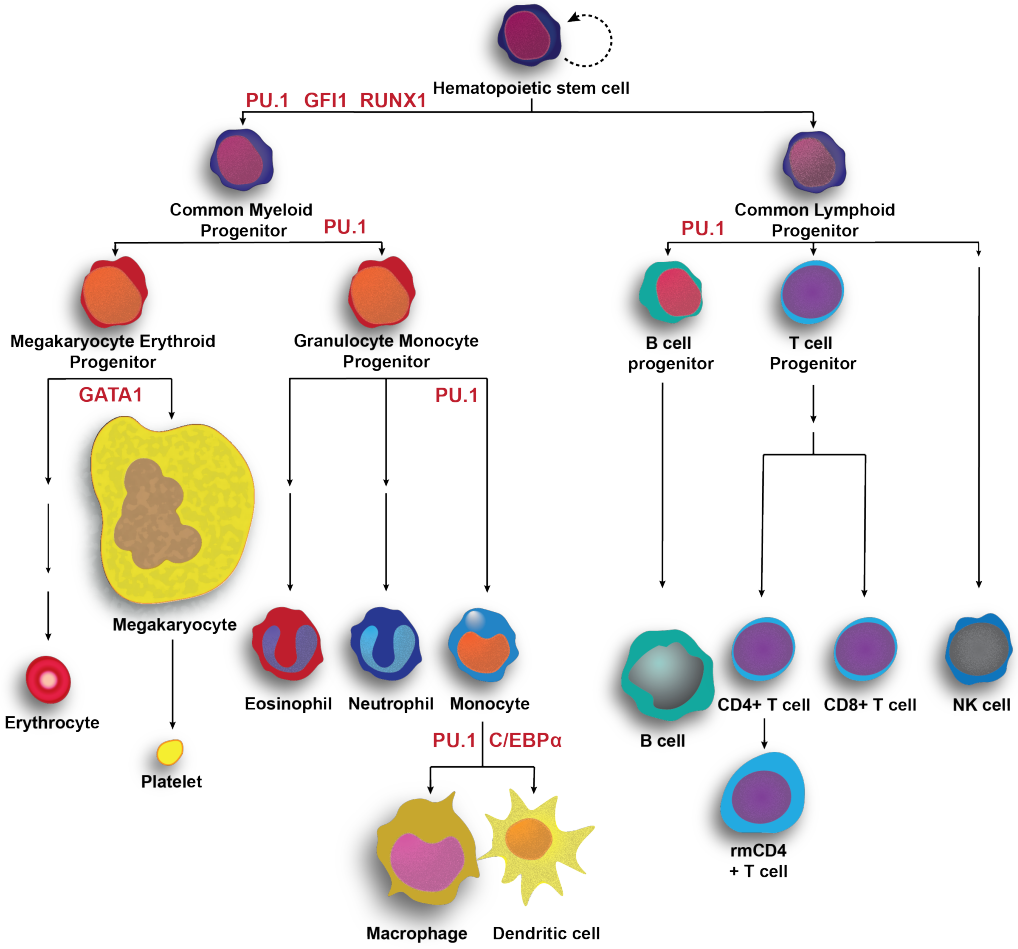

Figure 1.8. Many lineages in the hematopoietic compartment depend on PU.1.[9] Graphical representation of major steps in definitive hematopoiesis. Select essential transcription factors active at the depicted stages are shown in red.

HSPCs are rare, and HIV-infected HSPCs are even rarer (approximately 2.5 proviruses per million HSPCs), with less than 1 in a million HSPCs expected to carry a replication-competent provirus (Sebastian et al., 2017). Latency is preserved in the most

[9] This figure was adapted by Maria C. Virgilio from an earlier version by Thomas Zaikos.

quiescent HSPCs, and differentiation is associated with higher rates of active infection (Painter et al., 2017). A single HSPC harboring an intact virus has great potential to spread provirus through daughter cells and expressed virus to form new infections. The high proliferative potential, long lifespan, and capability to populate the body with infected daughter cells of all lineages, not just T cells, makes HSPCs a unique reservoir with exceptional potential for maintaining the viral reservoir.

$CD4^+$ T Lymphocytes

T cells mature towards one of two main differentiation trajectories that are identified by the surface expression of either CD8 or CD4 (**Figure 1.8**) (Germain, 2002). $CD8^+$ T lymphocytes are commonly referred to as cytotoxic T cells due to their function to kill other cells. Conversely, the $CD4^+$ T cells are commonly referred to as T helper cells because they are essential for protection against infection by helping to activate B cells to secrete antibodies, coordinating with macrophages to destroy pathogens, and helping to activate cytotoxic T cells to kill infected target cells (Alberts et al., 2002). $CD4^+$ T cells are the primary cellular target of HIV and represent the most abundant cellular reservoir of HIV proviruses. $CD4^+$ T cells also primarily express CXCR4 instead of CCR5, meaning viruses capable of binding and entering T cells are considered T-tropic.

Within the $CD4^+$ T cell lineage, there are several subsets of cells that are generated based on exposure to pathogens. Once T cells have committed to the CD4 lineage, the naïve T cells (T_N) have the greatest proliferative capacity of the $CD4^+$ T cells and lose their proliferative potential as they differentiate into the polarized, functional subsets: T_{H1}, T_{H2}, T_{H9}, T_{H17}, T_{FH}, T_{reg} [reviewed in (Liudahl and Coussens, 2018)]. After the peak of

infection and clearance of foreign antigen, greater than 90% of the T_H population dies. The remaining cells convert into long-lived memory cells that are primarily quiescent and do not rely on peptide-bound major histocompatibility complex II stimulation for maintenance [reviewed in (Pepper and Jenkins, 2011)]. T cells appear to be most susceptible to HIV infection after activation but before initiation of quiescence (Chun et al., 1995), while the latent reservoir in T cells is most likely established during the effector-to-memory transition (Shan et al., 2017). Central memory T cells, T_{CM}, are classically considered the primary reservoir of latent HIV infection. These memory cells then rely upon homeostatic proliferation for maintenance until they are re-activated. Although T cells are generally thought to circulate throughout the bloodstream, more in-depth studies of HIV burden and SIV models of infection, have shown T cells harboring HIV in lymphoid and other tissues (Estes et al., 2017). HIV-infected $CD4^+$ T cells are also in cervical mucosa, gut associated lymphoid tissue, genital tract, and lymph nodes (Ananworanich et al., 2012; Cantero-Pérez et al., 2019; Coombs et al., 2003; Günthard et al., 2001; Yukl et al., 2013).

Macrophages as target of HIV

Macrophages were first described by Elie Metchnikoff studying embryology and evolution in invertebrates in the late 1800s. He noted that specialized cells are capable of ingesting and attacking foreign material, which he called ‘phagocytes’ from Greek which loosely translates to ‘cells that eat’ (Kaufmann, 2008; Lazarov et al., 2023; Metschnikoff, 1891). Macrophages are tissue-resident cells that function to shape tissue

development, maintain local homeostasis, and are a critical component of the immune system.

Macrophages are terminally differentiated, non-dividing cells that express the HIV receptors, particularly CD4 and CCR5, and are often the first cells to become infected *in vivo* for both HIV and SIV infections (Cohen et al., 2011; Gupta et al., 2002; Hu et al., 2000; Zhang et al., 1999). In stark contrast to HIV infected $CD4^+$ T cells, which can only survive active HIV infection for a couple of days, macrophages are highly resistant to the cytotoxic effects of the virus (Gendelman et al., 1988; Orenstein et al., 1988). They have a long half-life while infected with HIV and can survive weeks to months (**Figure 1.6**) (Aquaro et al., 2002; Sharova et al., 2005). This is observed even in *in vitro* cultures. A common model of studying macrophages is to generate monocyte-derived macrophages (MDMs) *in vitro* through isolation of monocytes from peripheral blood and stimulate them with M-CSF and GM-CSF (Waki and Freed, 2010). HIV-infected MDMs also survive weeks in culture and can efficiently mediate cell-to-cell infection with autologous $CD4^+$ T cells in culture (Collins et al., 2015; Sharova et al., 2005). As antigen-presenting cells, macrophages come in close contact with $CD4^+$ T cells, creating ample opportunity to pass virus from macrophage directly to T cell.

HIV-infected tissue-resident macrophages are found in semen, foreskin, and vaginal, rectal, and cervical mucosa (Cummins et al., 2007; Ganor et al., 2019; Iijima et al., 2008; Quayle et al., 1997; Shehu-Xhilaga et al., 2007). Indeed, most viral transmission events occur at mucosal surfaces, then spread to secondary lymphoid tissue before dissemination (Cohen et al., 2011; Reinhart et al., 1997; Schacker et al., 2001). In several instances of infection studies in tissues, HIV was found in both macrophages and $CD4^+$

T cells. Furthermore, when HIV strains are introduced to tissue *in vitro*, spread is most observable if the strain used is macrophage-tropic rather than T cell-tropic, suggesting the capacity for macrophage infection is an important part of effective HIV spread within tissues (Greenhead et al., 2000). This coincides with observations on HIV transmission events. Viruses collected from newly infected individuals tend to be macrophage-tropic or dual-tropic, and their Env has a greater binding affinity for CCR5. With time, virus isolated from these same individuals have acquired a greater affinity towards CXCR4 and T cell infection (Joseph et al., 2015; Ochsenbauer et al., 2012). While macrophages were initially identified as an important cellular source of HIV (Gyorkey et al., 1985; Ho et al., 1986), later studies concluded that macrophage infection was not important in a natural infection. This is because experiments measuring HIV infection in macrophage cultures found less productive infection compared to $CD4^+$ T cell cultures when measured by Gag p24 ELISA of the culture supernatant (**Figure 1.6**) (Ochsenbauer et al., 2012). However, PLWH who progress to AIDS with minimal $CD4^+$ T cells still exhibit high viral loads with viral production originating from macrophages (Orenstein et al., 1997), indicating that the in vitro infection does not recapitulate the situation in vivo with respect to the importance of macrophages.

Macrophages introduce HIV to so-called immune privileged spaces like the central nervous system, in the later stages of AIDS, this causes encephalopathy (Gras and Kaul, 2010; Koenig et al., 1986). Analogous central nervous system pathology also occurs in rhesus macaques with SIV infection (Desrosiers et al., 1991).

Within the myeloid compartment, macrophages are the cells most permissive to HIV infection due to the downregulation of antiviral factors such as SAMHD1 after

differentiation, which is not observed in dendritic cells (Laguette et al., 2011; Lahouassa et al., 2012). Macrophages also express several antiviral factors that must be counteracted by the invading pathogens.

All myeloid roads lead to PU.1

Macrophages from primitive hematopoiesis

As terminally differentiated cells, macrophages arise from two main independent sources. The first is from the yolk sac during early development. With the help of critical lineage-determining transcription factors such as RUNX1 (Runt-related transcription factor 1) and PU.1, erythroid-myeloid progenitors in the yolk sac are pushed towards the macrophage phenotype (Gautier et al., 2012; Gomez Perdiguero et al., 2015; Gosselin et al., 2014; Gosselin and Glass, 2014). These yolk sac-derived macrophages arise before definitive hematopoiesis and find their way to developing tissues during fetal development. There they acquire tissue-specific functions and become specialized macrophages within their niche, relying on signals within their tissue microenvironment for tissue-specific differentiation programs [reviewed in (Cox et al., 2021)]. These include microglia in the brain (Alliot et al., 1999; Vilhardt, 2005), Kupffer cells in the liver (Ikarashi et al., 2013; Naito et al., 2004), osteoclasts in the bone (Jacome-Galarza et al., 2019), among many others. These tissue-resident macrophages are capable of self-renewal and remain resident in their respective tissues for life. Their importance for healthy development is underscored by the findings that embryos lacking macrophages are often embryonic lethal or suffer major developmental issues after birth.

Macrophages of definitive hematopoiesis

The second source of macrophages is from hematopoietic stem cells (HSCs) in the blood and bone marrow. During development, HSCs find their way to the fetal liver and eventually to the bone marrow, where they remain for life (Perdiguero and Geissmann, 2016). The bone marrow HSCs give rise to all hematopoietic lineages for the life of the individual, including lymphoid cells (such as T and B cells) and myeloid lineage cells (such as monocytes, macrophages, and dendritic cells).

Macrophages derived from HSCs go through many differentiation steps on their way to becoming terminally differentiated macrophages. Hematopoiesis progresses through the tightly regulated interplay between lineage-defining transcription factors. Some of these compete for binding to promoter regions of genes. Those factors that outcompete others drive the next stage of differentiation. As with macrophages arising from primitive hematopoiesis, those from definitive hematopoiesis also rely upon RUNX1 as an early lineage-determining transcription factor. RUNX1 is necessary in very early hematopoiesis for development of HSCs, and myeloid lineage cells as knockouts are embryonic lethal (**Figure 1.8**). However deletions of *RUNX1* in adult mice do not cause significant damage to the hematopoietic compartment but can lead to myeloproliferative disorders (Chen et al., 2009; Growney et al., 2005; Ichikawa et al., 2004; Putz et al., 2006).

As a necessary transcription factor for early hematopoiesis, RUNX1 is also responsible for the upregulation of other lineage-determining transcription factors, particularly PU.1. As with RUNX1, PU.1 is essential during development, and knockout mice are embryonic lethal. PU.1 is necessary for early HSC maintenance, but it is well

recognized as the master myeloid transcription factor. PU.1 regulates the expression of thousands of genes in macrophages, with 40,000 PU.1 binding motifs identified in macrophages, and PU.1 is understandably important for the development and maintenance of macrophages as part of a functioning immune system (Heinz et al., 2010).

PU.1 is the master myeloid transcription factor

PU box binding-1 (PU.1) is a member of the E-twenty-six (ETS) family of ancient metazoan transcription factors and is the product of the proto-oncogene *SPI1* (Degnan et al., 1993; Klemsz et al., 1990; Laudet et al., 1993; Li et al., 2020; Sharrocks, 2001). *SPI1* was first identified as an oncogene driving a murine erythroleukemia via retroviral insertion of the spleen focus forming virus (SFFV) upstream of a novel gene, and it was named *SPI1* for SFFV proviral integration 1 (Moreau-Gachelin et al., 1988). The putative DNA binding motif for PU.1 and other ETS family transcription factors is a purine-rich core DNA sequence GGAA/T (Karim et al., 1990).

Expression of PU.1 is tightly regulated within the hematopoietic compartment. High levels of PU.1 push cells towards macrophage differentiation, whereas lower levels of PU.1 push cells towards B cell development (DeKoter and Singh, 2000). Throughout hematopoiesis, PU.1 competes with other lineage-defining transcription factors. For instance, PU.1 competes with GATA1 binding, and when PU.1 wins, the cells are driven away from the erythroid lineage and further down the myeloid lineage (**Figure 1.8**) (Iwasaki et al., 2003; Rekhtman et al., 2003; Stopka et al., 2005; Zhang et al., 2000). Conversely, suppression of PU.1 by other transcription factors such as GFI1, prevents

differentiation into macrophages and drives the cells towards the B cell lineage (Dahl et al., 2007; Spooner et al., 2009).

Because high levels of PU.1 are synonymous with macrophage development and maintenance, it is no surprise that PU.1 is also an important transcription factor regulating macrophage function. Macrophages are sentinels of tissue, with the ability to fight off pathogens, mediate the immune response to infection, and maintain local tissue health and homeostasis. And macrophage function is largely regulated by PU.1 activity. PU.1 activates chemokines, cytokines, and cytokine receptors including factors such as macrophage colony stimulating factor (M-CSF), granulocyte-macrophage stimulating factor (GM-CSF), and the adhesion molecule CD11b; these are themselves lineage-determining factors (Hohaus et al., 1995; Smith et al., 1996; Zhang et al., 1996).

PU.1 coordinates with other transcription factors to regulate macrophage function

PU.1 regulates macrophage differentiation and function by binding to PU.1 motifs in the promoters of several genes either alone or through interactions with other transcription factors. For example, PU.1 co-regulates differentiation with C/EBPα and C/EBPβ and c-Jun to transactivate M-CSF and GM-CSF among others (**Figure 1.8**) (Behre et al., 1999). PU.1 coordinates expression of several genes, driving monocyte-to-osteoclast differentiation together with the Ten-Eleven Translocation 2 (TET2) protein (de la Rica et al., 2013). TET2 promotes DNA demethylation by converting 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5 hMC) (Ito et al., 2010). Mutations in TET2 are found in cases of myelodysplastic syndrome, indicating the importance of TET2 in maintaining normal myeloid function (Delhommeau et al., 2009; Feng et al., 2019). TET2 also

demethylates the promoter of IFITM3 in response to HIV infection of macrophages (Wang and Su, 2019).

PU.1 and Interferons

PU.1 regulates several genes involved in inflammation and the immune response, including several interleukins and tumor necrosis factor (TNF), but it is also responsible for the expression of several pattern recognition receptors (PRRs) and their co-factors, which play a pivotal role in sensing parts of pathogens known as PAMPs. In this case, PU.1 regulates the expression of several key cell surface molecules that sense foreign material and signal internally to alert the macrophage of the infection. Several of these molecules are pivotal in the macrophage innate immune response to HIV, including CD14, mannose receptor, and Toll-like Receptor 4 (TLR4) (Del Cornò et al., 2016; Kurt-Jones et al., 2000; Vigerust et al., 2005; Zanoni et al., 2011).

TLRs are transmembrane PRRs that form either homo- or hetero-dimers and sense PAMPs, triggering a signaling cascade, eventually leading to the upregulation of inflammatory response genes (Kumar et al., 2009). Various TLRs sense different PAMPs. TLR4 forms a homodimer anchored in the cell membrane and senses a canonical PAMP of Gram-negative bacterial lipopolysaccharide (LPS). TLR4 also recognizes viral proteins from dengue and respiratory syncytial virus (Kurt-Jones et al., 2000; Modhiran et al., 2015). The dimerization of TLR4 is mediated by the myeloid differentiation factor-2 (MD-2), and MD-2 binds LPS within the TLR4-MD2 complex (Nagai et al., 2002; Ohto et al., 2007). Both TLR4 and its requisite co-factor, MD-2 encoded by the gene *LY96*, are regulated by PU.1 in macrophages (Rehli et al., 2000; Roger et al., 2005; Tissières et al.,

2009). Interestingly, the monocyte-macrophage cell surface marker and PRR CD14 also binds LPS, triggering the co-internalization of CD14-TLR4 and an interferon response similarly to TLR4-MD2 (Wright et al., 1990; Zanoni et al., 2011).

Once TLR4-MD2 recognizes a PAMP, two intracellular signaling cascades proceed and are mediated through several important transcription factors including AP-1, NF-κB, IRF3 (interferon regulatory factor 3), and PU.1, upregulating proinflammatory cytokines and type I interferons (IFN-I) in the nucleus [reviewed in (Ghisletti et al., 2010; Molteni et al., 2016)]. IRF3 in particular induces expression of IFN-I, and may be translocated to the nucleus as part of the DNA damage response (Kim et al., 1999; Weaver et al., 2001). Whether recruitment of IRF3 to the nucleus contributes to the Vpr-mediated cell-cycle arrest is unknown.

One of the first proteins demonstrated to bind cooperatively with PU.1 was the interferon regulatory factor 4 (IRF4). Together, they upregulate immune response gene transcription and drive immunoglobulin expression in B cells (Pongubala et al., 1992, 1993). As with IRF4, IRF8 is a key transcription factor for myeloid development, can coordinate with PU.1 to induce gene upregulation, and can strongly upregulate Type I IFN genes (Honda and Taniguchi, 2006). The importance of IRF regulation of the immune response is evidenced by several viruses with the ability to counteract IRF protein function [reviewed in (Yanai et al., 2012)].

PU.1 and interferon stimulated genes

The IFN-I family of genes encode cytokines, primarily IFNα and IFNβ. Because these cytokines are secreted, they can induce an antiviral state both on the cells that

secrete them and nearby uninfected bystander cells [reviewed in (McNab et al., 2015)]. IFNs induce the expression of several interferon stimulated genes (ISG) and pathways that disrupt the HIV life cycle at several stages through both intrinsic and innate immune responses (Yan and Chen, 2012). IFNs also contribute to the adaptive immune response by recruiting T and B cells to the site of infection where antigen-presenting cells such as infected macrophages can initiate antigen memory. IFNα/β bind the interferon receptors (IFNAR) on the outer membrane of cells, activating the Janus kinase 1 (JAK1). This in turn leads to the phosphorylation of the cytosolic transcription factors signal and activator of transcription 1 and 2 (STAT1 and STAT2), which translocate to the nucleus, upregulating ISGs and gamma activated sequence (GAS)-containing genes and upregulating antiviral factors [reviewed in (Ivashkiv and Donlin, 2013)]. One of these ISGs is ISG15. The ubiquitin-like antiviral protein ISG15 is induced by IFN-I and LPS and has broad antiviral activity (Perng and Lenschow, 2018). ISG15 interferes with HIV Gag multimerization at the plasma membrane, inhibiting viral release (Okumura et al., 2006). Because of the strong antiviral activity of interferons and ISGs in limiting viral replication and spread, the interferon response is targeted by many viruses, including HIV, whose accessory proteins counteract interferon response mechanisms.

PU.1 and IFITMs

PU.1 alone and together with IRFs regulates the immune response to infection and induces the upregulation of another class of ISGs called IFITMs. IFITM proteins contribute to broad antiviral immunity. They restrict HIV-1, influenza A, West Nile virus, and dengue, among other viruses (Brass et al., 2009; Lu et al., 2011). IFITM proteins colocalize with

Gag and Env proteins in HIV-infected cells and can be incorporated into nascent HIV virions, limiting cell-free entry into new target cells; however they do not impede cell-to-cell transmission, consistent with this mode of transmission being most efficient due to protection from the host's antiviral mechanisms (Compton et al., 2014). IFITM3 in particular is a potent HIV restriction factor that targets HIV Env in macrophages for degradation, limiting spread, and recruiting other viral proteins to the lysosome for degradation (Spence et al., 2019). HIV Vpr counteracts IFITM3 in macrophages, limiting the impact IFITM3 has on viral spread (Wang and Su, 2019).

PU.1 and mannose receptor

Unlike the ISGs upregulated by PU.1 and IRFs in response to HIV infection, PU.1 also maintains several innate immune, antiviral molecules in macrophages. One of these is mannose receptor, a member of the C-type lectin family (CLEC) with eight C-type lectin domains, a transmembrane domain, and short cytoplasmic tail. Originally named the macrophage mannose receptor (MMR) because it is highly expressed in macrophages, it has since been identified at lower levels on other cell types such as dendritic cells and was therefore renamed mannose receptor (MR). MR localizes to the cell membrane and early endosomes, and traffics through the Golgi (Pontow et al., 1996; Schweizer et al., 2000). As a PRR, MR binds mannose, fructose, and *N*-acetylglucosamine residues that are typically found on pathogens, eventually mediating their presentation from the antigen-presenting cells to T cells (Largent et al., 1984; Taylor et al., 2005). There may be 100,000 copies of MR on the surface of each macrophage, and several times that number in internal compartments (Stahl et al., 1980). MR is extremely stable, with a half-

life of 33h and can be recycled many times to the cell surface to bind ligands, making MR an extremely abundant and consistent PRR for macrophages (Lennartz et al., 1989; Stahl et al., 1980).

As discussed earlier, HIV finds target cells through binding of the HIV Env glycoprotein gp120 to the necessary receptor, CD4, and one of two co-receptors, CCR5 and CXCR4 (Berger et al., 1999). Although the HIV receptors are well described, HIV entry is also helped by cell surface adhesion molecules to secure and stabilize HIV as it finds and binds the correct receptors (Guo and Hildreth, 1995; Hioe et al., 1998; Lifson et al., 1986; Montefiori et al., 1988; Orentas and Hildreth, 1993). The HIV Env has a high density of mannose residues with terminally mannosylated sugars accounting for nearly half of the glycosylation sites on gp120 (Mizuochi et al., 1988; Zhu et al., 2000). Studies investigating the role of MR in HIV infection have shown that blocking MR reduces the tethering of virus particles to monocyte derived macrophages (MDMs) in addition to reducing the amount of viral particles MDMs can pass to T cells, strongly suggesting MR is an important HIV restriction factor (Nguyen and Hildreth, 2003).

MR is present at high levels at the cell surface and may help stabilize HIV particles as they enter macrophages. Paradoxically, MR is also a restriction factor, because it can bind nascent virus particles exiting the infected macrophage. The ability to use MR on entry but remove MR before exit would greatly benefit HIV. Indeed others have suggested HIV Tat reduces MR expression in macrophages by interfering with PU.1 binding motifs in the MR promoter (Caldwell et al., 2000). Several PU.1 binding motifs have been identified in the MR promoter in both mice and rats, and PU.1 is essential for MR transcription (Egan et al., 1999; Eichbaum et al., 1997). Regulation of MR by PU.1 further

supports the critical role of PU.1 in myeloid differentiation and function. The myeloid-specific phenotype of MR is also supported by the transcriptional requirement of a myeloid specific transcription factor. Vpr and Nef proteins target MR, supporting viral replication and spread (Lubow et al., 2019), as described in Chapter 2.

Bioinformatics and single cell sequencing to understand HIV biology

Each cell within an organism has the same DNA sequence, or genotype, yet the phenotype of each cell can fundamentally differ from any other across time and space. For example, consider the morphology and function of a neuron compared to a red blood cell to understand the variations that are possible between cells within a single organism.

Although the genotype is the same across cells, the spatial-temporal expression of those genes drives phenotypic differences. Regulating the expression of the approximately 18,000 genes in the human genome varies widely as cells differentiate, maintain homeostasis, and respond to external stimuli. Therefore, different cells will express different portions of the genome from each other. The central dogma of molecular biology states that DNA is transcribed into RNA and then translated into protein – retroviruses are the exception, as was discussed above. Because RNA is transcribed from DNA, it is possible to infer gene expression through quantification of RNA transcripts. This is accomplished by sequencing cDNAs converted from the mRNA transcripts, i.e., transcriptomic measurements. Furthermore, quantifying protein abundance is much more challenging than quantifying RNA, therefore, RNA is used for a quantifiable intermediate for determining the phenotypic state that is defined by protein expression.

The genome is three billion bases long and yet can fit into the nucleus of a single cell because it is tightly wound around histones. Chemical modifications to histones determine how tightly wound the DNA is in that region of the genome. The more loosely organized the histones, the more accessible the DNA becomes, allowing access to gene expression factors. Therefore, chromatin accessibility can also be a proxy for regions of the genome that are actively being expressed, were recently expressed, or potentially could be soon. Transcription proceeds through a multistep process involving the recruitment of transcription factors, the opening of the chromatin, recruitment of polymerases and other factors. The result is that genomic regions necessary for expression become accessible.

Single cell transcriptomic and epigenetic measurements

Traditionally, transcriptomic and epigenetic measurements are performed 'in bulk,' meaning the measurements are averages across all the cells sampled. While such measurements can be sufficient, they also mask heterogeneity, obscuring the link between genotype and phenotype (**Figure 1.9**). Complex tissues such as the brain or even blood are a mixture of highly diverse cells with distinct phenotypes. Using these cells for bulk analysis prevents the determination of specific genetic and phenotypic changes within cellular subsets. To overcome this challenge, single-cell transcriptomic (scRNA-seq) and epigenomic (scATAC-seq) techniques were developed, allowing for the assessment of the transcriptomic or epigenetic, and even both (multiomic) profile of individual cells within a heterogeneous population (**Figure 1.9**) [basics of single cell data analysis reviewed in (Lee et al., 2019)]. Single-cell resolution is particularly useful in

measuring rapidly changing cell populations, such as the hematopoietic compartment, where HSCs differentiate into daughter cells in response to various stimuli, or more differentiated cells activate in response to invading pathogens such as HIV.

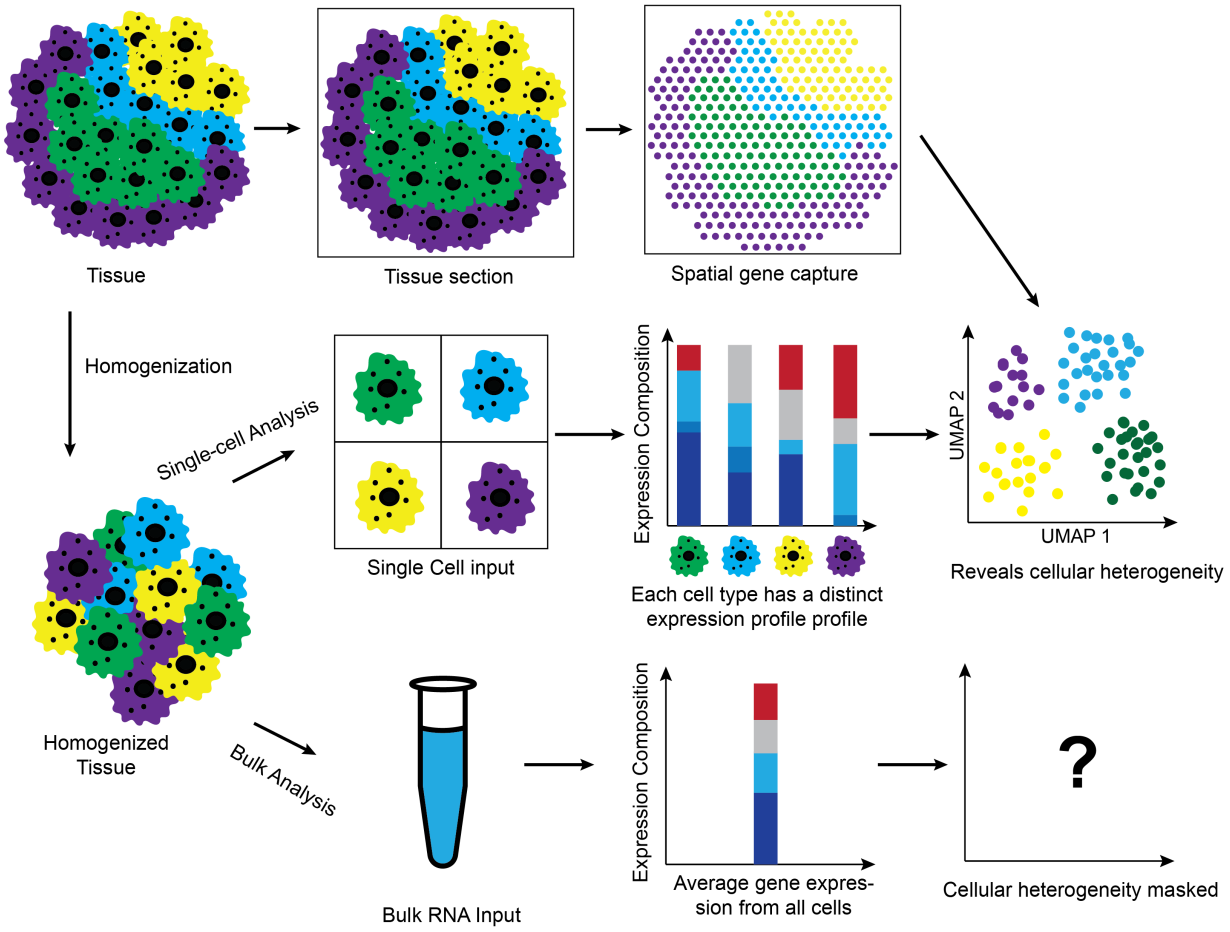

Figure 1.9. Single-cell and spatial-transcriptomic approaches provide higher resolution over bulk RNA-sequencing approaches.[10] Spatial gene capture from sectioned tissue provides spatial-transcriptomic level data in 2D (top). Single-cell analysis relies on homogenized tissues to provide single-cell level information to distinguish individual cell types. Traditional bulk approaches such as RNA-sequencing provide average gene expression level information across all cells originating from homogenized tissue.

The most common method currently in use to capture single cell measurements is droplet-based microfluidics approaches. The microfluidic devices use a controlled fluid

[10] This figure was created by Maria C. Virgilio

flow to guide single-cell suspension cells through channels in a microchip through a stream of oil droplets such that the number oil droplets exceeds the number of cells in the stream (see step 1 in **Figure 1.10**). These oil droplets contain beads coated with synthetic oligonucleotides (oligos) containing barcoding information. Typically, the oligos coating each bead have one barcode that is shared by all the oligos and unique to that single bead (see step 2 in Fig. 1.10). The second barcode is unique to each oligo. This is called a UMI or unique molecular identifier because it uniquely barcodes each individual RNA molecule for scRNA-seq from each individual cell. On one side of the barcoded oligo is a universal sequence for downstream processing and sequencing and on the other side, a polydT sequence that is used to capture the polyA tails of mRNA molecules (see step 2 in Fig. 1.10). Once RNA molecules have annealed to the polydT oligo, the RNA is reverse transcribed within the oil droplet because the necessary components including reverse transcriptase are also included in the oil droplet. The number of RNA molecules from an individual cell is small and capture rates are imperfect, so the combined cDNA molecules from all the individual cells which are now properly barcoded are usually lightly PCR-amplified and processed before moving to massively parallel sequencing.

Similarly, single cell or single nucleus ATAC sequencing is performed like scRNA-seq with a few modifications. ATAC sequencing relies on Tn5 endonucleases to fragment accessible regions of genomic DNA, and in the process, attach barcodes. Cells for scRNA-seq are generally processed as whole cells such that both cytoplasmic and nuclear RNA is tagged. ATAC barcoding reagents must be able to access the genomic DNA (gDNA) in the nucleus that is inaccessible in whole cells. Therefore, cells are gently lysed to remove the plasma membrane without significantly disturbing the inner nuclear

membrane. Nuclei are collected and mixed with tagged-Tn5 endonucleases, then passed through the fluidics microchip for processing with barcoded beads that capture tagged genomic fragments from individual nuclei. Libraries are processed similarly as with scRNA-seq. For multiome processing, cells are treated similarly to scATAC-seq, but the beads used in the microchip contain oligos to capture both RNA and DNA molecules.

High throughput single cell measurements are generally performed to capture RNA (10X Genomics, DROP-seq) and gDNA; however there are single-cell sequencing approaches to capture genomic, epigenetic and proteomic profiles. These techniques can provide data on single genome sequences, chromatin accessibility (scATAC-seq), DNA methylation (snmC-seq), cell surface proteins (CITE-seq, FACS), internal proteins (PEA), small RNAs, histone modifications (scChIP-seq), chromosome conformations (scHi-C-seq), single-gene perturbations (Perturb-seq), spatial position (MERFISH), and cell trajectory (MultiVelo, Monocle, Velocyto) (**Figure 1.9**) [single-cell techniques reviewed in (Stuart and Satija, 2019)].

Analysis of single-cell data

Once the data are collected, they must be properly processed and interpreted. This involves demultiplexing, gene expression quantification, data filtering, data normalization and applying statistical techniques to identify biological differences between cells (see steps 3 and 4, **Figure 1.10**). Demultiplexing involves taking matching the thousands of transcripts from the library to the cell source. This is done by identifying the two barcodes for each cDNA sequenced – the UMI and the cell barcode (**Figure 1.10**). Part of this

process involves allowing for minor sequencing errors within the barcodes to form a single consensus sequence for each cell.

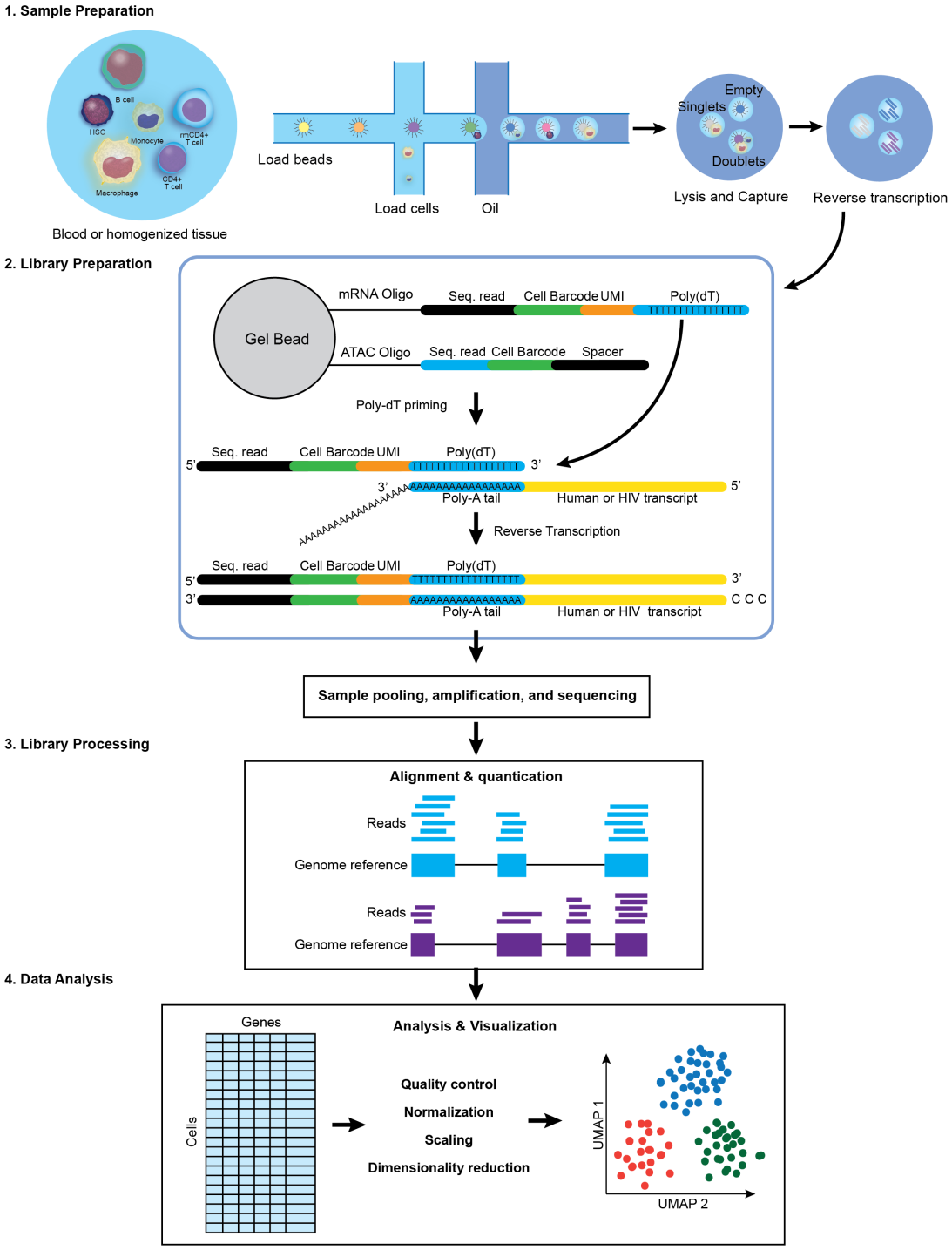

Figure 1.10. Overview of single-cell transcriptomic data generation and analysis.[11] Graphical depiction of the major steps involved in single-cell RNA or ATAC sequencing. (1) single-cell suspensions of cells are prepared and loaded into the fluidics chip for barcoding and amplification. Individual cell libraries are pooled for highly parallel sequencing. All reads must be aligned to a reference genome. Expression quantification, quality control, and normalization is used to process the data before visualization and analysis.

Demultiplexed reads are also used to estimate the gene expression level of each gene in each cell. Gene expression estimates require alignment of barcoded sequences to a reference genome. Once the genomic location is determined, the total number of transcripts for each gene from each cell can be counted and organized in a matrix of integer counts for genes vs. cells.

Data filtering is then used to remove several artifacts. For example, doublets are often generated with single cell data, which means that two cells or two nuclei accidentally were processed and barcoded with the same bead. Such doublets can often be filtered out because they have a much higher number of transcripts compared to other cells, or they have transcripts that map to two very different cell types. Conversely, low quality cell samples that result from either dead/dying cells or ambient transcripts, which are free RNAs released from dead cells, that were barcoded, often contain degraded RNA, resulting in a low fraction of mapped reads during alignment. Low quality cells might also contain a higher percentage of ribosomal or mitochondrial RNA, which are more resistant to degradation.

Once the sequences have been preliminarily trimmed, the data must then be normalized. The inherent nature of single-cell molecule-capture ensures there will be

[11] This figure was made by Maria C. Virgilio and inspired by figures that appeared in:
Lee, H., Yu, H., Welch, J., 2019. A beginner's guide to single-cell transcriptomics. Biochemist 41, 34–38. https://doi.org/10.1042/bio04105034

technical variation in terms of the total number of transcripts captured per cell, which if left unadjusted, would overwhelm real biological variation. Therefore, the gene expression matrix must be normalized to account for this. This involves log-transforming the expression matrix to down weight extreme values, followed by scaling to ensure genes expressed or captured at different levels are comparable. The normalized data must then undergo statistical analysis and dimensionality reduction to highlight important biological differences among the cells.

Dimensionality reduction involves transforming the high dimensional data matrix into low-dimensional space, where different sources of variation can be represented across different axes. The result is a reduction of noise in the data, and it highlights interpretable features. Some commonly used dimensionality reduction techniques include principal component analysis (PCA), independent component analysis (ICA), non-negative matrix factorization (NMF), and deep neural networks. Each of these techniques has advantages and disadvantages, and they function as data visualization aids, compressing multidimensional data into interpretable 2D or 3D spaces.

The most common visualization of multidimensional data are t-SNE (t-distributed stochastic neighbor embedding) and UMAP (uniform manifold approximation and projection). Within these plots, data from individual cells are presented as a compressed summary of all the information presented within that cell and projected across space, such that cells with similar gene expression patterns are grouped into ‘clusters’ together that are then distinct from cells exhibiting patterns in a different cluster (**Figures 1.9** and **1.10**). Once clusters are determined, statistical tests are then applied to find the defining

features of each cluster. These features can be used as biomarkers and can help to identify biological properties of the cells such as functionality and regulatory mechanisms.

LIGER

Integrative computational tools are needed to combine individual single-cell datasets into a unified shared analysis. Integrating multiple samples can be challenging, given the immense heterogeneity across individual datasets that can vary due to factors such as the number of cell samples, the depth of sequencing, donor variability, and the type of data collected (i.e., RNA vs DNA). While reducing the noise in the data arising from differences between samples is important, it is also critical not to eliminate any real biological differences of interest. There are several high quality computational methods to integrate single cell samples, including LIGER (linked inference of genomic experimental relationships) (Welch et al., 2019) and Seurat (Hao et al., 2021).

LIGER accepts multiple single-cell datasets as input which may come from different experimental parameters such as time points, species, or molecular measurements. LIGER uses integrative NMF (iNMF) to create a low-dimensional space, where each cell is defined by a set of dataset-specific factors that correspond to a biologically interpretable signal (i.e., lineage-defining factors). Representation of the data is a combination of a set of batch-specific factors and a set of shared factors. Clustering is achieved by searching for shared factors, where cells with similar profiles are grouped, then normalized to match a chosen reference dataset to achieve batch correction. Overall, LIGER is able to integrate different data modalities well while highlighting the biological differences between and within samples.

Understanding HIV biology through single-cell sequencing approaches

Single-cell experiments have been used broadly to address biological questions. The resolution provided by single-cell information has allowed unparalleled resolution and specificity of biological sample analysis. Single-cell sequencing techniques have recently been used to better understand HIV infection. Single-cell resolution is particularly helpful in HIV experimentation because HIV is capable of infecting many cell types within the hematopoietic compartment, changing the biology of not only the bona fide infected cells, but also bystander cells, and HIV behaves very differently even in very similar cells. Perhaps part of the variation in HIV behavior and transcriptional activation is due to the semi-random nature of lentiviral integration, but integration site variability is unlikely to account for all the observations associated with the broad pattern of HIV behavior. It is also important to remember single-cell experiments capture a single moment in time, but cells are dynamics spaces. Newer techniques have been developed to predict cell fate and the dynamics of transcription and RNA but will not be discussed here. Nevertheless, it is hard to understate how transformative single-cell approaches have been to many fields, including the study of HIV.

Summary of Dissertation

Several previous studies from our laboratory have generated significant evidence for an unknown macrophage-specific restriction factor targeting HIV Env and counteracted by Vpr. In Chapter 2, I present evidence that mannose receptor (MR) is a macrophage-specific restriction factor targeting HIV Env for lysosomal degradation. Several experiments demonstrate that in the absence of Vpr, MR binds HIV Env and mediates Env degradation, limiting viral spread. Previous findings that HIV Nef dysregulates MR trafficking are confirmed. Vpr acts to remove MR from infected macrophages by reducing the expression of the mannose receptor gene, *MRC1,* in a process that does not involve direct contact between Vpr and MR. Together, Nef and Vpr target MR through distinct mechanisms to rescue Env expression, virion release, and spread from macrophages to T cells. In Chapter 3, single-cell RNA sequencing and computational approaches were used to demonstrate a Vpr-mediated shift in the transcriptional landscape of HIV-infected macrophages. PU.1-regulated genes are suppressed by Vpr in infected macrophages, including several genes encoding factors that would otherwise target Env for degradation, including MR and IFITM3 – solving the mystery from Chapter 2. Vpr also limits the TLR and IFN-I immune response in both infected and bystander macrophages. The ability of Vpr to target PU.1 is evolutionarily conserved and accomplished by mediating the proteasomal degradation of PU.1, ultimately enhancing spread from macrophages. Finally, Chapter 4 provides an in-depth discussion of these results, their relevance, and suggests future experiments that will continue to illuminate the molecular mechanisms of innate antiviral immunity and the role Vpr plays in HIV infection.

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CHAPTER 2

Mannose Receptor Is an HIV Restriction Factor Counteracted by Vpr in Macrophages[1]

Abstract

Earlier publications provide significant evidence of the existence of a macrophage-specific restriction factors that targets Env and, by extension, the HIV replication cycle. This factor degrades Env, degrades Env-associated virions, restricts virion release, and inhibits formation of virological synapses between infected macrophages and uninfected $CD4^+$ T cells. Crucially, this factor is absent in $CD4^+$ T cells and is counteracted in macrophages by Vpr, which rescues the previously mentioned restrictions. Here, we report that the macrophage mannose receptor (MR), is also a restriction factor targeting Env, in primary human monocyte-derived macrophages. Vpr acts synergistically with HIV Nef to target distinct stages of the MR biosynthetic pathway and dramatically reduce MR expression. Silencing MR or deleting mannose residues on Env rescues Env expression in HIV-1-infected macrophages lacking Vpr. However, we also show that disrupting interactions between Env and MR reduces initial infection of macrophages by cell-free virus. Together these results reveal a Vpr-Nef-Env axis that hijacks a host mannose-MR

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response system to facilitate infection while evading MR's normal role, which is to trap and destroy mannose-expressing pathogens.

Introduction

Vpr is a highly conserved HIV accessory protein that is necessary for optimal replication in macrophages (Balliet et al., 1994) but its mechanism of action is poorly understood. Studies using human lymphoid tissue (HLT), which are rich in both T cells and macrophages, have found that loss of Vpr decreases virus production (Rucker et al., 2004) but only when the virus strain used is capable of efficiently infecting macrophages (Eckstein et al., 2001). These studies provide evidence that Vpr enhances infection of macrophages and increases viral burden in tissues where macrophages reside. Because Vpr is packaged into the virion (Cohen et al., 1990) and localizes to the nucleus (Lu et al., 1993), it may enhance early viral replication events. However, in mononuclear phagocytes *vpr*-null virus in which Vpr protein is provided by trans-complementation in the producer cells replicates poorly compared to wild-type virus (Connor et al., 1995), indicating that Vpr's role in the HIV replication cycle continues into late stages.

Previous work by our group demonstrated that Vpr counteracts an unidentified macrophage-specific restriction factor that targets Env and Env-containing virions for lysosomal degradation (Mashiba et al., 2014). This restriction could be conferred to permissive 293T cells by fusing them with MDM to create 293T-MDM heterokaryons. A follow up study demonstrated that by increasing steady state levels of Env, Vpr increases formation of virological synapses between infected MDM and autologous uninfected T cells, enhancing HIV infection of T cells (Chapter 2). This enhances spread to T cells and

dramatically increases levels of Gag p24 in the culture supernatant. This finding helps explain the paradoxical observations that Vpr is required for maximal infection of T cells *in vivo* (Hoch et al., 1995) but numerous studies have shown Vpr only marginally impacts infection of pure T cell cultures in vitro [e.g. (Mashiba et al., 2014)].

Our goal in the current study was to identify and characterize the myeloid restriction factor targeting Env that is counteracted by Vpr. We reasoned that macrophage-specific Env-binding proteins, including the carbohydrate binding protein mannose receptor (MR), were candidates. MR is expressed on several types of macrophages *in vivo* (Liang et al., 1996; Linehan et al., 1999) and is known to mediate innate immunity against various pathogens (Macedo-Ramos et al., 2014; Subramanian et al., 2019). MR recognizes mannose rich structures including high-mannose glycans, which are incorporated in many proteins during synthesis. In eukaryotic cells most high-mannose glycans are cleaved by α-mannosidases and replaced with complex-type glycans as they transit through the secretory pathway. By contrast, in prokaryotic cells, high-mannose residues remain intact, making them a useful target of pattern recognition receptors including MR. Some viral proteins, including HIV-1 Env, evade mannose trimming (Coss et al., 2016) and retain enough high-mannose to bind MR (Lai et al., 2009; Trujillo et al., 2007). There is evidence that HIV-1 proteins Nef and Tat decrease expression of MR based on studies performed in monocyte derived macrophages (MDM) and the monocytic U937 cell line, respectively (Caldwell et al., 2000; Vigerust et al., 2005). Nef dysregulates MR trafficking using an SDXXLΦ motif in MR's cytoplasmic tail (Vigerust et al., 2005), which is similar to the sequence in CD4's tail that Nef uses to remove it from the cell surface (Bresnahan et al., 1998; Cluet et al., 2005; Greenberg et al., 1998).

Whether MR or its modulation by viral proteins alters the course of viral replication has not been established.

Here we confirm that Nef reduces MR expression in primary human MDM, although in our system, the effect of Nef alone was relatively small. In contrast, we report that co-expression of Vpr and Nef dramatically reduced MR expression. In the absence of both Vpr and Nef, MR levels normalized indicating that Tat did not play a significant, independent role in MR downmodulation. Deleting mannose residues on Env or silencing MR alleviated mannose-dependent interactions between MR and Env and reduced the requirement for Vpr. Although the post-infection interactions between MR and Env reduced Env levels and inhibited viral release, we provide evidence that these same interactions were beneficial for initial infection of MDM. Together these results reveal that mannose residues on Env and the accessory proteins Nef and Vpr are needed for HIV to utilize and then disable an important component of the myeloid innate response against pathogens intended to thwart infection.

Results

Identification of a restriction factor counteracted by Vpr in primary human monocyte-derived macrophages.

Because we had previously determined that Vpr functions in macrophages to counteract a macrophage specific restriction factor that targets Env, we reasoned that Env-binding proteins selectively expressed by macrophages were potential candidate restriction factors. To determine whether any factors fitting this description were targeted by Vpr, we cultured macrophages under conditions that achieve a saturating infection by

both wild-type and Vpr-null mutant viruses (**Figures 2.1A and B**). We found that mannose receptor (MR), which is highly expressed on macrophages and has been previously shown to bind Env (Fanibunda et al., 2008; Lai et al., 2009; Trujillo et al., 2007), was significantly decreased by wild-type HIV 89.6 but not by 89.6 *vpr*-null (**Figures 2.1C and D**, $p<0.01$). In contrast, we observed no significant effect of Vpr on the expression of GAPDH. We also observed that stimulator of interferon genes (STING) was unaffected by Vpr (**Figure 2.2**). Relative expression of known restriction factors GBP5 and IFITM3 varied in infected MDM from multiple donors (**Figure 2.2**), but unlike MR they were not consistently reduced in the wild-type condition, indicating they are not targeted by Vpr.

To confirm the effect of Vpr on Env during HIV infection of primary human macrophages in which MR was downmodulated, we performed quantitative western blot analysis. As shown in **Figures 2.1E and F**, we confirmed that amounts of Vpr sufficient for MR downmodulation were also sufficient for stabilizing expression of Env (gp160, gp120, gp41). Compiled data from nine donors clearly demonstrated results that were similar to our prior publication (Mashiba et al., 2014); under conditions of matched infection in which there was no significant difference in HIV Gag pr55 levels between wild-type and *vpr*-null infections, all three forms of Env were significantly more abundant in the wild-type infection (gp160: 4-fold, $p<0.002$; gp120: 6-fold, $p<0.002$; gp41: 3-fold, $p<0.001$).

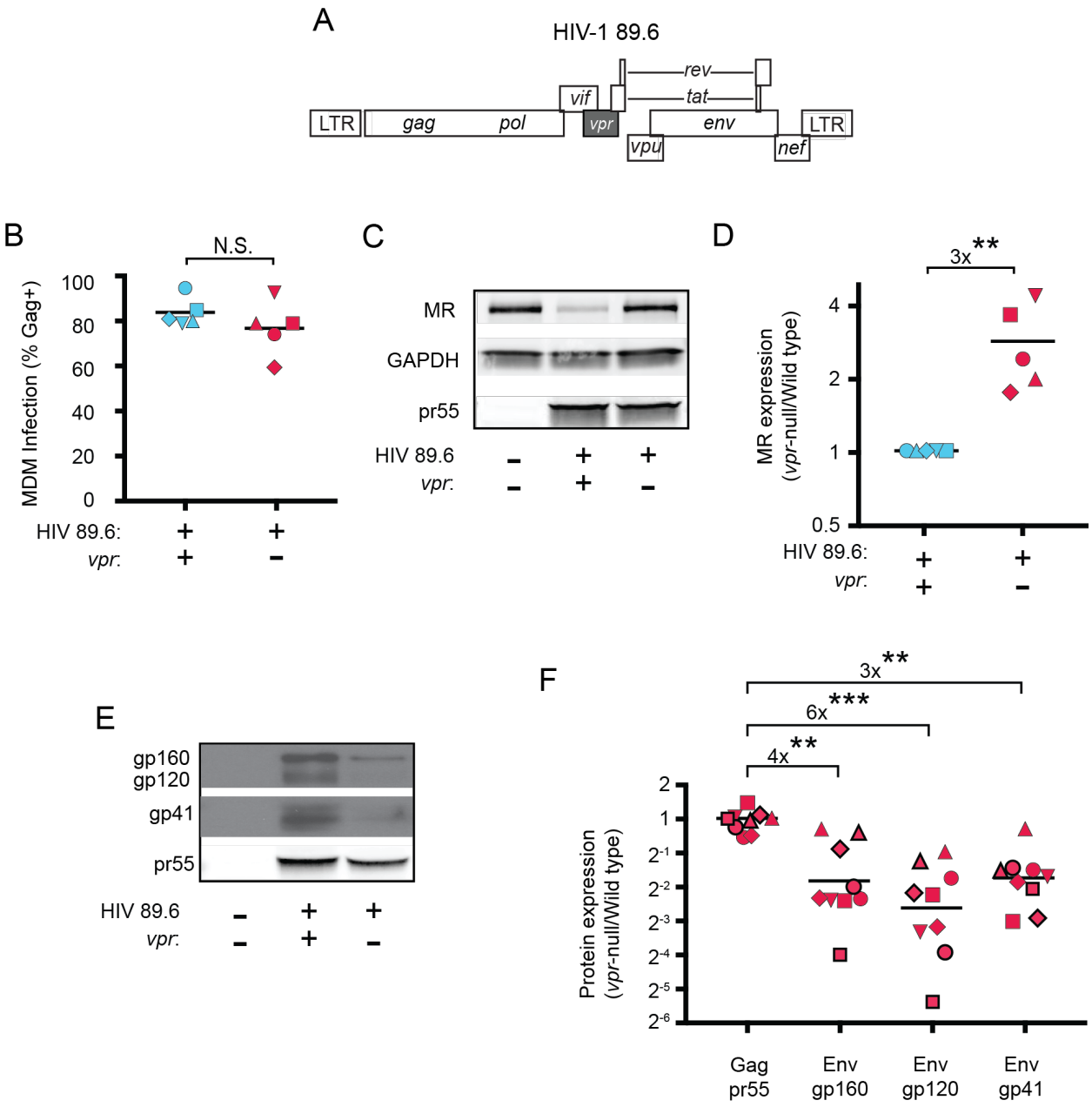


Figure 2.1: HIV Vpr reduces steady state levels of host mannose receptor in MDM and increases steady state levels of viral Env protein[2]**. (A)** Diagram of the HIV 89.6 proviral genome. The shaded box shows the location of *vpr,* which was disrupted by a frame shift mutation to create the Vpr-null version (Mashiba et al., 2014). HIV-1 89.6 is a dual CXCR4/CCR5-tropic HIV molecular clone isolated from the peripheral blood of an AIDS patient (Collman et al., 1992). **(B)** Summary graph depicting MDM infected by HIV 89.6 wild-type and *vpr*-null with matched infection frequencies of at least 50% 10 days post infection as measured flow cytometrically by intracellular Gag p24 staining. This subset with high frequencies of infection was selected to examine potential effects on host factors. **(C)** Western blot analysis of whole cell lysates from

[2] The data in this figure was generated by Jay Lubow.

MDM prepared as in B. **(D)** Summary graph displaying relative expression of MR in wild-type and mutant 89.6 from blots as shown in C. Western blot protein bands were quantified using a Typhoon scanner. Values for MR expression in MDM infected with Vpr-null HIV were normalized to GAPDH and then to wild-type for each donor. Statistical significance was determined using a two-tailed, ratio *t*-test. ** p=0.005 **(E)** Western blot analysis of HIV protein expression in MDM infected as in B. **(F)** Summary graph of HIV protein expression from western blot analysis as in E and quantified as described in methods. The ratio of expression in wild-type to *vpr*-null infection is shown. Data from 9 independent donors with similar frequencies of infection (within 2-fold) following ten days of infection are shown. Statistical significance was determined using a two-tailed, ratio *t*-test, N.S. – not significant, p=0.31, ** p<0.01, *** p<0.001. Data from each donor is represented by the same symbol in all charts. Mean values are indicated.

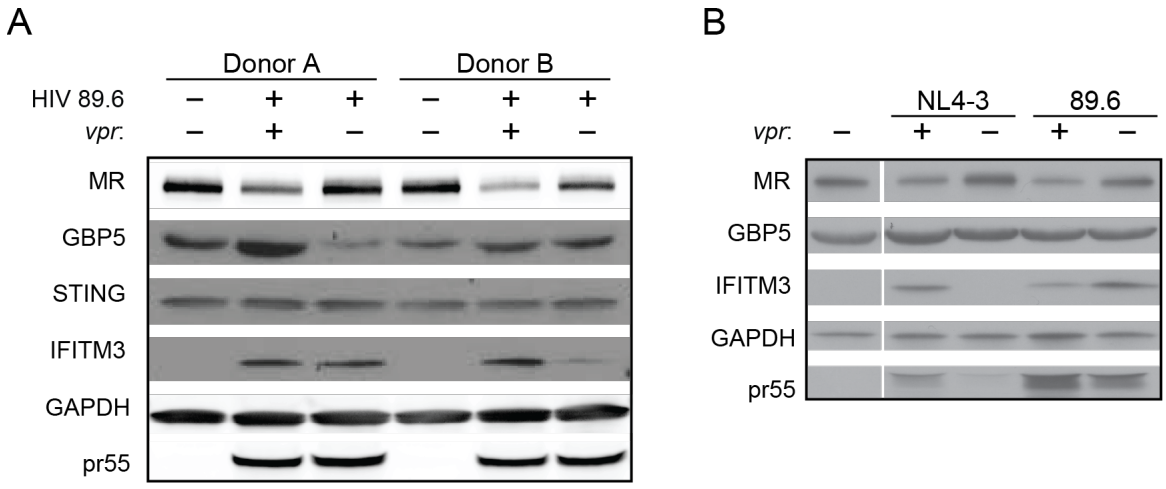


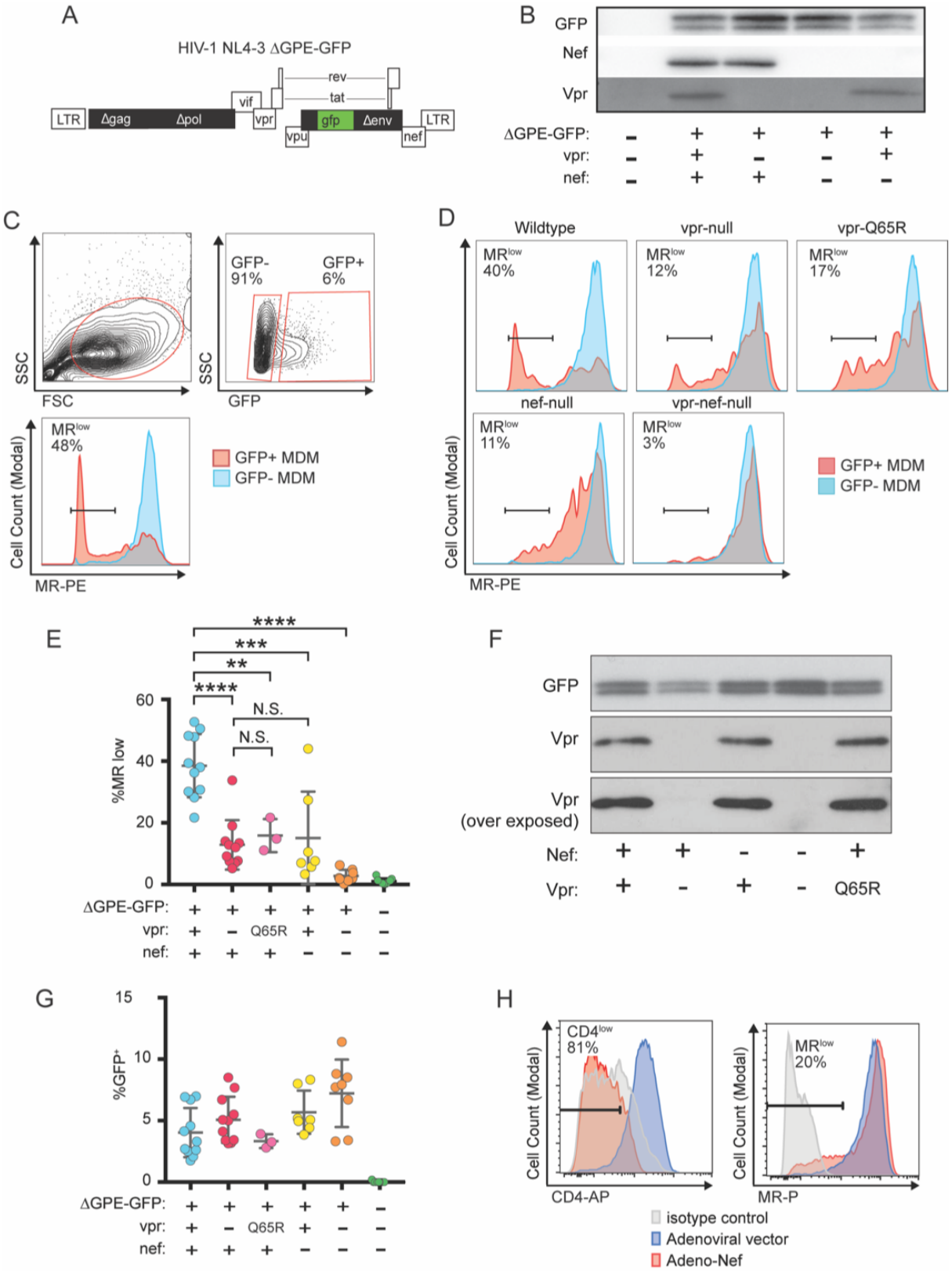
Figure 2.2: HIV Vpr reduces steady state levels of MR but not GBP5, STING or IFITM3[3]. (A) Western blot analysis of whole cell lysates from MDM infected with wild-type or *vpr*-null HIV-1 89.6 for 10 days. **(B)** Western blot analysis of whole cell lysates from MDM infected with wild-type or *vpr*-null HIV-1 89.6 and YU2-pseudotyped NL4-3 for 10 days. *n*=3 independent donors.

Vpr and Nef counteract MR expression in infected macrophages via independent and additive mechanisms.

Because an earlier report indicated that Nef decreases surface expression of MR (Vigerust et al., 2005), we asked whether Nef was playing a role in MR downmodulation in our systems. Because HIVs lacking Vpr and Nef spread too inefficiently in MDM to observe effects on host proteins by western blot analysis, we utilized a replication

[3] The data in this figure was produced by Jay Lubow and Zana Lukic.

defective HIV with a GFP marker (NL4-3 ΔGPE-GFP, **Figure 2.3A**) to allow measurement of MR expression via flow cytometry following single-round transduction. This construct has the additional advantage that it eliminates potentially confounding effects of differences between wild-type and mutant HIV viral spread. We generated truncation mutations in *nef* and *vpr* and confirmed that these mutations only affected expression of the altered gene product in transfected 293T (**Figure 2.3B**). For these experiments, primary MDM were harvested earlier than the experiments described in **Figure 2.1** (five days versus ten days) because the viruses could not replicate and the GFP marker allowed identification of transduced cells (**Figure 2.3**). Under these conditions, we found that MR expression was dramatically reduced in a subset of GFP^+ cells when both Vpr and Nef were expressed (**Figure 2.3C-E**). Both Nef and Vpr contributed to MR downmodulation; loss of function mutation in either Vpr or Nef reduced the severity of MR downmodulation similarly, and there was no statistical difference between MR levels in macrophages expressing either Vpr or Nef alone (**Figure 2.3E**). In addition, complete elimination of downmodulation required mutation of both Vpr and Nef (**Figure 2.3C-E**). These results indicate that both Vpr and Nef are required for maximal MR downmodulation in HIV-infected macrophages and that neither alone is sufficient.


A
HIV-1 NL4-3 ΔGPE-GFP
LTR
Δgag
Δpol
vif
vpr
rev
tat
gfp
Δenv
vpu
nef
LTR
B
GFP
Nef
Vpr
ΔGPE-GFP:
vpr:
nef:
C
SSC
FSC
GFP-
91%
GFP+
6%
SSC
GFP
MRlow
48%
Cell Count (Modal)
MR-PE
GFP+ MDM
GFP- MDM
D
Wildtype
vpr-null
vpr-Q65R
MRlow
40%
MRlow
12%
MRlow
17%
nef-null
vpr-nef-null
MRlow
11%
MRlow
3%
Cell Count (Modal)
MR-PE
GFP+ MDM
GFP- MDM
E
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N.S.
N.S.
%MR low
60
40
20
0
ΔGPE-GFP:
vpr:
nef:
Q65R
F
GFP
Vpr
Vpr
(over exposed)
Nef:
Vpr:
Q65R
G
%GFP+
15
10
5
0
ΔGPE-GFP:
vpr:
nef:
Q65R
H
CD4low
81%
Cell Count (Modal)
CD4-AP
MRlow
20%
Cell Count (Modal)
MR-P
isotype control
Adenoviral vector
Adeno-Nef

Figure 2.3: Combined effects of Nef and Vpr completely remove MR from a significant proportion of infected cells at early time points[4]**. (A)** Diagram of HIV NL4-3 ΔGPE-GFP. **(B)** Western blot analysis of whole cell lysates from 293T cells transfected with the indicated viral expression construct. **(C)** Flow cytometry plots indicating the gating strategy used to identify live GFP^+ vs GFP^- cells and the fraction of cells that are MR^{low}. **(D)** Representative flow cytometric analysis of MDM at five days post transduction by the indicated virus. The percentage of GFP^+ cells that fell into the MR^{low} gate is indicated in each panel **(E)** Summary graph depicting the percentage of GFP^+ cells that fell into the MR^{low} gate in transduced MDM. For the uninfected column the results from GFP^- cells are displayed. (each dot indicates an independent donor, range 3-11). **(F)** Western blot analysis of whole cell lysates from 293T cells transfected with the indicated viral expression construct. **(G)** Summary graph depicting the frequency of transduced (GFP^+) MDM at the time of harvest. **(H)** Representative flow cytometric plots of MDM transduced with the indicated adenoviral vector (*n*=3 independent donors). Mean +/- standard deviation is shown. Statistical significance was determined by a two-tailed, paired *t*-test. N.S. not significant, ** p<0.01, *** p<0.001, **** p<0.0001.

Vpr was previously demonstrated to interact with a cellular co-factor called DCAF1, a component of the cellular DCAF1-DDB1-CUL4 E3 ubiquitin ligase complex. (Hrecka et al., 2007; Lahouassa et al., 2016; Le Rouzic et al., 2007; McCall et al., 2008; Wu et al., 2016; Zhou et al., 2016). The interaction between Vpr and DCAF1 can be disrupted through a Vpr mutation (Q65R) that inhibits many Vpr-dependent functions, including reversal of Env degradation in macrophages (Mashiba et al., 2014). To determine whether this mutant is defective at MR downmodulation, we generated the mutation in the NL4-3 ΔGPE-GFP parent (**Figure 2.3A**), confirmed expression in transfected 293T cells (**Figure 2.3F**) and tested the effect of the mutation on MR levels in macrophages. As expected, we found that in transduced MDM the *vpr*-Q65R mutant behaves similarly to *vpr*-null (**Figure 2.3E**). These results indicate interactions between Vpr and DCAF1 are required to mediate Vpr's effects on MR.

The differences in MR downmodulation we observed using this system were not due to variations in multiplicity of infection of the different viral constructs as MDM

[4] The data in this figure was produced by Jay Lubow and Brian Peterson.

transduced with the mutant viral constructs had roughly similar transduction rates as the parental construct (**Figure 2.3G**) but demonstrated less MR downmodulation (**Figure 2.3E**).

To determine whether the relatively modest effect of Nef alone on MR levels was due to using HIV to deliver Nef as compared to an adenoviral vector delivery system used in a prior publication (Vigerust et al., 2005), we repeated the experiment using an adenoviral vector expressing Nef. These experiments confirmed that levels of Nef sufficient to downmodulate the HIV receptor, CD4, on nearly all MDM in the culture achieved only modest effects on MR in a subset of cells (**Figure 2.3H**) similar to what was observed using the HIV reporter construct (**Figure 2.3E**). Thus, Nef and Vpr have modest but significant effects on MR when expressed individually, however the combined effects of both proteins can achieve nearly complete downmodulation at least in a subset of infected cells.

While the effect of Nef has been previously reported and found to be due to disruption of MR intracellular trafficking (Vigerust et al., 2005), the effect of Vpr on MR is a novel observation. Vpr is known to target cellular proteins involved in DNA repair pathways for proteasomal degradation via interactions with Vpr binding protein [DCAF1, (McCall et al., 2008)]. Using this mechanism, Vpr degrades the uracil deglycosylases UNG2 and SMUG1 in 293T cells following co-transfection (Schrofelbauer et al., 2007; Schrofelbauer et al., 2005). To determine whether Vpr directly targets MR using a similar strategy, we co-transfected NL4-3 ΔGPE-GFP or a *vpr*-null derivative with expression vectors encoding an UNG2-FLAG fusion protein or MR (Liu et al., 2004)] in 293T cells. We then analyzed expression of MR or UNG2 by flow cytometry and western blot (**Figure**

2.4). We found that Vpr in 293T cells virtually eliminated UNG2 expression when measured by flow cytometry and noticeably reduced UNG2 by western blot. However, Vpr had no effect on expression of MR measured by either method. Thus, we concluded that Vpr does not degrade MR by the direct, proteasomal mechanism it uses to degrade UNG2. Because MR expression in this system is controlled by a heterologous CMV promoter; the lack of effect by Vpr suggested its action may depend on MR's native promoter.

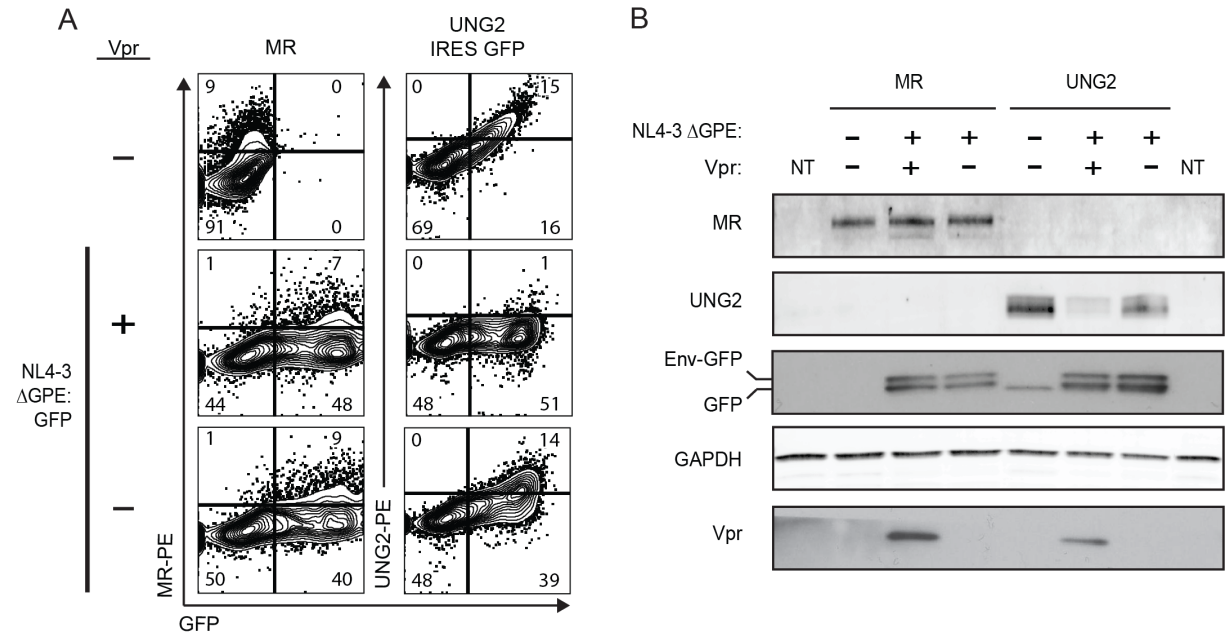

Figure 2.4: HIV Vpr reduces steady state levels of UNG2 but not MR in co-transfected 293T cells[5]**. (A)** Flow cytometric plots of 293T cells co-transfected with NL4-3 ΔGPE-GFP, pCDNA3.1-hMR, and pMSCV 3x FLAG UNG2 IRES-GFP as indicated. **(B)** Western blot analysis of 293T cells co-transfected exactly as in A. Env-GFP indicates the location of the fusion protein containing the N terminus of Env followed by GFP as described in Methods.

[5] The data in this figure was produced by Madeline Merlino and Jay Lubow.

Vpr reduces transcription of MRC1.

In addition to targeting proteins for degradation, Vpr also functions to inhibit transcription of genes such as *IFNA1* (Laguette et al., 2014; Mashiba et al., 2014). Therefore, we hypothesized that Vpr may reduce MR expression via inhibition of transcription. To examine this, we assessed transcriptional activity in primary human MDM transduced with the wild-type or Vpr-null reporter virus (**Figure 2.5A**) using cells isolated based on GFP expression (**Figure 2.5B**). We found that the MR gene (*MRC1*) was consistently reduced in cells transduced by *vpr*-competent virus compared to cells transduced by *vpr*-null virus (**Figure 2.5C and D**, p=0.001). In contrast, any effects of Vpr on the housekeeping genes *ACTB* (β-actin*)* and *POL2A* (RNA polymerase 2A*)* were significantly smaller (**Figure 2.5D**, p<0.01). Similar results were obtained when each gene was normalized to *ACTB* instead of *GAPDH* (**Figure 2.6A-B**). The magnitude of the effect on *MRC1* is consistent with prior reports of HIV-1 inhibiting *MRC1* transcription– though this was not previously linked to Vpr (Koziel et al., 1998; Sukegawa et al., 2018). Relative *MRC1* expression in untransduced MDM was heterogeneous, varying over a ten-fold range. When compiled across donors, *MRC1* levels in mock-transduced samples were not significantly different than transduced (**Figure 2.6C-F**).

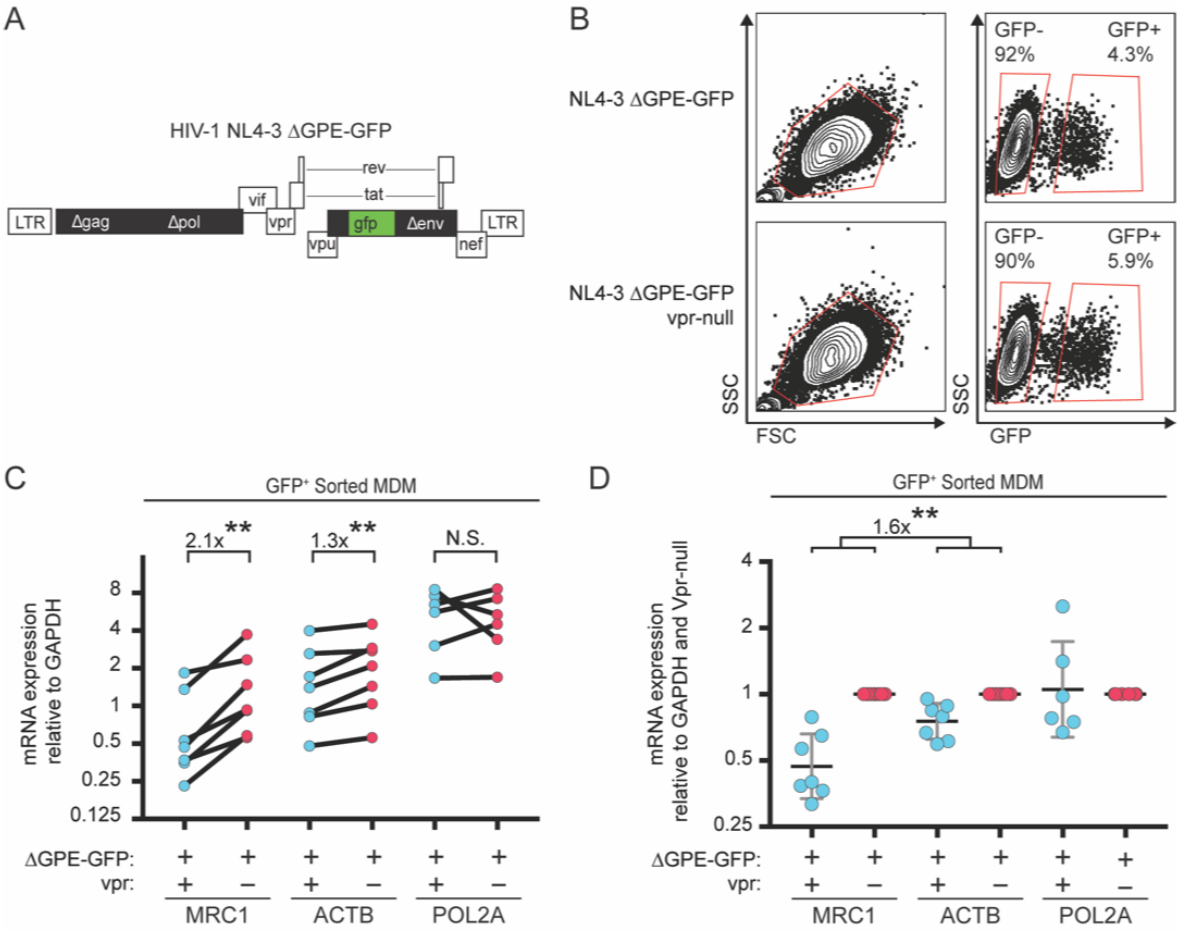

Figure 2.5: Vpr reduces transcription of *MRC1*[6]. (A) Diagram of HIV NL4-3 ΔGPE-GFP. **(B)** Flow cytometry plots indicating the gating strategy used to sort live GFP$^+$ vs GFP$^-$ cells for subsequent qPCR analysis. **(C)** Summary graph of mannose receptor (*MRC1*), β-actin (*ACTB*) and RNA Polymerase 2A (*POL2A*) mRNA expression in MDM transduced with the indicated HIV reporter and sorted for GFP expression by FACS. All data are normalized to *GAPDH* mRNA expression. **(D)** Summary graph of *MRC1*, *ACTB* and *POL2A* expression normalized to the Vpr-null condition in each donor. (*n*=7 independent donors). Geometric mean +/- geometric standard deviation is shown. Statistical significance was determined by a two-tailed, ratio *t*-test. N.S. = not significant p=0.81, ** p<0.01.

[6] The data in this figure was generated by Jay Lubow, Maria C. Virgilio, Francisco Gomez-Rivera, and Gretchen Zimmerman.

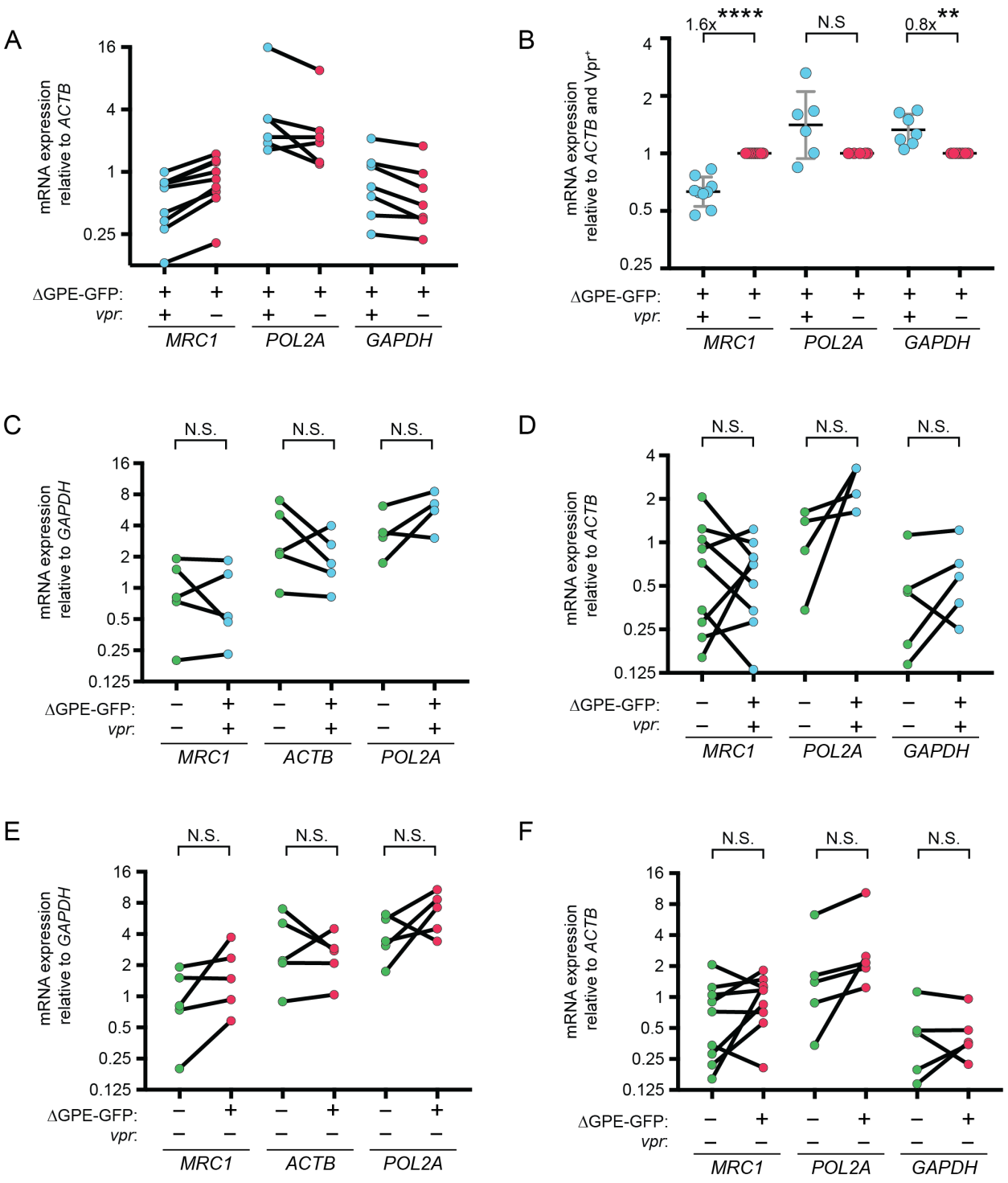

Figure 2.6: Vpr does not reduce transcription of housekeeping genes[7]**. (A)** Summary graph of mannose receptor (*MRC1*), RNA Polymerase 2A (*POL2A*) and *GAPDH* mRNA expression in MDM transduced with Vpr-competent or Vpr-null HIV NL4-3 ΔGPE-GFP and sorted for GFP

[7] The data in this figure was generated by Jay Lubow, Maria C. Virgilio, Francisco Gomez-Rivera, and Gretchen Zimmerman.

expression by FACS. All data are normalized to *ACTB* mRNA expression. **(B)** Summary graph of same data as A normalized to the Vpr+ condition in each donor. (*n*=8 independent donors). Geometric mean is indicated by the line. **(C)** Summary graph of *MRC1*, *ACTB* and *POL2A* mRNA expression in untransduced MDM and MDM transduced with Vpr-competent HIV NL4-3 ΔGPE-GFP. All data are normalized to *GAPDH*. **(D)** Summary graph of *MRC1*, *POL2A* and *GAPDH* mRNA expression in untransduced MDM and MDM transduced with Vpr-competent HIV NL4-3 ΔGPE-GFP. All data are normalized to *ACTB.* **(E)** Summary graph of *MRC1*, *ACTB* and *POL2A* mRNA expression in untransduced MDM and MDM transduced with Vpr-null HIV NL4-3 ΔGPE-GFP. All data are normalized to *GAPDH*. **(F)** Summary graph of *MRC1*, *POL2A* and *GAPDH* mRNA expression in untransduced MDM and MDM transduced with Vpr-null HIV NL4-3 ΔGPE-GFP. All data are normalized to *ACTB*. Statistical significance was determined by a two-tailed, ratio *t*-test. N.S. = not significant, ** p<0.01, **** p<0.0001.

Combined effect of Vpr and Nef dramatically enhances Env levels in primary human MDM.

To determine whether the striking downmodulation of MR we observed with expression of both Nef and Vpr affected viral spread in MR^+ macrophages, we generated additional mutations in HIV-1 89.6 to create a *nef*-null mutant and a *vpr-nef*-null double mutant. As expected, in transfected 293T cells these mutations did not alter Env protein levels (**Figure 2.7A**) or release of virions as assessed by measuring Gag p24 into the supernatant by ELISA (**Figure 2.7B**). However, in primary human MDM infected with these HIVs, the mutants demonstrated defects in viral spread, with the double mutant having the greatest defect (**Figure 2.7C and D**). The defect in spread was caused in part by diminished virion release, which we previously showed occurred in the absence of Vpr (Mashiba et al., 2014); MDM infected with the HIV mutants released less Gag p24 even after adjusting for the frequency of infected cells (**Figure 2.7D**, right panel).

To determine whether combined effects of Nef and Vpr on MR expression affected Env restriction, we assessed Env levels in primary human MDM infected with each construct. Because the frequency of infected cells as assessed by intracellular Gag staining (**Figure 2.7C**) and Gag pr55 western blot (**Figure 2.7E**) was lower in the mutants

than in the wild-type infection, lysate from the wild-type sample was serially diluted to facilitate comparisons. Remarkably, we found that the *vpr-nef*-null double mutant, which retains near normal MR levels, exhibited the greatest defect in Env expression (**Figure 2.7E**, compare lanes with similar Gag as indicated). In sum, Vpr and Nef-mediated downmodulation of MR correlated inversely with Env levels, consistent with MR being the previously described but unidentified HIV restriction factor that targets Env for lysosomal degradation in macrophages and is counteracted by Vpr (Mashiba et al., 2014). Combined effects of Nef on MR and other Env binding proteins including CD4 (Aiken et al., 1994) and chemokine receptors (Michel et al., 2006) may also play a role in stabilization of Env.

Mannose-containing glycans in Env are required for macrophage restriction of HIV in the absence of Vpr.

A particularly dense mannose containing structure on Env, known as the mannose patch, may mediate interactions between Env and MR . This structure is present on all HIV Env proteins that require Vpr for stability in macrophages [89.6, NL-43 and AD8 (Mashiba et al., 2014)]. Interestingly, a macrophage tropic strain YU-2, which was isolated from the CNS of an AIDS patient (Li et al., 1991), lacks a mannose patch. This structure is the target of several broadly neutralizing antibodies including 2G12, to which YU-2 is highly resistant (Trkola et al., 1996). If Vpr targets MR to counteract detrimental interactions between MR and mannose residues on Env, we hypothesized that HIV Envs lacking a mannose patch would have a reduced requirement for Vpr. To test this hypothesis, we first examined the extent to which virion release and Env expression were

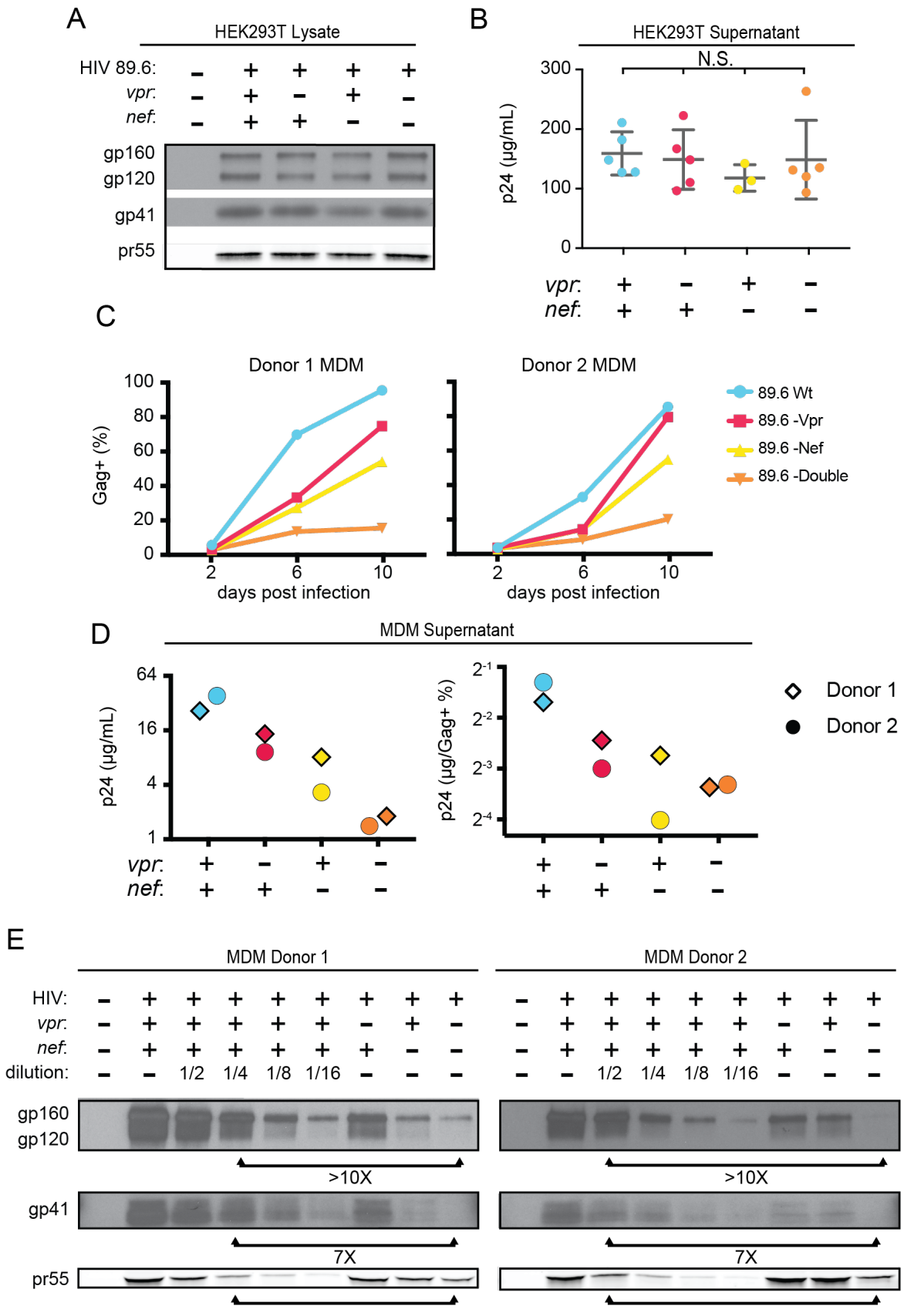
A
HEK293T Lysate
HIV 89.6:
vpr:
nef:
gp160
gp120
gp41
pr55
B
HEK293T Supernatant
N.S.
p24 (µg/mL)
300
200
100
0
vpr:
nef:
C
Donor 1 MDM
Donor 2 MDM
Gag+ (%)
100
80
60
40
20
0
2
6
10
days post infection
89.6 Wt
89.6 -Vpr
89.6 -Nef
89.6 -Double
D
MDM Supernatant
p24 (µg/mL)
64
16
4
1
p24 (µg/Gag+ %)
2-1
2-2
2-3
2-4
Donor 1
Donor 2
E
MDM Donor 1
MDM Donor 2
HIV:
vpr:
nef:
dilution:
1/2
1/4
1/8
1/16
gp160
gp120
gp41
pr55
>10X
7X

Figure 2.7: Combined effect of Vpr and Nef dramatically enhances Env levels in primary human MDM[8]. (A) Western blot analysis of whole cell lysate from 293T transfected with the indicated HIV construct. **(B)** Summary graph of virion release from 293Ts transfected as in A as measured by Gag p24 ELISA. (*n* = 5 independent transfections). The mean +/- standard deviation is shown. Statistical significance was determined by one-way ANOVA. (N.S. – not significant) **(C)** Frequency of infected primary human MDM infected with the indicated HIV and analyzed over time by flow cytometric analysis of intracellular Gag. (For parts C-E, *n*= 2 independent donors) **(D)** Virion release by primary human MDM infected with the indicated HIV and analyzed by Gag p24 ELISA 10 days post infection. In the right panel, virion release was adjusted for frequency of infected cells as measured in part C. **(E)** Western blot analysis of whole cell lysate from primary human MDM infected with the indicated HIV. Within each donor, lanes 2-6 are a serial dilution series of the wild-type sample. The arrows below the Gag pr55 bands indicate the dilution of wild-type that has approximately the same amount of Gag pr55 as the *vpr*-*nef*-null double mutant.

influenced by Vpr in primary human MDM infected with YU-2 or 89.6 HIVs. Consistent with our hypothesis, we observed no significant difference in Gag p24 release between wild-type and *vpr*-null YU-2 infection of MDM (**Figure 2.8A**). Moreover, the *vpr*-null mutant of YU2 displayed only a minor defect in Env expression compared to Vpr null versions of 89.6 and NL4-3 (**Figure 2.8B**).

Because there are a number of other genetic differences between YU-2 and the other HIVs, we constructed a chimeric virus, which restricted the differences to the *env* open reading frame. As shown in **Figure 2.8C**, a fragment of the YU-2 genome containing most of *env* but none of *vpr* (**Figure 2.8C**, shaded portion) was cloned into NL4-3 and NL4-3 *vpr*-null. As expected, these genetic alterations did not affect Env protein levels or virion release in transfected 293T cells (**Figures 2.8D and E**). To confirm that the chimeric Env was still functional, we examined infectivity in T cells prior to performing our analyses in primary human MDM. Conveniently, sequence variation within the gp120 region allows YU-2 Env to only utilize the co-receptor CCR5 for entry, whereas NL4-3 can only utilize CXCR4. Thus, we expected the NL4 3*env*^{YU2} chimera would switch from being CXCR4-

[8] The data in this figure was generated by Jay Lubow.

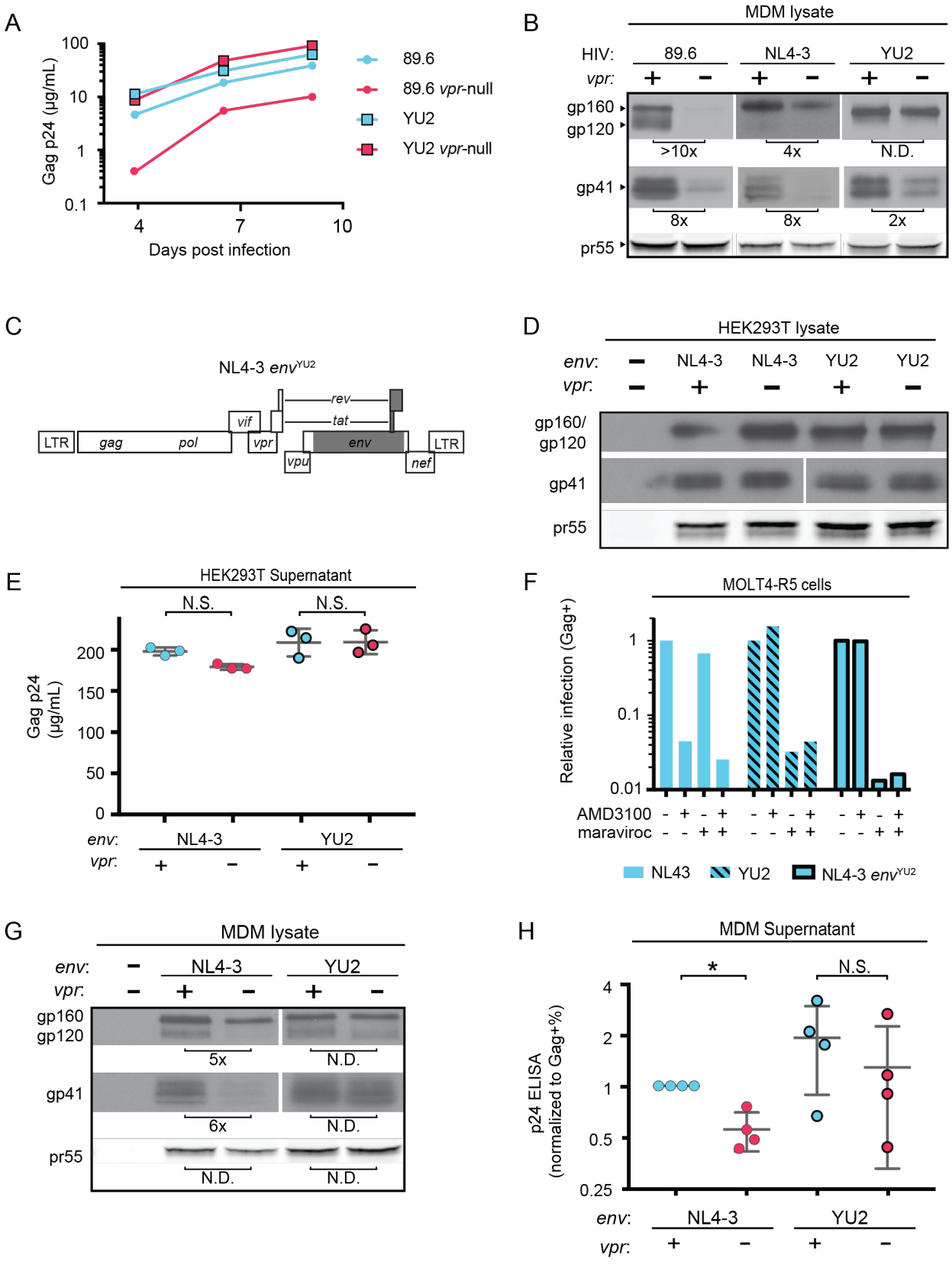
A
Gag p24 (µg/mL)
100
10
1
0.1
4
7
10
Days post infection
89.6
89.6 vpr-null
YU2
YU2 vpr-null
B
MDM lysate
HIV:
89.6
NL4-3
YU2
vpr:
+ − + − + −
gp160
gp120
>10x
4x
N.D.
gp41
8x
8x
2x
pr55
C
NL4-3 env^YU2
rev
tat
vif
LTR
gag
pol
vpr
env
LTR
vpu
nef
D
HEK293T lysate
env:
NL4-3
NL4-3
YU2
YU2
vpr:
gp160/
gp120
gp41
pr55
E
HEK293T Supernatant
N.S.
N.S.
Gag p24
(µg/mL)
200
150
100
50
0
env:
NL4-3
YU2
vpr:
+ − + −
F
MOLT4-R5 cells
Relative infection (Gag+)
1
0.1
0.01
AMD3100 - + - + - + - + - + - +
maraviroc - - + + - - + + - - + +
NL43
YU2
NL4-3 env^YU2
G
MDM lysate
env:
NL4-3
YU2
vpr:
− + − + −
gp160
gp120
5x
N.D.
gp41
6x
N.D.
pr55
N.D.
N.D.
H
MDM Supernatant
*
N.S.
p24 ELISA
(normalized to Gag+%)
4
2
1
0.5
0.25
env:
NL4-3
YU2
vpr:
+ − + −

Figure 2.8: HIV YU2, which lacks a mannose rich patch, does not require Vpr for robust Env protein expression and spread in MDM[9]**. (A)** Virion release over time by primary human MDM infected with the indicated HIV as measured by ELISA (*n*=2 independent donors). **(B)** Western blot analysis of whole cell lysate from MDM infected for 10 days with the indicated HIV. Because NL4-3 infects MDM poorly, NL4-3 was pseudotyped with a YU-2 Env expression plasmid co-transfected in the producer cells as described in Methods. Subsequent spread was blocked in all samples by the addition of entry inhibitors AMD3100 and maraviroc initially added 48 hours post-infection and maintained throughout the culture period. **(C)** Diagram of the HIV NL4-3 genome. The shaded portion represents the sequence that was replaced with sequence from HIV YU2 to create the NL4-3 *env*$^{YU-2}$ chimera. **(D)** Western blot analysis of 293T cells transfected with the indicated HIV constructs. YU-2 gp41 is detected by the monoclonal antibody z13e1 and NL4-3 gp41 is detected by the monoclonal antibody CHESSIE-8. **(E)** Virion release from 293T transfected as in E as measured by p24 ELISA. (*n*=3 experimental replicates). **(F)** Relative infection of MOLT4-R5 cells 48 hours after inoculation by the indicated viruses and treated with entry inhibitors as indicated. The frequency of infected cells was measured by intracellular Gag stain and normalized to the untreated condition for each infection. **(G)** Western blot analysis of primary human MDM infected for 10 days with the indicated virus as in B. (*n*=2 independent donors) **(H)** Summary graph showing virion release as measured by p24 ELISA for primary human MDM infected as in G. Virus production was adjusted for infection frequency as determined flow cytometrically using an intracellular Gag stain. The mean +/- standard deviation is shown. (*n*=4 independent donors). N.D. – no difference. Statistical significance was determined using a two-tailed, ratio *t*-test. N.S. – not significant, * $p<0.05$.

to CCR5-tropic. To test this, we utilized a T cell line expressing both chemokine receptors (MOLT4-R5) and selectively blocked entry via CXCR4 and CCR5 entry inhibitors [AMD3100 and maraviroc, respectively (**Figure 2.8F**)]. As expected, entry of MOLT4-R5 cells by NL4-3 was blocked by AMD3100 but not maraviroc, indicating CXCR4-tropism. The chimeric NL4-3 *env*^{YU2} and wild-type YU-2 demonstrated the inverse pattern, indicating CCR5-tropism. These results demonstrated that we had made the expected changes in the chimeric Env without disrupting its capacity to infect cells.

[9] The data in this figure was generated by Jay Lubow.

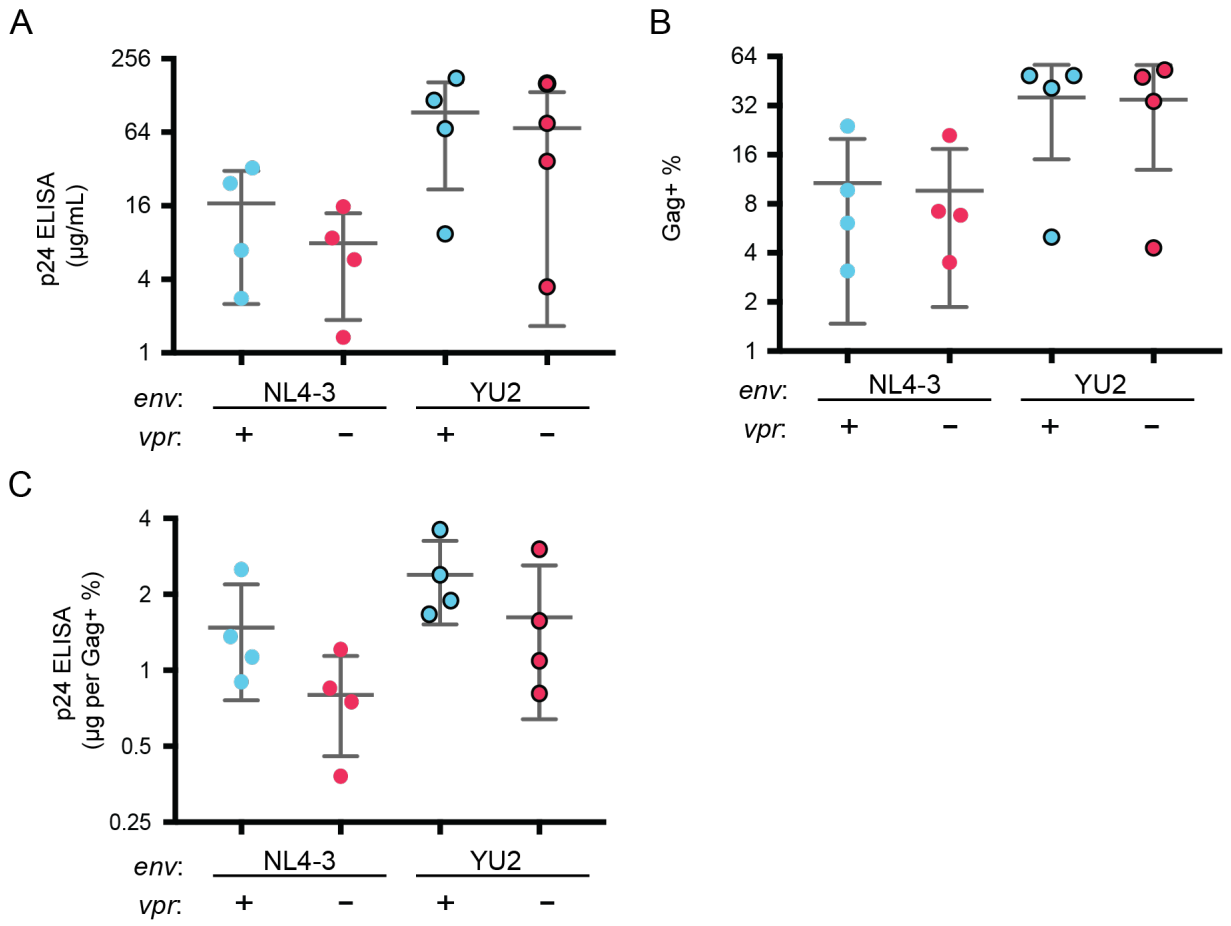

Figure 2.9: Raw p24 ELISA and intracellular gag data 10 days post infection by NL43*env*^{YU2}.[10] **(A)** Summary graph showing Gag p24 concentration of supernatant from MDM cultures 10 days post infection with the indicated virus. **(B)** Summary graph showing the fraction of MDM that are Gag+ 10 days post infection with the indicated virus. **(C)** Summary graph showing the p24 concentration normalized to the fraction of cells that are Gag+ for each donor. *n*= 4 independent donors.

To determine whether swapping a limited portion of YU-2 containing Env into NL4-3 alleviated the requirement for Vpr, we examined Env expression and virion release in primary human MDM infected with these viruses. Because the parental NL4-3 virus required pseudotyping with a macrophage-tropic Env for entry and was unable to spread in MDM, all infections were treated with entry inhibitors AMD3100 and maraviroc starting at 48 hours after inoculation and maintained throughout the culture period to block

[10] The data in this figure was generated by Jay Lubow.

subsequent rounds of infection. Consistent with our hypothesis that YU-2 Env lacked determinants necessary for the restriction that was alleviated by Vpr, we observed that wild-type NL4-3 Env but not chimeric NL4-3 *env*^{YU2} required Vpr for maximal expression (**Figure 2.8G**). Moreover, MDM infected with the chimeric HIV had a reduced requirement for Vpr for maximal virion release (**Figure 2.8H and Figure 2.9**). This experiment provides strong evidence that the requirement for Vpr can be alleviated by genetic changes within the *env* open reading frame. These results are consistent with a model in which YU-2 *env* confers resistance to the effects of MR due to the absence of the mannose rich structure on the YU-2 Env glycoprotein.

Deletion of N-linked glycosylation sites in Env reduces Env restriction in HIV infected human primary MDM and diminishes the need for Vpr and Nef.

To more directly assess the role of mannose in restricting expression of Env in HIV-1 infected primary human MDM, we engineered a version of 89.6 Env in which two N-linked glycosylation sites, N230 and N339 (HIV HxB2 numbering) were deleted by substituting non-glycosylated amino acids found at analogous positions in YU-2 Env (**Figure 2.10A**). The glycosylation sites N230 and N339 were selected because they contain high-mannose glycan structures (Leonard et al., 1990) that are absent in YU-2 Env. Loss of N230 limits neutralization by glycan specific antibodies (Huang et al., 2014). Loss of N339 decreases the amount of oligomannose ($Man_9GlcNAc_2$) present on gp120 by over 25%, presumably by opening up the mannose patch to processing by α-mannosidases (Pritchard et al., 2015). These substitutions (N230D and N339E) in 89.6 did not alter virion production (**Figure 2.10B**) or Env protein expression (**Figure 2.10C**) in transfected 293T cells.

To confirm that mutation of N230 and N339 disrupted the mannose patch on Env, we assayed the ability of 2G12, which recognizes epitopes in the mannose patch (Sanders et al., 2002; Scanlan et al., 2002) to neutralize wild-type and mutant Env. As shown in **Figure 2.10D**, wild-type but not mannose deficient N230D N339E Env was neutralized by 2G12. In addition, we found that these substitutions did not disrupt infection of a T cell line that does not express MR (**Figure 2.10E**). However, somewhat unexpectedly, we found that HIV containing the N230D N339E Env substitutions was approximately 40% less infectious to primary human macrophages expressing MR than the wild-type parental virus (**Figure 2.10E**, p=0.002). This macrophage-specific difference in infectivity suggested that mannose on Env may facilitate initial infection through interactions with MR, which is highly expressed on differentiated macrophages. To examine this possibility further, we asked whether soluble mannan, which competitively inhibits MR interactions with mannose containing glycans (Shibata et al., 1997), was inhibitory to HIV infection of macrophages. As a negative control, we tested 89.6 ∆env pseudotyped with vesicular stomatitis virus G-protein Env (VSV-G) which has only two N-linked glycosylation sites, both of which contain complex-type rather than high-mannose glycans (Reading et al., 1978). Therefore VSV-G should not bind MR or be inhibited by mannan. As expected, we found that infection of a T cell line lacking MR was not sensitive to mannan (**Figure 2.10F**, left panel). However, infection of MDM by wild-type HIV-1 was inhibited up to 16-fold by mannan (**Figure 2.10F**, right panel). This was specific to HIV Env because mannan did not inhibit infection by HIV lacking *env* and pseudotyped with heterologous VSV-G Env.

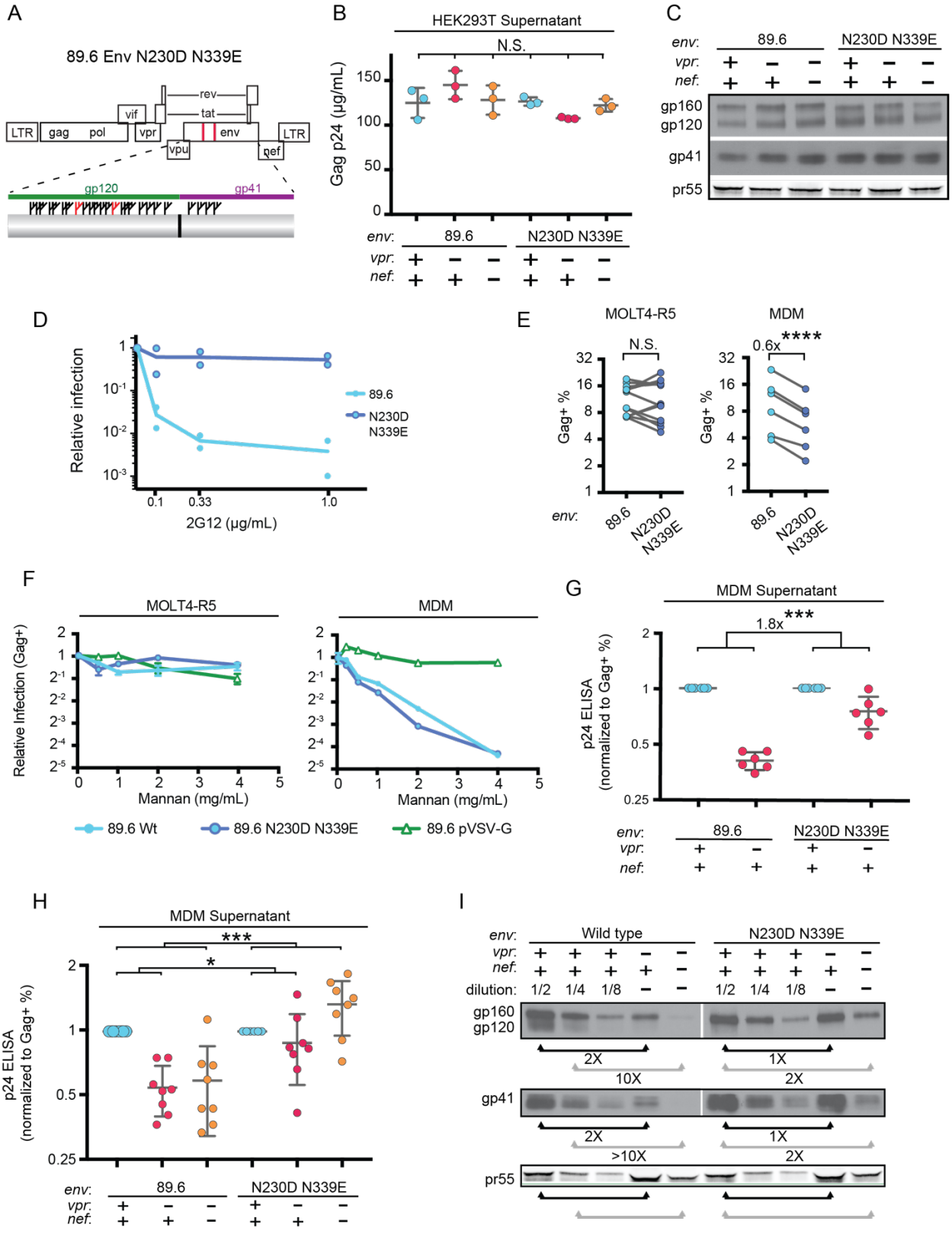
A
89.6 Env N230D N339E
LTR
gag
pol
vif
vpr
tat
rev
env
vpu
nef
LTR
gp120
gp41
B
HEK293T Supernatant
N.S.
Gag p24 (µg/mL)
150
100
50
0
env:
89.6
N230D N339E
vpr:
nef:
C
env:
89.6
N230D N339E
vpr:
nef:
gp160
gp120
gp41
pr55
D
Relative infection
1
10-1
10-2
10-3
89.6
N230D N339E
0.1
0.33
1.0
2G12 (µg/mL)
E
MOLT4-R5
MDM
N.S.
0.6x
****
Gag+ %
32
16
8
4
2
1
env:
89.6
N230D N339E
F
MOLT4-R5
MDM
Relative Infection (Gag+)
2
1
2-1
2-2
2-3
2-4
2-5
0
1
2
3
4
5
Mannan (mg/mL)
89.6 Wt
89.6 N230D N339E
89.6 pVSV-G
G
MDM Supernatant
1.8x
***
p24 ELISA
(normalized to Gag+ %)
2
1
0.5
0.25
env:
89.6
N230D N339E
vpr:
nef:
H
MDM Supernatant
***
*
p24 ELISA
(normalized to Gag+ %)
2
1
0.5
0.25
env:
89.6
N230D N339E
vpr:
nef:
I
env:
Wild type
N230D N339E
vpr:
nef:
dilution:
1/2
1/4
1/8
gp160
gp120
gp41
pr55
2X
10X
1X
2X
>10X

Figure 2.10: Deletion of N-linked glycosylation sites in *env* reduces the requirement for Vpr and Nef for virion release and Env expression in HIV-1 infected primary human MDM[11]. (A) Upper panel, diagram of HIV genome encoding the mutations N230D and N339E (indicated in red) to prevent N-linked glycosylation at those sites. Lower panel, diagram of HIV 89.6 N230D N339E mutant Env protein. Branched symbols represent N-linked glycans. **(B)** Summary graph showing virion release from 293Ts transfected with the indicated HIV construct as measured by p24 ELISA. (*n*=3 experimental replicates). Statistical significance was determined by one-way ANOVA. **(C)** Western blot analysis of 293T transfected as in B. **(D)** Summary graph showing relative infection frequency of MOLT4-R5 T cells by the indicated HIV following treatment as indicated with the neutralizing antibody 2G12. The percentage of infected cells was measured by intracellular Gag stain and normalized to the untreated condition for each virus. (n= 2 independent experiments, both are plotted). **(E)** Summary graphs of relative infection of the indicated cell type by mutant or parental wild-type HIV. The frequency of infected cells was measured flow cytometrically by intracellular Gag stain and normalized to the wild-type virus. (*n*=5 experimental replicates for MOLT4-R5; *n*=2 experimental replicates for MDM from 4 independent donors). **(F)** Summary graph depicting relative infection of the indicated cell type by each virus plus or minus increasing concentrations of mannan as indicated. The frequency of infected cells was measured by intracellular Gag stain and normalized to the uninhibited (0 mg/mL mannan) condition for each virus. 89.6 pVSV-G indicates 89.6 ∆env pseudotyped with VSV-G protein. (*n*=2 independent donors for 89.6 wild-type and 89.6 ∆env pVSV-G; *n*=1 donor for 89.6 *env* N230D N339E) **(G)** Summary graph of virion release from primary human MDM following 10 days of infection by the indicated HIV as measured by p24 ELISA. Virion release was normalized to the infection frequency assessed flow cytometrically by intracellular Gag stain. The result for each *vpr*-null mutant was normalized to the *vpr*-competent virus encoding the same *env*. (*n*=6 independent donors) **(H)** Summary graph of virion release from primary human MDM following 10 days of infection by the indicated HIV as measured by p24 ELISA. Virion release was normalized to the infection frequency assessed flow cytometrically by intracellular Gag stain. For this single round infection assay, all viruses were pseudotyped with YU2 Env and viral spread was blocked 48 hours later by addition of AMD3100 and maraviroc. (*n*=8 independent donors) The result for each *vpr*-null or *vpr*-*nef*-null mutant was normalized to the *vpr*- and nef-competent virus encoding the same *env*. **(I)** Western blot analysis of MDM infected as in E. The lysates from the *vpr*-competent and *nef*-competent infections were diluted to facilitate comparisons to *vpr*- and *nef*-null mutants. (*n*=2 independent donors) For summary graphs, the means +/- standard deviation is shown. In panels G and H statistical significance was determined by a two-tailed, paired *t*-test * p=0.01, ** p<0.01, *** p<0.001.

Interestingly, mannan also inhibited baseline macrophage infection by mannose-deficient Env (89.6 Env N230D N339E), indicating that N230D N339E substitutions did not completely abrogate glycans on Env that are beneficial to initial infection. In sum, our results demonstrate that interactions with mannose binding receptors are advantageous for initial HIV infection of macrophages and that the glycans remaining on Env N230D

[11] The data in this figure was generated by Jay Lubow.

N339E retain some ability to bind glycan receptors on macrophages that facilitate infection.

While interactions between high-mannose residues on Env and MR were advantageous for viral entry, we hypothesized that they interfered with intracellular Env trafficking and were deleterious to egress of Env-containing virions in the absence of Vpr and/or Nef. To test this, we examined virion release and Env expression by HIVs encoding the mannose-deficient Env N230D N339E in the presence or absence of Vpr. In a spreading infection of MDM, we found that virus expressing mannose-deficient Env had a reduced requirement for Vpr for maximal virus release compared with the parental wild-type virus (**Figure 2.10G**, $p<0.001$). In addition, in single-round infections of MDM, the mannose-deficient Env had a reduced requirement for both Nef and Vpr (**Figure 2.10H** and **Figure 2.11**, $p<0.001$). Single round infection assays cultured for ten days were used to assess the *vpr-nef* double mutant because depletion of mannose on Env did not rescue spread under conditions that were most comparable to our ten day spreading infections. The defect in spread is likely due to pleiotropic effects of Nef that disrupt interference by the HIV receptors, CD4, CXCR4 and CCR5 (Lama et al., 1999; Michel et al., 2005; Venzke et al., 2006) combined with the reduced infectivity of the mannose deficient Env.

Finally, we asked whether the mannose-deficient Env had increased stability in primary human MDM lacking Vpr and/or Nef by western blot analysis. We found that the Env mutant (N230D. N339E) was more stable in the absence of Vpr (**Figure 2.10I**, right side, black bars) and Nef (**Figure 2.10I**, right side, gray bars) once differences in infection frequency were accounted for by matching pr55 expression in the dilution series. These data provide strong support for a model in which MR restricts Env expression via direct

interaction with high-mannose residues on Env and this restriction is counteracted by Vpr and Nef.

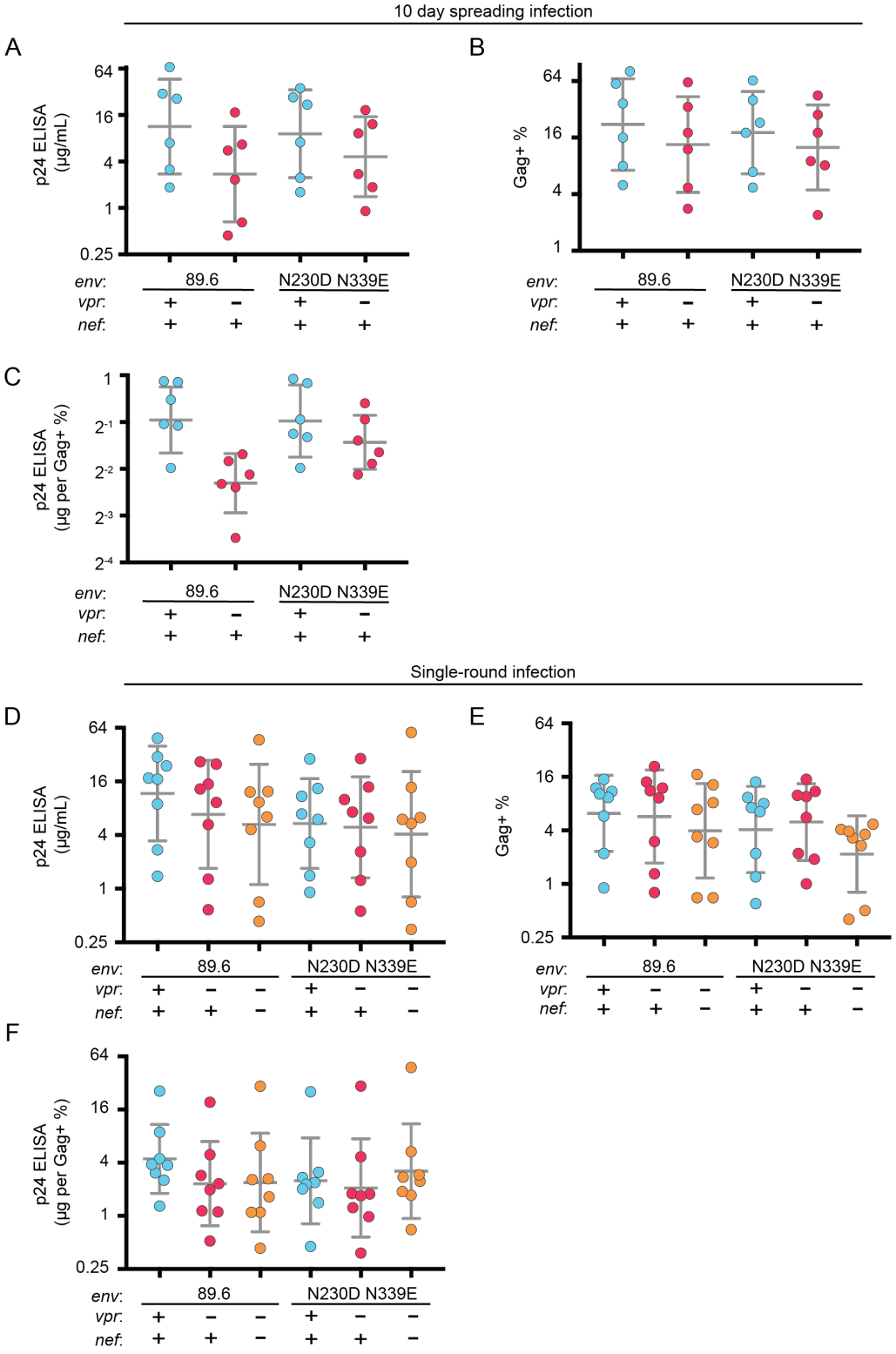

Figure 2.11: Raw p24 ELISA and intracellular gag data 10 days post infection by 89.6 Env N230D N339E[12] **(A)** Summary graph showing Gag p24 concentration of supernatant from MDM cultures 10 days post infection with the indicated virus, which were allowed to spread in culture. Data correspond to Figure 2.10G. **(B)** Summary graph showing the fraction of MDM that are Gag+ 10 days post infection with the indicated virus. **(C)** Summary graph showing the p24 concentration normalized to the fraction of cells that are Gag+ for each donor. A-C *n*= 6 independent donors. **(D)** Summary graph showing Gag p24 concentration of supernatant from MDM cultures 10 days post infection with the indicated virus. Viral replication was blocked by AMD3100 and maraviroc 48 hours post infection. Data correspond to Figure 2.10H. **(E)** Summary graph showing the fraction of MDM that are Gag+ 10 days post infection with the indicated virus. **(F)** Summary graph showing the p24 concentration normalized to the fraction of cells that are Gag+ for each donor. D-F *n*= 8 independent donors.

Silencing MR alleviates restriction of Env in primary human MDM lacking Vpr.

To directly test the hypothesis that MR is a restriction factor in MDM that is counteracted by Vpr, we examined the effect of MR silencing on Env expression in HIV-infected MDM lacking Vpr. Consistent with our hypothesis, we observed that silencing MR stabilized Env relative to Gag pr55 (**Figure 2.12A**). These results support the conclusion that the Env restriction observed in the absence of Vpr is dependent on expression of MR.

Previous work in our laboratory demonstrated that restriction of Env in primary human MDM disrupted formation of virological synapses and cell-to-cell spread of HIV from infected MDM to T cells. Expression of Vpr alleviated these effects, dramatically increasing viral transmission – especially under conditions of low initial inoculum of free virus. To expand on these findings, we measured Vpr-dependent HIV-1 spread from primary human MDM to autologous T cells, as diagrammed in **Figure 2.13A**. Co-cultured cells were stained for CD3 to distinguish T cells and CD14 to distinguish MDM as shown in **Figure 2.13B**, accounting for differences in autofluorescent background in the two cell

[12] The data in this figure was generated by Jay Lubow.

types by using isotype controls (**Figure 2.13C**) We confirmed our prior finding that Vpr enhances HIV-1 89.6 spread from MDM to T cells (**Figure 2.13D**) and extended this finding to the transmitted/founder (T/F) clone REJO (**Figure 2.13E**). Consistent with our previous findings, we observed that a higher frequency of T cells became infected following co-culture with infected MDM as compared to incubation with high titer cell free virus [(47-fold (89.6, p=0.0002) and 38-fold (REJO, p=0.048)].

To determine whether Vpr stimulated spread from macrophages to T cells by counteracting MR restriction, we measured spread to T cells from macrophages in which MR had been silenced as diagrammed in **Figure 2.12B**. Using the gating strategy shown in **Figure 2.13B**, infected MDM and infected T cells were identified by intracellular Gag stain (**Figure 2.12C**). We found that silencing MR reduced the difference between wild type and Vpr-null infected macrophage spread to T cells from 7-fold (p=0.003) to 2-fold (p=0.02) (**Figure 2.12D**). These results provide strong evidence that MR is the previously described but unidentified restriction factor in macrophages that reduces HIV spread from macrophages to T lymphocytes in the absence of Vpr.

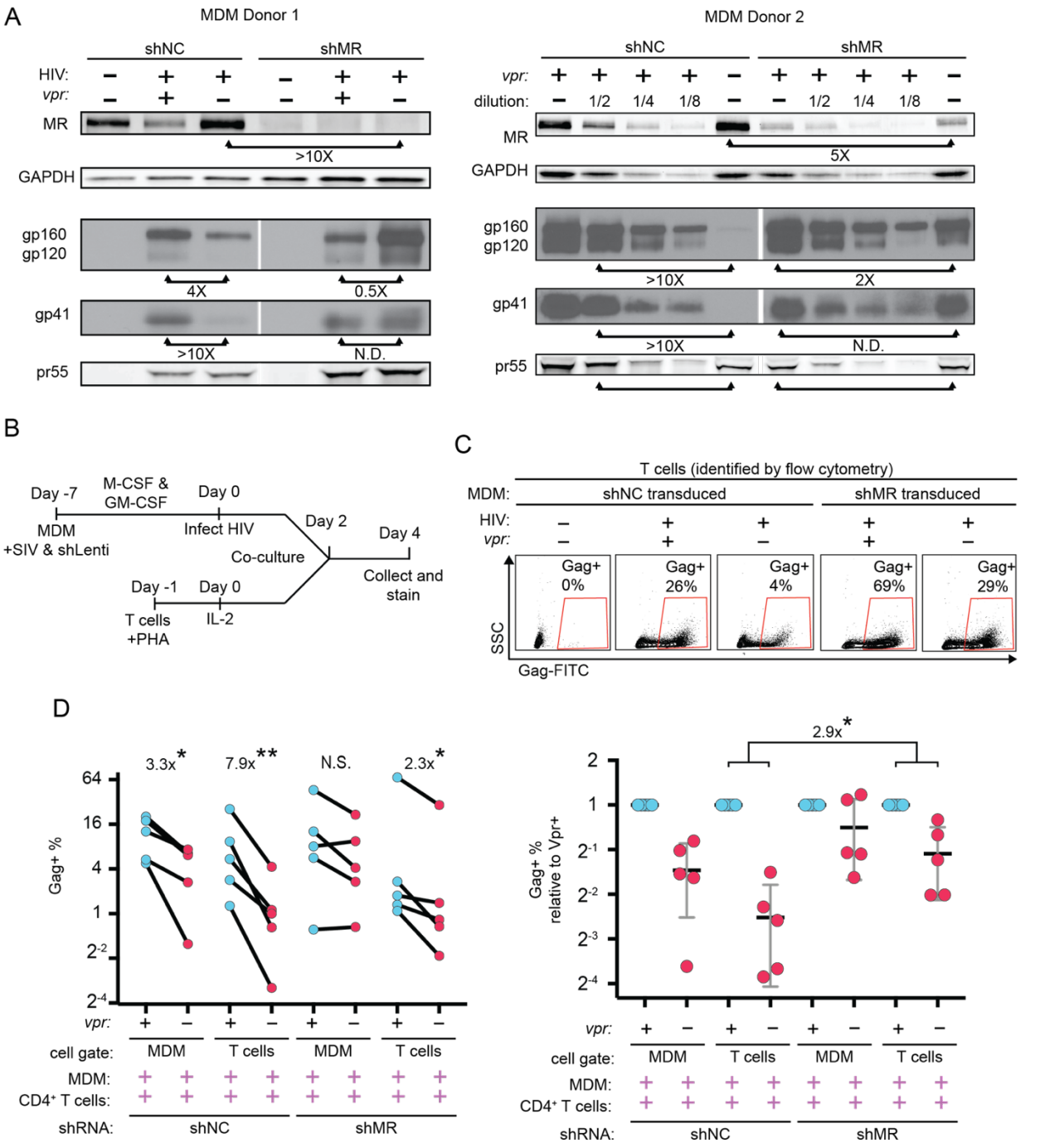

Figure 2.12: Knockdown of MR enhances Env expression and spread to T cells in *vpr*-null infection of MDM[13]. (A) Western blot analysis of MDM from two independent donors treated with the indicated silencing vector and infected with the indicated HIV for 10 days. The shRNA sequences encoded by the negative control vector (shNC) and the MR silencing vector (shMR) are described in Methods. **(B)** Schematic diagram of experimental protocol used for silencing experiments. **(C)** Representative flow cytometric plots showing frequency of infected (Gag^+)

[13] The data in this figure was generated by Jay Lubow, Maria C. Virgilio, and Valeri Terry.

primary T cells following two days of co-culture with autologous, HIV 89.6 infected primary MDM. T cells were identified in co-culture by gating on $CD3^+$ $CD14^-$ cells as shown in Figure 2.13B. **(D)** Summary graph displaying relative infection of MDM and T cells as measured in C (*n*=5 independent donors). Data in the left panel are unnormalized. In the right panel the data have been normalized to the wild-type condition for each donor and shRNA.

Discussion

We previously reported that Env and Env-containing virions are degraded in macrophage lysosomes in the absence of Vpr, impairing virion release, virological synapse formation, and spread of HIV to T cells (Mashiba, Collins et al. 2014). Moreover, this requirement for Vpr was conferred to heterokaryons comprised of macrophages and permissive cells, suggesting the existence of a previously unidentified host restriction factor that is counteracted by Vpr in macrophages (Mashiba, Collins et al. 2014). Results presented here clearly define mannose receptor (MR) as the HIV restriction factor counteracted by Vpr in macrophages to enhance viral dissemination. We provide strong evidence that Env mannosylation is required for HIV restriction of Env and virion release in macrophages in the absence of Vpr, and that MR silencing relieves a requirement for Vpr to overcome this restriction. Moreover, we confirm and extend a prior report that Nef also acts to downmodulate MR from the macrophage cell surface (Vigerust, Egan et al. 2005) and demonstrate that Vpr and Nef cooperate to counteract MR in an additive fashion through independent mechanisms.

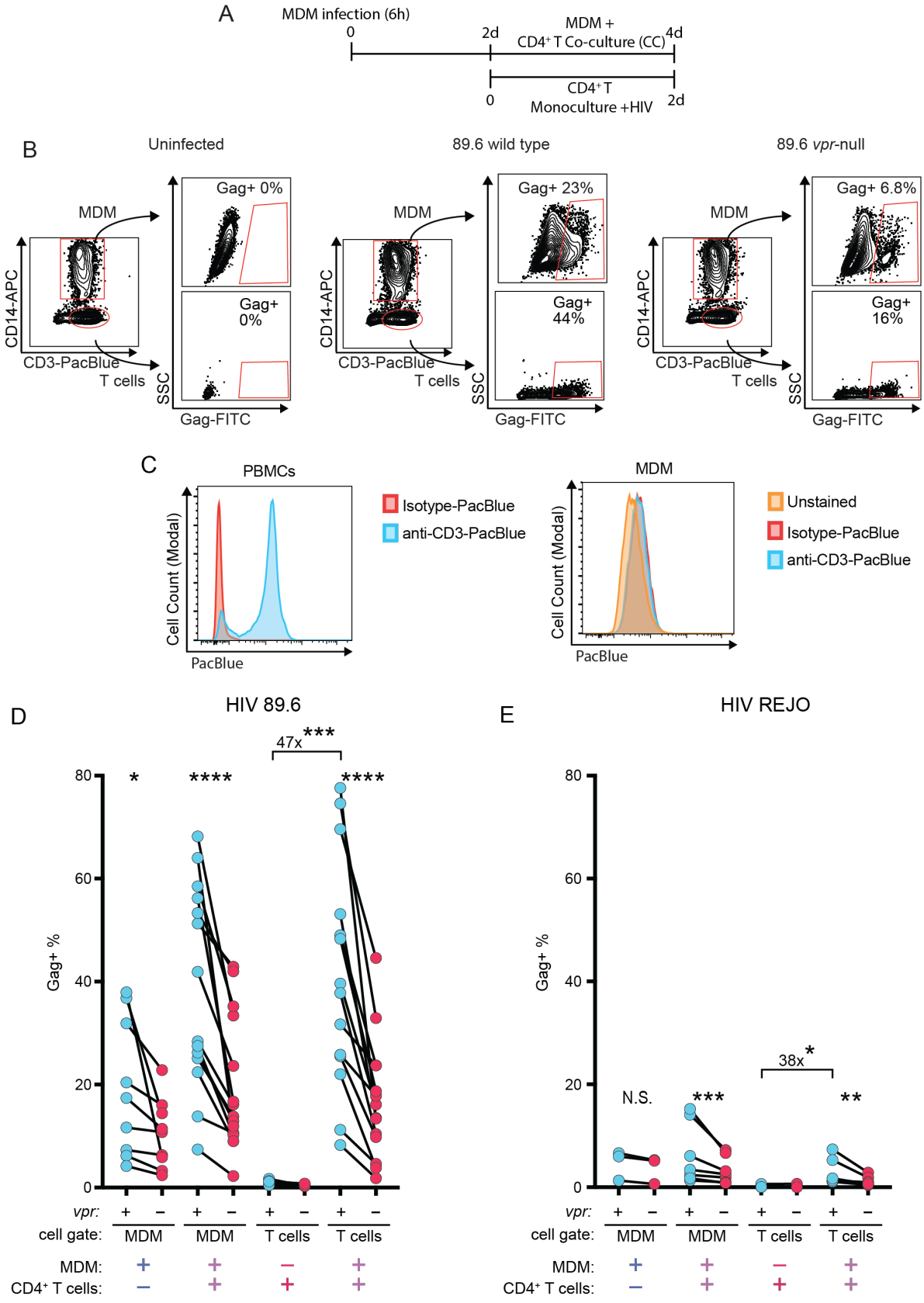
A
MDM infection (6h)
0
2d
MDM +
CD4+ T Co-culture (CC)
4d
CD4+ T
Monoculture +HIV
0
2d
B
Uninfected
89.6 wild type
89.6 vpr-null
MDM
CD14-APC
CD3-PacBlue
T cells
SSC
Gag-FITC
Gag+ 0%
Gag+
0%
Gag+ 23%
Gag+
44%
Gag+ 6.8%
Gag+
16%
C
PBMCs
MDM
Cell Count (Modal)
PacBlue
Isotype-PacBlue
anti-CD3-PacBlue
Unstained
D
HIV 89.6
47x
***
*
****
****
Gag+ %
vpr:
cell gate:
MDM
T cells
MDM:
CD4+ T cells:
E
HIV REJO
38x
*
N.S.
***
**

Figure 2.13: Cell-to-cell infection from macrophages to autologous CD4+ T cells is highly efficient and enhanced by Vpr[14]. (A) Diagram of the MDM and T cell co-culture experiments depicted in parts B, D, and E. **(B)** Representative flow cytometric plots and gating strategy used to identify MDM and T cells in co-culture and the fraction of Gag+ cells of both types. **(C)** Flow cytometric histograms illustrating the PacBlue signal detected in the indicated cell type following treatment with the indicated antibody. **(D)** Summary graph of the percentage of cells of the indicated type that are Gag+ following infection by HIV-1 89.6 **(E)** Summary graph of the percentage of cells of the indicated type that are Gag+ following infection by HIV-1 T/F clone REJO.

Other investigators have reported that HIV inhibits *MRC1* transcription in macrophages and that MR inhibits virion egress upon exogenous expression in 293T cells (Sukegawa et al., 2018). In contrast to results we report here, the prior study observed effects on virions that were Env-independent and did not examine effects of Vpr on MR. In primary macrophages, Vpr-sensitive virion restriction only occurs when virions contain Env (Mashiba et al., 2014) and genetic changes in the *env* open reading frame – especially those that alter N-linked glycosylation sites – critically affect the requirement for Vpr. The effect of MR on Env and Env-containing virion release reported here helps explain previous observations that primate lentivirus infection reduces MR activity in humans (Koziel et al., 1998; Koziel et al., 1993) and monkeys (Holder et al., 2014). By confirming and extending our prior finding that Vpr-mediated stabilization of Env promotes macrophage to T cell spread we also provide an explanation for how Vpr increases infection of human lymphoid tissue *ex vivo* (Eckstein et al., 2001; Rucker et al., 2004), which contain macrophages and T cells in a highly physiological, three-dimensional environment.

As Nef had already been shown to reduce MR surface expression (Vigerust et al., 2005), the observation that HIV encodes a second protein, Vpr, to reduce MR expression

[14] The data in this figure was generated by Jay Lubow and Valeri Terry.

was unanticipated, but not unprecedented; other host proteins are known to be affected by more than one lentiviral accessory protein. The HIV receptor, CD4, is simultaneously targeted by Vpu, Nef and Env in HIV-1 (Chen et al., 1996) and tetherin is alternately targeted by Vpu, Nef, or Env in different strains of primate lentiviruses (Harris et al., 2012). Nef has also been shown to downmodulate the viral co-receptors CXCR4 (Venzke et al., 2006) and CCR5 (Michel et al., 2005), which may also interfere with Env expression and viral egress in infected cells. Nef's activity against CXCR4, CCR5, and MR presumably has the same ultimate purpose as its activity against CD4, namely to stabilize Env, enhance virion release and prevent superinfection of the producer cell (Lama et al., 1999; Ross et al., 1999). The impact of these deleterious interactions is clearly demonstrated by the profound loss of Env we observed in HIV-infected macrophages lacking both Vpr and Nef.

The need for both Vpr and Nef to counteract MR may be explained by the high level of MR expression, estimated at 100,000 copies per macrophage (Stahl et al., 1980). The potent combined effect likely derives from synergistic targeting of MR at two different stages of MR synthesis. Nef was shown to alter MR trafficking (Vigerust et al., 2005) and we show Vpr inhibits MR transcription.

In addition, our results suggest that maximal MR downmodulation is time-dependent in macrophages, which have the capacity to survive while infected for weeks; western blot analysis of whole cell lysates from saturated, ten-day infected cultures achieved a more striking reduction than was observed by flow cytometric analysis of five day cultures of macrophages infected with non-spreading viruses expressing GFP. This time dependency is potentially explainable in part by very long half-life of MR [33 hours

(Lennartz et al., 1989)] combined with the large amount of MR expressed per cell discussed above.

In sharp contrast to the effect we observed in MDM, Vpr did not affect MR protein levels when MR was expressed via a heterologous promoter in the 293T cell line, which is derived from human embryonic kidney cells and is not a natural target of HIV. The cell type selectivity in these experiments is likely due to differences in the promoters driving MR expression, however, we cannot rule out the existence of other macrophage specific pathways required to recreate the effect of Vpr on MR. Further work will be needed to examine these questions and determine other mechanistic details.

Our findings also implicate the Vpr binding protein [VprBP/DCAF1 (McCall et al., 2008)], a component of the cellular DCAF1-DDB1-CUL4 E3 ubiquitin ligase complex in downmodulation of MR by Vpr. This complex is required for most of the known functions of Vpr, including: disruption of the cell cycle, disruption of cellular DNA repair pathways in dividing cells (Belzile et al., 2007; Hrecka et al., 2007; Lahouassa et al., 2016; Le Rouzic et al., 2007; Wen et al., 2007; Wu et al., 2016; Zhou et al., 2016) and transcriptional inhibition of type I interferons in response to infection in macrophage cultures (Laguette et al., 2014; Mashiba et al., 2014). Additional research is now needed to determine how interactions between Vpr and DCAF1 mediate these pleiotropic effects.

Deleterious interactions between MR and Env that are alleviated by Vpr and Nef, likely occur along the secretory pathway and continue at the cell surface. This is based on previously published work showing that Env-containing virions are retained at the cell surface and targeted to lysosomes in macrophages lacking Vpr. Our prior studies also provided evidence that unprocessed Env gp160 is affected and targeted to lysosomal

compartments albeit to a lesser degree (Mashiba et al., 2014). Because Env processing occurs via furin-mediated cleavage in the trans-Golgi network (TGN), the effect on unprocessed Env provides evidence that in addition to acting at the surface, MR likely also interacts with Env along the secretory pathway prior to its arrival and processing in the TGN.

MR's interaction with Env appears to be mediated by the unusually high density of N linked glycosylation sites on Env that retain high-mannose glycans, which is a known pathogen-associated molecular pattern (McGreal et al., 2006; Stahl and Ezekowitz, 1998). Here, we show that selective deletion of mannose residues alleviated the requirement for Vpr. Deletion of individual glycosylation sites is known to lead to changes in the processing of neighboring glycans and deletions at certain sites lead to larger than expected losses of oligomannose (Balzarini, 2007) presumably because their removal allows greater access to mannosidases and facilitates trimming of surrounding glycans. Selective pressure to maintain mannose residues on Env may be due to the enhanced attachment they mediate. Indeed, we provide strong evidence that Env's interaction with MR boosts initial infection of MDM. This finding is supported by a prior report that MR enhances HIV-1 binding to macrophages and transmission of the bound virus to co-cultured T cells (Nguyen and Hildreth, 2003). Our study adds to these findings by providing evidence that interactions with mannose binding receptors also enhance direct infection of macrophages. Moreover, the capacity of Vpr and Nef to mitigate the effect of detrimental intracellular interactions during viral egress limits the negative impact of retaining high-mannose on Env. In addition, the dense glycan packing, which is privileged

from antibody recognition through immune tolerance, is believed to play a role in evasion of the antibody response (Stewart-Jones et al., 2016).

Because MR has both positive and negative effects on infection, the interpretation of some experiments examining spreading infection in the setting of MR silencing or mutations in Env that reduced mannose content were complex to interpret. Some donors had increased infection resulting from MR silencing whereas others had a small decrease at the ten-day time point (data not shown). By using viral systems that allowed us to focus independently on viral entry and exit, we nevertheless clearly discerned that MR can serve as a positive factor for entry and a negative factor for egress.

Thus far, all viral Envs we have tested (NL4-3, AD8 and 89,6) require Vpr for stable expression in macrophages except YU2. We show here that genetically altering the mannose patch on 89.6 so that it mirrored changes in the YU-2 mannose patch altered the behavior of 89.6 to resemble that of YU-2 with respect to Vpr phenotypes. This is strong evidence supporting our model that Vpr alleviates deleterious interactions caused by the Env mannose patch. Interestingly, YU-2 was cloned from the central nervous system and 89.6 was directly cloned from peripheral blood. Because the blood-brain barrier limits exposure to antibodies, CNS isolates may have a diminished requirement for high mannose residues, which protect from antibody responses.

Here we also confirm and extend our prior observation in Chapter 2 that co-culturing T cells with infected MDM boosted HIV infection compared to direct infection of T cells with cell-free virus. Similar to clone 89.6, T cell infection by the transmitter/founder virus, REJO, was enhanced by co-culture with MDM, and spread from MDM to T cells was enhanced by Vpr. In the context of natural person-to-person transmission,

accelerated spread to T cells may be critical to establishing a persistent infection before innate and adaptive immune responses are activated. The strong selective pressure to retain Vpr despite its limited effect on T cell-only cultures indicates there is more to learn about the role of Vpr, macrophages and T/F viruses in HIV transmission and pathogenesis. Collectively, these studies suggest that novel therapeutic approaches to inhibit the activity of Vpr and Nef in macrophages would potentially represent a new class of antiretroviral drug that could be an important part of a treatment or prophylactic cocktail.

Materials and Methods

Viruses, viral vectors, and expression plasmids

The following molecular clones were obtained via the AIDS Reagent Program: p89.6 [cat# 3552 from Dr. Ronald G. Collman), pNL4-3 (cat# 114 from Dr. Malcolm Martin), pREJO.c/2864 (cat# 11746 from Dr. John Kappes and Dr. Christina Ochsenbauer) and pYU2 (cat# 1350 from Dr. Beatrice Hahn and Dr. George Shaw). *Vpr*-null versions of 89.6, NL4-3, and YU2 were created by cutting the AflII site within *vpr* and filling in with Klenow fragment. The *vpr*-null version of REJO was created using by doing the same at the AvrII site. A *nef*-null version of 89.6 was created by deleting *nef* from its start codon to the XhoI site. To do this, a PCR amplicon was generated from the XhoI site in *env* to *env*'s stop codon. The 3' reverse primer added a XhoI site after the stop codon. The 89.6 genome and the amplicon were digested with XhoI and ligated together. (5' primer CACCATTATCGTTTCAGACCCT and 3' primer TCTCGAGTTTAAACTTATAGCAAAG CCCTTTCCA). The NL4-3 env^{YU2} chimera consists of the pNL4-3 plasmid in which the fragment from the KpnI site in *env* to the BamHI site in *env* has been replaced with the equivalent fragment of pYU-2. Because the KpnI site is not unique within the plasmid, the fragment from the SalI site to BamHI site (which are unique) was cloned into pUC19, the change was made in *env*, and the fragment from SalI to BamHI was inserted back into pNL4-3. To generate p89.6 N230D N339E a synthetic DNA sequence (ThermoFisher, Waltham, Massachusetts) was purchased commercially. The synthetic gene contained the following nucleotide mutations, counting from the start of 89.6 *env*: 694 A>G, 701 C>A, 1018 A>G, 1020 T>A. This sequence was substituted into p89.6 using the KpnI and BsaBI sites within *env*.

pSIV3+, pSPAX2, pAPM-1221 and pMD2.G were obtained from Dr. Jeremy Luban (Pertel et al., 2011). pSIV3+ *vpr*-null was generated using a synthesized DNA sequence (ThermoFisher) containing a fragment of the SIV genome in which the Vpr start codon was converted to a stop codon (TAG). This was substituted into pSIV3+ using the sites BstBI and SapI. pYU2 env was obtained from Dr. Joseph Sodroski (Sullivan et al., 1995). Creation of pNL4-3 ΔGPE-GFP was described previously (McNamara et al., 2012; Zhang et al., 2004). Notably, the transcript containing the *gfp* gene retains the first 42 amino acids of *env*, including the signal peptide, which creates a fully fluorescent Env-GFP fusion protein. The *vpr*-Q65R mutant of NL4-3 ΔGPE-GFP was created using the Q5 site-directed mutagenesis kit from New England Biolabs (Ipswich, MA). The forward primer was AGAATTCTG*C**G**A*CAACTGCTG and the reverse primer TATTATGGCTTCCACTCC. After synthesis by PCR, the entire provirus was confirmed by sequencing.

pCDNA.3.hMR was obtained from Dr. Johnny J. He (Liu et al., 2004). pPROA-3FLAG-UNG2-EYFP was obtained from Dr. Marit Otterlei (Akbari et al., 2010) and 3x FLAG tagged UNG2 was amplified using the 5' primer CTAGCTCGAGACCATGGACTACAAAGACCATGAC, which added an XhoI site, and the 3' primer GTTAACTCACAGCTCCTTCCAGTCAATGGGCTT, which added an HpaI site. The amplicon was cloned into the XhoI and HpaI sites of pMSCV IRES-GFP (Van Parijs et al., 1999) to generate pMSCV 3xFLAG UNG2 IRES-GFP.

Primary MDM and T cell isolation and culture

Leukocytes isolated from anonymous donors by apheresis were obtained from the New York Blood Center Component Laboratory. The use of human blood from anonymous, de-identified donors was classified as non-human subject research in accordance with federal regulations and thus not subjected to formal IRB review. Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll density gradient. $CD14^+$ monocytes were positively selected using a CD14 sorting kit (cat# 17858, StemCell Technologies, Vancouver, Canada) following the manufacturer's instructions. Monocyte-derived macrophages (MDM) were obtained by culturing monocytes in R10 [RPMI-1640 with 10% certified endotoxin-low fetal bovine serum (Invitrogen, ThermoFisher)], penicillin (10 Units/mL), streptomycin (10 µg/mL), L-glutamine (292 µg/mL), carrier-free M-CSF (50 ng/mL, R&D Systems, Minneapolis, Minnesota) and GM-CSF (50 ng/mL, R&D Systems) for seven days. Monocytes were plated at $5x10^5$ cells/well in a 24 well dish, except for those to be transduced with lentivirus and puromycin selected, which were plated at 1 $x10^6$ cells/well.

CD4+ T lymphocytes were prepared from donor PBMCs as follows: anti-CD8 Dynabeads (cat# 11147D, ThermoFisher) were used to deplete CD8+ T lymphocytes and the remaining cells, which were mainly CD4+ lymphocytes, were maintained in R10 until the time of stimulation. Lymphocytes were stimulated with 5 µg/mL phytohemagglutinin (PHA-L, Calbiochem, Millipore Sigma, Burlington, Massachusetts) overnight before addition of 50 IU/mL recombinant human IL-2 (R&D Systems).

Cell Lines

The 293T cell line was obtained from ATCC and independently authenticated by STR profiling. It was maintained in DMEM medium (Gibco) supplemented with 100 U/mL penicillin, 100 □g/mL streptomycin, 2 mM glutamine (Pen-Strep-Glutamine, Invitrogen), 10% fetal bovine serum (Invitrogen), and 0.022% plasmocin (Invivogen). The MOLT-R5 cell line was obtained from the NIH AIDS Reagent Repository, which confirmed the lot is mycoplasma negative. It was maintained in RPMI-1640 medium (Gibco) supplemented with 100 U/mL penicillin, 100 □g/mL streptomycin, 2 mM glutamine (Pen-Strep-Glutamine, Invitrogen), 10% fetal bovine serum (Invitrogen), and 0.022% plasmocin (Invivogen).

Silencing by shRNA

Sequences within *MRC1* suitable for shRNA-based targeted were identified using the program available at http://katahdin.mssm.edu/siRNA/RNAi.cgi?type=shRNA maintained by the laboratory of Dr. Ravi Sachidanandam. The sequence chosen, 5'-AGTAACTTGACTGATAATCAAT-3' was synthesized as part of larger DNA oligonucleotides with the sequences TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAGTAACTTGACTG ATAATCAATTAGTGAAGCCACAGATGTAATTGATTATCAGTCAAGTTACTTGCCTAC TGCCTCGG (forward) and AATTCCGAGGCAGTAGGCAAGTAACTTGACTGATAATCAATTACATCT GTGGCTTCACTAATTGATTATCAGTCAAGTTACTCGCTCACTGTCAACAGCAATATA CCTTC (reverse). These oligos were annealed, which created overhangs identical to

those produced by digestion with the enzymes EcoRI and XhoI. This double stranded DNA oligomer was inserted into the EcoRI and XhoI sites of pAPM-1221 to generate pAPM-MRC1-C.

Short hairpin RNA-mediated silencing was performed as previously described (Mashiba et al., 2014; Pertel et al., 2011). Briefly, we spinoculated freshly isolated primary monocytes with VSV-G-pseudotyped SIV3+ *vpr*-null at 2500 rpm for 2 hours with 4 μg/mL polybrene to allow Vpx-dependent degradation of SAMHD1. Cells were then incubated overnight in R10 with M-CSF (50 ng/mL) and GM-CSF (50 ng/mL) plus VSV-G-pseudotyped lentivirus containing an shRNA cassette targeting luciferase (pAPM-1221 or "shNC") or MR (pAPM-MRC1-C or "shMR"). The following day, media was removed and replaced with fresh R10 with M-CSF (50 ng/mL) and GM-CSF (50 ng/mL). Three days later 10 μg/mL puromycin was added and cells were cultured for 3 additional days prior to HIV-1 infection. shRNA target sequences used: *Luciferase*: 5'-TACAAACGCTCTCATCGACAAG-3', *MRC1:* 5'-ATTGATTATCAGTCAAGTTACT-3'

Virus production

Virus stocks were obtained by transfecting 293T cells (ATCC, Manassas, Virginia) with viral DNA and polyethylenimine (PEI). Cells were plated at $2.5x10^6$ cells per 10cm dish and incubated overnight. The following day 12 μg of total DNA was combined with 48 μg of PEI, mixed by vortexing, and added to each plate of cells. For NL4-3 ΔGPE-GFP cells were transfected with 4 μg viral genome, 4 μg pCMV-HIV, and 4 μg pHCMV-V (VSV-G expression plasmid). For SIV3+ *vpr*-null the cells were transfected with 10.5 μg of viral genome and 1.5 μg pHCMV-V. For shLentivirus (shNC or shMR) cells were transfected

with 6μg pAPM-1221 or pAPM-MRC1-C, 4.5μg pSPAX2, and 1.5μg pMD2.G. Viral supernatant was collected 48 hours post-transfection and centrifuged at 1500 rpm 5 min to remove cellular debris. SIV3+ *vpr*-null was pelleted by centrifugation at 14,000 rpm for 4 hours at 4°C and resuspended at 10x concentration. Virus stocks were aliquoted and stored at -80°C.

Co-transfections

Co-transfections of HIV and MR or UNG2 were performed in 293T cells. Cells were plated at $1.6x10^5$ per well in a 12-well dish. The following day 10 ng of pcDNA.3.hMR or 10ng of pMSCV 3xFLAG UNG2 IRES-GFP, 250 ng of NL4-3 ΔGPE-GFP, and 740 ng pUC19 plasmid was combined with 4μg PEI, mixed by vortexing, and added to each well. 48 hours later, cells were lifted using enzyme free cell dissociation buffer (ThermoFisher, cat# 13150016) and analyzed by flow cytometry or lysed in 500μL blue loading buffer (cat# 7722, Cell Signaling Technology, Danvers, Massachusetts) and analyzed by western blot.

HIV infections of MDM

Prior to infection, 500μL of medium was removed from each well and this "conditioned" medium was saved to be replaced after the infection. MDM were infected by equal inocula of HIV as measured by Gag p24 mass in 500μL of R10 for 6 hours at 37°C. After 6 hours, infection medium was removed and replaced with a 1:2 mixture of conditioned medium and fresh R10. Where indicated, HIV spread was blocked by AMD3100 (10μg/mL, AIDS Reagent Program cat# 8128) and/or maraviroc (20μM, AIDS

Reagent Program cat# 11580) added 48 hours post-infection and replenished with each media change every three days.

Spin transduction of MDM with NL4-3 ΔGPE-GFP

MDM were centrifuged at 2500rpm for 2 hours at 25°C with equal volume of NL4-3 ΔGPE-GFP or an isogenic mutant in 500uL total medium. Following infection, medium was removed and replaced with a 1:2 mixture of conditioned medium and fresh R10.

Adenoviral transduction of MDM

Adenovirus was prepared by the University of Michigan Vector Core, and the transduction of MDM was performed as previously described (Leonard et al., 2011) at an MOI of 1000 based on 293T cell infection estimations and the concentration of particles as assessed by OD_{280}.

Infection of T cells

Activated T cells were infected by two methods as indicated. For direct infection, 5 $x10^5$ cells were plated per well with 50µg HIV p24 in 500µL R10 +50IU/mL of IL-2 and incubated at 37°C for 48 hours. For co-culture with autologous, infected MDM medium was removed from MDM wells and 5 $x10^5$ T cells were added in 1mL R10 + 50IU/mL of IL-2. All T cell infections were collected 48 hours post infection.

Flow cytometry

Intracellular staining of cells using antibodies directed against HIV Gag p24, MR and FLAG-UNG2 was performed by permeabilizing PFA-fixed cells with 0.1% Triton-X in PBS for 5 min, followed by incubation with antibody for 20 minutes at room temperature. For Gag and MR, PE-conjugated primary antibodies were used. For FLAG-UNG2 cells were stained with a PE-conjugated goat anti-mouse IgG1 secondary antibody for 20 minutes at room temperature. Surface staining for CD4, CD3 and CD14 was performed before fixation as described in Chapter 2. Flow cytometric data was acquired using a FACSCanto instrument with FACSDiva collection software (BD, Franklin Lakes, New Jersey) or a FACScan (Cytek, BD) with FlowJo software (TreeStar, Ashland, Oregon) and analyzed using FlowJo software. Live NL4-3 ΔGPE-GFP transduced cells were sorted using a FACSAria III (BD) or MoFlo Astrios (Beckman Coulter) and gating on GFP^+ cells.

Quantitative RT-PCR

MDM sorted as described above in "Flow cytometry" were collected into tubes containing RLT buffer (Qiagen, Hilden, Germany) and RNA was isolated using RNeasy Kit (Qiagen) with on-column DNase I digestion. RNA was reverse transcribed using qScript cDNA SuperMix (Cat #95048, Quantabio, Beverly, Massachusetts). Quantitative PCR was performed using TaqMan Gene Expression MasterMix (ThermoFisher, cat# 4369016) on an Applied Biosystems 7300 Real-Time PCR System using TaqMan Gene Expression primers with FAM-MGB probe. The primer/probe sets for *ACTB* (Hs99999903), *MRC1* (Hs00267207), *POL2A* (Hs02786624), and *GAPDH* (Hs00172187) were purchased from ThermoFisher. Reactions were quantified using ABI Sequence

Detection software compared to serial dilutions of cDNA from mock-treated cells. Measured values for all genes were normalized to measured values of *GAPDH* or *ACTB* as indicated.

Immunoblot

MDM cultures were lysed in Blue Loading Buffer (cat# 7722, Cell Signaling Technology), sonicated with a Misonix sonicator (Qsonica, LLC., Newtown, Connecticut), boiled for 5 min at 95°C and clarified by centrifugation at 8000 RPM for 3 minutes. Lysates were analyzed by SDS-PAGE immunoblot. The proteins MR, GAPDH and pr55 were visualized using AlexFluor-647 conjugated secondary antibodies on a Typhoon FLA 9500 scanner (GE, Boston, Massachusetts) and quantified using ImageQL (GE). The proteins gp160, gp120, gp41, Nef, Vpr, GFP, Env-GFP, STING, GBP5, and IFITM3 were visualized using HRP-conjugated secondary antibodies on film. Immunoblot films were scanned and the mean intensity of each band, minus the background, was calculated using the histogram function of Photoshop CC (Adobe, San Jose, California).

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). Gag p24 antibody (clone 183-H12-5C, AIDS Reagent Program cat# 1519 from Dr. Bruce Cheseboro and Dr. Hardy Chen) was bound to Nunc MaxiSorp plates (ThermoFisher cat# 12-565-135) at 4°C overnight. Lysed samples were captured for 2 hr and then incubated with biotinylated antibody to Gag p24 (clone 31-90-25, ATCC cat# HB-9725) for 1 hr. Clone 31-90-25 was biotinylated

with the EZ-Link Micro Sulfo-NHS-Biotinylation Kit (ThermoFisher cat# PI-21925). Clones 31-90-25 and 182-H12-5C were purified using Protein G columns (GE Healthcare, cat# 45-000-054) following the manufacturer's instructions. Samples were detected using streptavidin-HRP (Fitzgerald, Acton, Massachusetts) and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma cat# T8665-IL). CAp24 concentrations were measured by comparison to recombinant CAp24 standards (cat# 00177-V, ViroGen, Watertown, Massachusetts).

Antibodies

Antibodies to CAp24 (clone KC57-PE cat# 6604667 and KC57-FITC cat# 6604665, Beckman Coulter, Brea, California), CD3 (clone OKT3-Pacific Blue, cat# 317313, BioLegend, San Diego, California), CD14 (clone HCD14-APC, cat# 325608, BioLegend), CD4 (clone OKT4, cat#17-0048-42, Invitrogen, ThermoScientific), FLAG (clone M2, cat#F3165, Sigma), and MR (clone 19.2-PE, cat# 555954, BD) were used for flow cytometry. Antibodies to the following proteins were used for immunoblot analysis: MR (cat# ab64693, Abcam, Cambridge, Massachusetts), GAPDH (clone 3C2, cat# H00002597-M01, Abnova, Taipei, Taiwan), Gag pr55 (HIV-Ig AIDS Reagent Program cat# 3957), Env gp160/120 (AIDS Reagent Program cat# 288 from Dr. Michael Phelan), 89.6 and YU-2 Env gp41 (clone z13e1, AIDS Reagent Program cat# 11557 from Dr. Michael Zwick), NL4-3 Env gp41 (clone CHESSIE-8, AIDS Reagent Program cat# 526 from Dr. George Lewis), Vpr (AIDS Reagent Program cat# 3951 from Dr. Jeffrey Kopp), GFP (cat# ab13970, Abcam), Nef (AIDS Reagent Program cat# 2949 from Dr. Ronald Swanstrom), FLAG (clone M2, cat# F3165, Sigma), STING (D2P2F, cat# 13647, Cell

Signaling Technology), GBP5 (sc-160353, which was a generous gift from Dr. Frank Kirchhoff), and IFITM3 (cat# 11714-1-AP, Proteintech, Rosemont, IL). Neutralizing antibody 2G12 (AIDS Reagent Program cat# 1476 from Dr. Hermann Katinger) was used at a 1 μg/mL at the time of infection. Antibody clone CHESSIE-8 was purified using Protein G columns (GE Healthcare, cat# 45-000-054) following the manufacturer's instructions.

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CHAPTER 3

HIV-1 Vpr combats the PU.1-driven antiviral response in primary human macrophages[1]

Abstract

HIV-1 Vpr promotes efficient spread of HIV-1 from macrophages to T cells by transcriptionally downmodulating restriction factors that target HIV-1 Envelope protein (Env). Here we find that Vpr induces broad transcriptomic changes by targeting PU.1, a transcription factor necessary for expression of host innate immune response genes, including those that target Env. Consistent with this, we find silencing PU.1 in infected macrophages lacking Vpr rescues Env. Vpr downmodulates PU.1 through a proteasomal degradation pathway that depends on physical interactions with PU.1 and DCAF1, a component of the Cul4A E3 ubiquitin ligase. The capacity for Vpr to target PU.1 is highly conserved across primate lentiviruses. In addition to impacting infected cells, we find that Vpr suppresses expression of innate immune response genes in uninfected bystander cells, and that virion-associated Vpr can degrade PU.1. Together, we demonstrate Vpr counteracts PU.1 in macrophages to blunt antiviral immune responses and promote viral spread.

[1] This chapter is modified from an existing preprint for an accepted publication in Nature Communications: Virgilio, M.C., Ramnani, B., Chen, T., Disbennett, W.M., Lubow, J., Welch, J.D., Collins, K.L., 2024. HIV-1 Vpr combats the PU.1-driven antiviral response in primary human macrophages. https://doi.org/10.1101/2023.03.21.533528

Introduction

The HIV-1 genome encodes several accessory proteins that counteract innate and adaptive antiviral responses. Although HIV accessory proteins have been widely studied, the role of the Vpr accessory protein remains enigmatic. Vpr is highly conserved amongst lentiviruses and is necessary for optimal replication in macrophages (Balliet et al., 1994). In addition, it is unique amongst HIV accessory proteins in that it is packaged in the virus particle at high levels through specific interactions with p6Gag (Lu et al., 1995). Studies using human lymphoid tissue, which is rich in both T cells and macrophages, have shown that loss of Vpr decreases virus production, but only when the virus strain is capable of efficiently infecting macrophages (Balliet et al., 1994; Connor et al., 1995; Dedera et al., 1989; Eckstein et al., 2001; Rücker et al., 2004). These studies provide evidence that Vpr enhances infection of macrophages and increases viral burden in tissues containing macrophages. Vpr localizes to the nucleus (Lu et al., 1993), where it induces G2 cell cycle arrest by targeting a variety of host factors involved in post-replication DNA repair (Ahn et al., 2010; Laguette et al., 2014; Lahouassa et al., 2016; Romani et al., 2015; Wang et al., 2013; Withers-Ward et al., 2000; Yan et al., 2019, 2018). To target these factors, Vpr requires the host protein DCAF1 (also known as Vpr binding protein (VprBP)) (Zhang et al., 2001). Through DCAF1, Vpr interacts with damaged DNA binding protein 1 (DDB1) as part of the Cul4A E3 ubiquitin ligase complex where host proteins recruited by Vpr are ubiquitylated and degraded (Belzile et al., 2007; Hakata et al., 2014; Hrecka et al., 2016; McCall et al., 2008; Romani and Cohen, 2012; Schröfelbauer et al., 2007). However, the effects of Vpr on DNA repair and cell cycle arrest do not provide a clear explanation for how Vpr enhances HIV infection of macrophages.

Macrophages are terminally differentiated antigen-presenting cells that are critical for many immune functions, including antiviral innate immune responses (Lavin et al., 2015; Silvin and Manel, 2015). Macrophage identity is tightly controlled through the timed expression of myeloid transcription factors, particularly PU.1 [reviewed in (Turkistany and Dekoter, 2011)]. PU.1 is a hematopoietic-specific and ETS family transcription factor that is essential for lymphoid and myeloid development (Dakic et al., 2005; Fisher and Scott, 1998; Gupta et al., 2009; Turkistany and Dekoter, 2011). ETS family proteins bind to purine-rich DNA domains with a central GGAA/T core consensus (Graves and Petersen, 1998). Macrophages require early and continuously high levels of PU.1 expression [reviewed in (Fisher and Scott, 1998; Turkistany and Dekoter, 2011)] to maintain normal functionality. PU.1 regulates many essential macrophage genes, including those for cytokine receptors M-CSF and GM-CSF and the adhesion molecule CD11b. PU.1 also coordinates with other transcription factors such as IRF4 and C/EBPa, and TET methyl cytosine dioxygenase 2 (TET2; also known as ten-eleven translocation 2) to regulate gene expression (de la Rica et al., 2013; Imperato et al., 2015; Marecki and Fenton, 2000; Turkistany and Dekoter, 2011).

We previously reported that Vpr counteracts accelerated degradation of the HIV Env protein, which occurs in HIV infected macrophages but not T cells (Collins et al., 2015; Mashiba et al., 2014). We hypothesized that Vpr disables a macrophage-specific restriction factor that detects and degrades HIV Env. We recently identified this factor as the macrophage mannose receptor (MR) (Lubow et al., 2020), which is highly expressed in macrophages but not T cells (Liang et al., 2019; Linehan et al., 1999). MR senses HIV Env via densely packed high mannose residues that serve as pathogen-associated

molecular patterns (PAMPs) because they are normally absent from host cellular proteins. MR recognition of HIV Env disrupts infection by promoting lysosomal degradation of Env and Env-containing viral particles (Collins et al., 2015; Lubow et al., 2020). Although the canonical targeting of host factors by Vpr involves the binding of Vpr to DCAF1, leading to ubiquitylation and degradation via the proteosome, Vpr does not directly interact with MR (Lubow et al., 2020). Instead, Vpr suppresses expression of the MR gene, *MRC1* (Lubow et al., 2020).

In this study, we examine the mechanism by which Vpr suppresses the expression of *MRC1* and demonstrate that Vpr additionally exerts suppressive effects on other important genes in macrophages, enhancing virion assembly and spread. Single-cell RNA sequencing (scRNA-seq) of primary human macrophages infected with infectious wild type or Vpr-null HIV allowed us to distinguish the effects of Vpr on both infected and virally exposed, uninfected cells in the same culture. Within infected cells, Vpr selectively downregulated genes controlled by the macrophage-selective transcription factor, PU.1. By targeting PU.1, Vpr systemically disrupted several antiviral factors, including *MRC1,* and *IFITM3,* a previously reported target of Vpr that is also capable of disrupting Env function (Wang and Su, 2019). Consistent with this, silencing PU.1 in macrophages infected with Vpr-null virus rescued Env production. Vpr caused a systemic reduction of genes implicated in Toll-like receptor (TLR) and type I interferon (IFN-I) signaling that affected all the cells in the culture. Thus, we provide a systems level explanation for the positive effect of Vpr on HIV spread in macrophages. Finally, we found that Vpr-mediated downmodulation of PU.1-regulated gene expression is mediated by protein-protein interaction between Vpr and PU.1, resulting in accelerated proteasomal degradation of

PU.1. This activity of Vpr is conserved among all HIV-1 molecular clones tested as well as HIV-2 and SIV. Remarkably, both the interaction between PU.1 and Vpr as well PU.1's subsequent degradation requires the Vpr interacting protein, DCAF1. The PU.1 transcriptional co-factor, TET2, is co-recruited with PU.1 to DCAF1 by Vpr. This aligns our results with other reports that Vpr targets TET2 (Lv et al., 2018; Wang and Su, 2019). Together, our data support a model in which Vpr promotes HIV spread via systemic detrimental effects on the host innate antiviral response to infection.

Results

Single-cell RNA sequencing of HIV-1 infected MDMs reveals Vpr-dependent transcriptional changes.

Vpr counteracts a number of host factors, primarily those implicated in DNA repair and restriction of HIV Env (Lubow et al., 2020; Wang and Su, 2019). Recently we demonstrated that Vpr decreases transcriptional expression of the host restriction factor, mannose receptor (*MRC1*), which we hypothesize results from Vpr targeting a macrophage-specific transcription factor (**Figure 3.1A**). To identify the transcription factor targeted by Vpr, we undertook an unbiased approach to characterize the genome-wide effects HIV-1 Vpr in monocyte derived macrophages (MDMs). For these studies, we used an infectious HIV molecular clone (89.6) that has or lacks an intact *vpr* gene (**Figure 3.1B**). We infected primary human $CD14^+$ MDMs from three independent healthy human donors and allowed the infection to spread for 10 days before harvesting cells (uninfected, 89.6^{wt} or $89.6^{\Delta vpr}$ infected) and prepared them for single-cell gene expression analysis (**Figure 3.1C**). LIGER (linked inference of genomic experimental relationships) (Welch et

al., 2019) was used to integrate all nine data sets. This analysis facilitated alignment despite significant transcriptomic differences between donors and sample types. Uniform manifold approximation and projection (UMAP) visualization of all donor sets showed no significant donor or batch-specific differences amongst the samples (**Figure 3.1D**). Based on gene expression patterns in each of the clusters, we determined the main cluster to be pro-inflammatory macrophages (Cluster 0), and we identified a minor population of cycling cells (Cluster 1) within the virus-treated cells (**Figure 3.1E**, **F**). These two clusters do not appear to have an infection phenotype because cells from cultures exposed to HIV were distributed across both the clusters (**Figure 3.1E**, **G**).

To separate the bona fide infected cells from bystander cells, we computationally segregated cells into a subset that expressed both *tat* and *gag* transcripts from those that expressed neither (**Figure 3.1H, Supplementary Table 3.1**). From a total of 13,639 WT-Vpr and 35,780 Vpr-*null* exposed MDMs, 6156 and 8699 were identified as gag^{+}/tat^{+}, respectively. The proportion of infected cells identified by donor using this analysis was similar to that found by the standard method of identifying infected cells by intracellular Gag staining and detection by flow cytometry (**Supplementary Table 3.2**). A comparison of gene expression profiles in cells with and without Vpr revealed that Vpr expression boosted HIV gene expression as previously reported (Zhang and Bieniasz, 2020) and caused a significant transcriptional shift in host gene expression (**Figure 3.1I**). Using a two-sided Wilcoxon rank-sum test and false discovery rate correction, we identified 3150 genes with statistically significant two-fold or greater differential expression between 89.6^{wt} and $89.6^{\Delta vpr}$-infected cells.

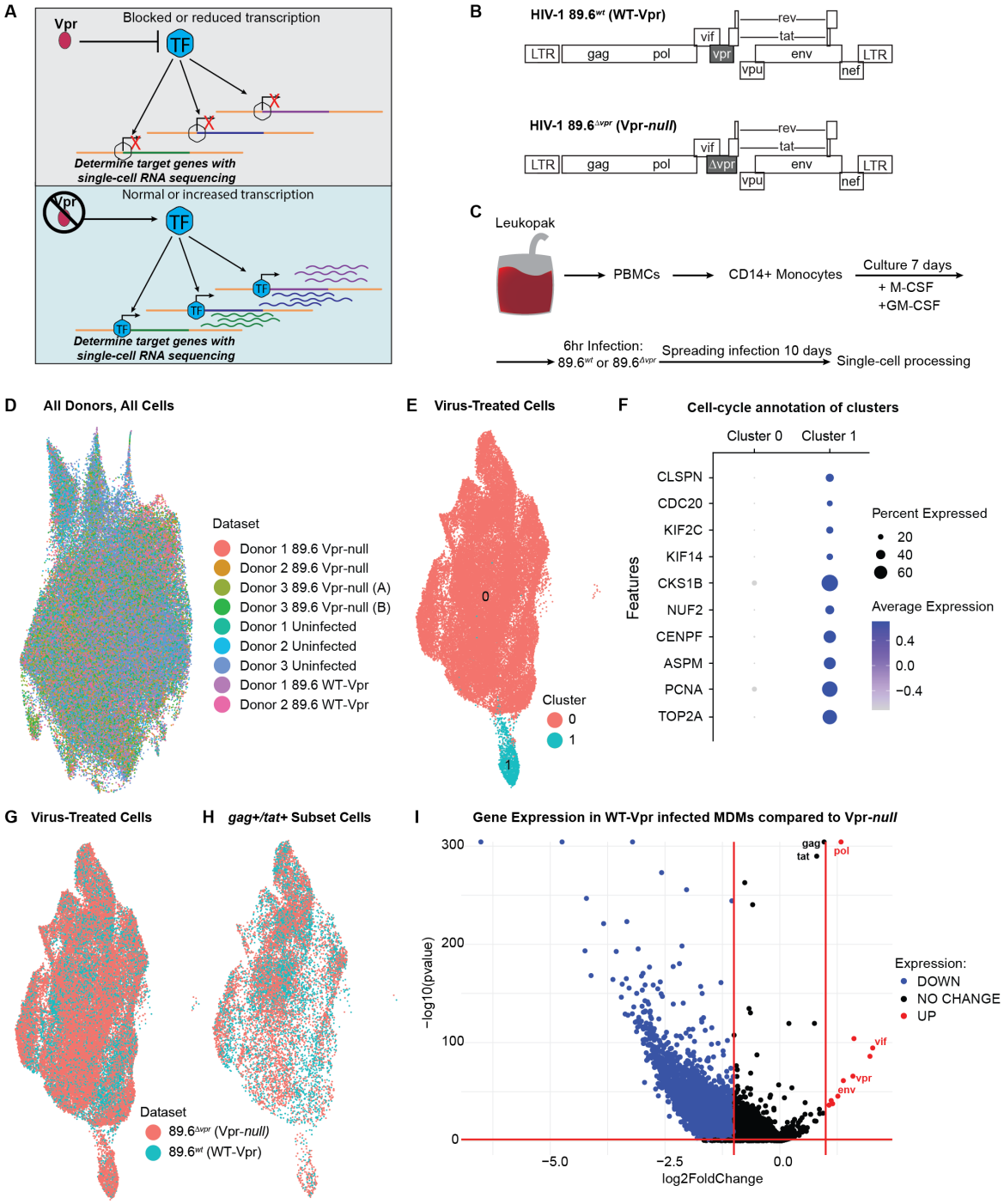

Figure 3.1. Single-cell RNA sequencing of HIV-1 infected MDMs reveals Vpr-dependent transcriptional changes.[2] (A) Schematic diagram illustrating the objective of single-cell RNA sequencing; the identification of Vpr-targeted transcription factor(s) in HIV infected primary

[2] This figure was created by Maria C. Virgilio

macrophages. (B) Genome maps for full-length 89.6^{wt} HIV-1 (top) and the same viral genome with a premature stop-codon in *vpr* (bottom). (C) Experimental setup for the generation of the scRNA-seq datasets. (D) UMAP representation of LIGER-integrated scRNA-seq data from MDM samples treated as shown in (C) and listed in Dataset. (E, G, H) UMAP representations of LIGER-integrated scRNA-seq data from MDMs treated as indicated. (E) Colors indicate individual clusters. Cluster 0 = pro-inflammatory macrophages, Cluster 1 = cycling cells. (F) Dot plot representation of cell-cycle genes used to determine clusters in (E). The size of the dot equates to the percentage of cells within the population expressing the feature, and the color indicates the average expression of the feature across all cells in each cluster. (G, H) Cells are colored according to whether they were exposed to 89.6 WT-Vpr virus (blue) or Vpr-null virus (pink). (H) Bona fide infected cells were identified based on expression of HIV *tat* and *gag*. (I) Volcano plot of differentially expressed genes from HIV 89.6^{wt} verses 89.6$^{\Delta vpr}$ infected MDMs from (H) as determined by two-sided Wilcoxin Rank Sum. Significance determined as greater than 1 log2 fold change and false discovery rate adjusted p-value of $p < 0.05$ (red-bars). Blue colored genes indicate genes less highly expressed in HIV 89.6^{wt} verses 89.6$^{\Delta vpr}$ infected MDMs, red colored genes indicate genes more highly expressed, and black colored genes indicate no significant difference between datasets.

Vpr-repressed genes in MDMs include targets of the transcription factor, PU.1.

To identify the transcription factor(s) targeted by Vpr, we used the DNA motif identification software, HOMER (hypergeometric optimization of motif enrichment), to scan for common transcription factor-binding motifs in the promoters of genes downregulated in the presence of Vpr. Our analysis revealed several candidate transcription factors (TFs) and TF-binding families, including many members of the ETS family of TFs (**Figure 3.2A; Supplementary Figure 3.9A**). However, only PU.1 (encoded by the gene *SPI1*) was highly expressed in our data sets (**Figure 3.2B**). Moreover, PU.1 appeared twice in the list – as both a member of the ETS family and as a co-factor in the ETS/IRF (interferon regulatory factor) family of transcription factors, a relationship that is well documented (Marecki and Fenton, 2000) (**Figure 3.2A**). PU.1 is the master myeloid transcription factor (reviewed in (Buckland, 2002; Dakic et al., 2005; Pham et al., 2013)). It is required for terminal differentiation of macrophages and is necessary to maintain macrophage immune function (Turkistany and Dekoter, 2011). From the list of most downregulated genes in Vpr-expressing MDMs, HOMER identified 316 genes with a PU.1

binding motif in their promoter and 670 PU.1-IRF co-regulated genes, for a combined 840 distinct PU.1-regulated genes identified (**Figure 3.2C, E**). One of the genes identified with a PU.1 binding motif was interferon induced transmembrane protein 3 (*IFITM3)*. IFITM3 is an important host restriction factor that is similar to mannose receptor (MR), in that IFITM3 targets HIV Env and has reduced gene expression (by reverse transcription quantitative PCR (RT-qPCR)) in the presence of Vpr in HIV-infected MDMs (Wang and Su, 2019). Gene ontology analysis of the 840 genes targeted by PU.1 revealed that they are enriched for genes involved in regulation of the immune system and defense response (**Figure 3.2D**).

Because we had previously demonstrated that the gene encoding mannose receptor (*MRC1*) was transcriptionally downmodulated approximately two-fold by Vpr in HIV infected primary human macrophages using RT-qPCR (Lubow et al., 2020), we examined our data set to specifically identify *MRC1* transcripts. In the scRNA-seq data set shown here, we identified a Vpr-dependent change in *MRC1* transcripts (log2FC = 0.932) that is statistically significant (false-discovery rate corrected two-sided Wilcoxon rank-sum p-value = 0.002) but fell just below our arbitrary cutoff of a two-fold change (**Figures 3.1H, 3.2C**). Consistent with the pattern we observed for other Vpr-suppressed genes, we identified several PU.1 binding sites in the promoter region of *MRC1* (**Supplementary Figure 3.10A**) when we manually scanned for PU.1 binding motifs, some of which have been previously described (Caldwell et al., 2000). Furthermore, MDMs transduced with a short hairpin RNA (shRNA)-expressing lentivirus targeting *SPI1* transcripts reduced both PU.1 and MR protein (**Supplementary Figure 3.10B**). While the effect of Vpr on *MRC1* transcriptional expression is modest, it combines synergistically

with HIV Nef-dependent disruption of MR trafficking, reversing mannose receptor-dependent lysosomal degradation of Env in HIV infected primary macrophages (Lubow et al., 2020).

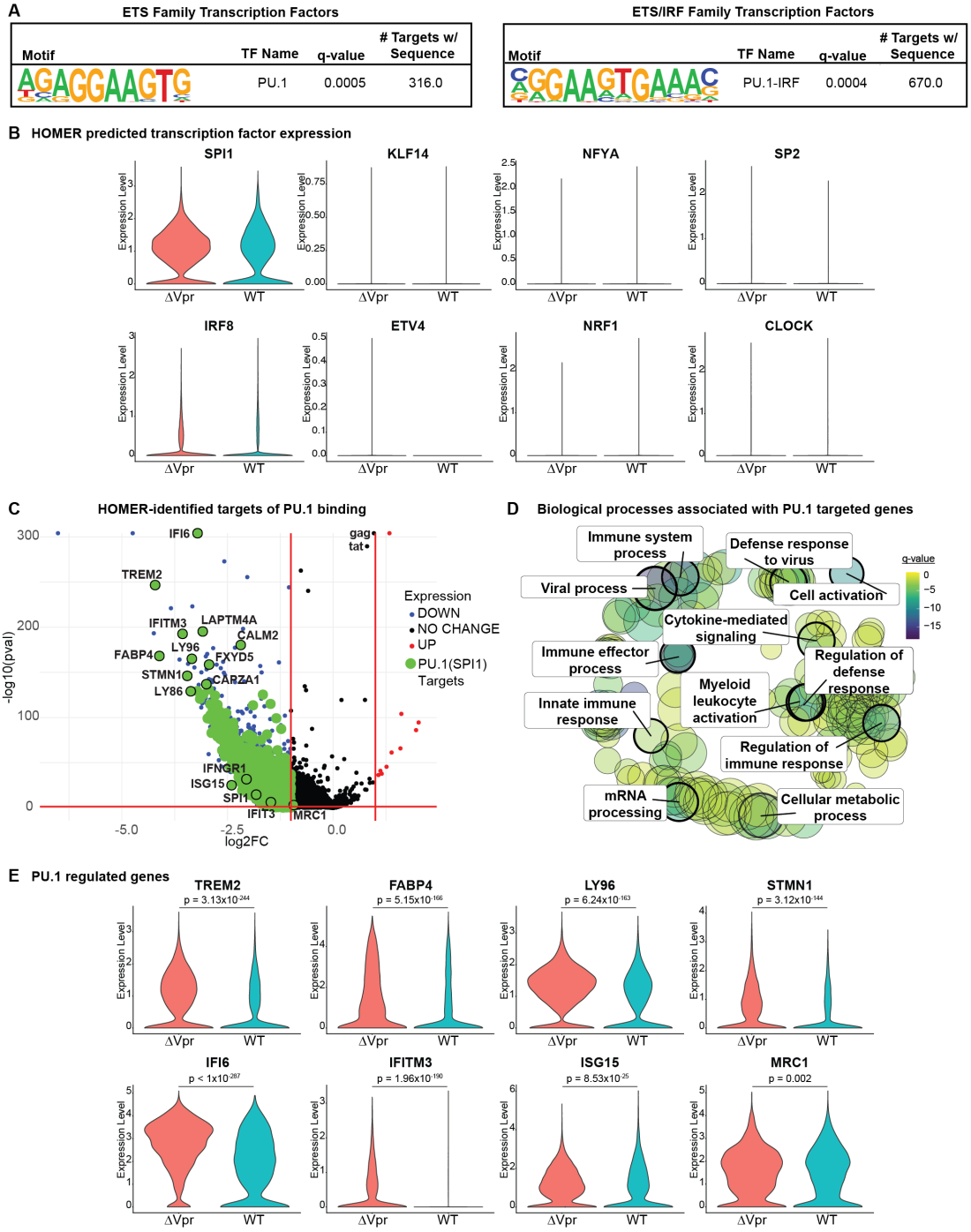

Figure 3.2. Vpr downmodulates PU.1-dependent transcription.[3] (A) PU.1 motifs identified by HOMER as present in the promoters of Vpr-downmodulated genes (**Figure 3.1I**, Blue). (B) Violin plots displaying RNA abundance of the indicated transcription factor genes in MDMs infected with the indicated virus. (C) Volcano plot as in **Figure 3.1I** except that genes containing a PU.1 or PU.1-IRF binding motif in their promoter region are highlighted in green (See also Supplementary **Figure 3.10**). (D) Biological processes associated with the PU.1 targeted genes from (C). Size of circles indicates the relative number of GO terms associated with the process. FDR adjusted q-values associated with GO terms are indicated by the color. Bolded rings are associated with biological processes listed. (E) Violin plots displaying RNA abundance of the indicated genes in MDMs infected with the indicated virus. HIV-1 89.6^{wt} (WT); HIV-1 $89.6^{\Delta vpr}$ (ΔVpr). False-discovery rate corrected two-sided Wilcoxon rank-sum p-values are shown above the conditions being compared.

Vpr suppresses PU.1 regulated genes implicated in Toll-like receptor and IFN-I responses.

PU.1 regulated genes that are downmodulated in Vpr-expressing cells include several factors implicated in Toll-like receptor (TLR) signaling pathways (**Figure 3.3A**). TLRs are evolutionarily conserved, pattern recognition receptors (PRRs) that recognize PAMPs (Beutler, 2009; Medzhitov et al., 1997; Molteni et al., 2016), including HIV. To determine whether these effects of Vpr are restricted to infected cells within the culture, we compared gene expression profiles amongst all the sample types collected from our single-cell analysis: uninfected (virus naïve), 89.6^{wt} and $89.6^{\Delta vpr}$-infected, and 89.6^{wt} and $89.6^{\Delta vpr}$ virally exposed but uninfected (bystander) cells. This was accomplished by computationally segregating cells into subsets that expressed both *tat* and *gag* transcripts from those that were virally exposed but appeared uninfected (*tat* and *gag*-negative) (**Figure 3.3B**).

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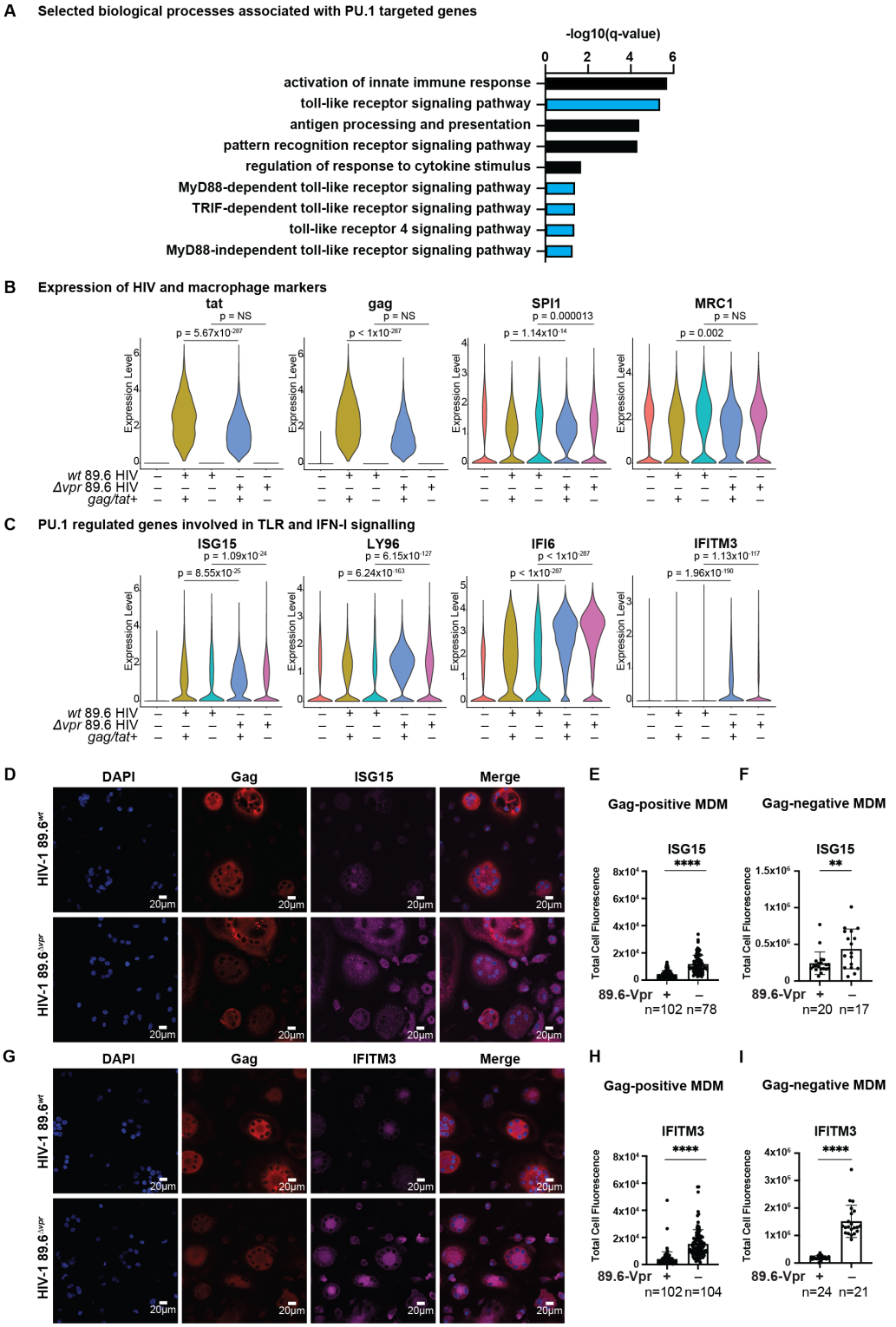
A
Selected biological processes associated with PU.1 targeted genes
-log10(q-value)
0
2
4
6
activation of innate immune response
toll-like receptor signaling pathway
antigen processing and presentation
pattern recognition receptor signaling pathway
regulation of response to cytokine stimulus
MyD88-dependent toll-like receptor signaling pathway
TRIF-dependent toll-like receptor signaling pathway
toll-like receptor 4 signaling pathway
MyD88-independent toll-like receptor signaling pathway
B
Expression of HIV and macrophage markers
tat
p = NS
p = 5.67x10-287
gag
p = NS
p < 1x10-287
SPI1
p = 0.000013
p = 1.14x10-14
MRC1
p = NS
p = 0.002
Expression Level
wt 89.6 HIV
Δvpr 89.6 HIV
gag/tat+
C
PU.1 regulated genes involved in TLR and IFN-I signalling
ISG15
p = 1.09x10-24
p = 8.55x10-25
LY96
p = 6.15x10-127
p = 6.24x10-163
IFI6
p < 1x10-287
p < 1x10-287
IFITM3
p = 1.13x10-117
p = 1.96x10-190
Expression Level
wt 89.6 HIV
Δvpr 89.6 HIV
gag/tat+
D
DAPI
Gag
ISG15
Merge
HIV-1 89.6wt
HIV-1 89.6Δvpr
20μm
E
Gag-positive MDM
ISG15
****
Total Cell Fluorescence
89.6-Vpr + –
n=102 n=78
F
Gag-negative MDM
ISG15
**
Total Cell Fluorescence
89.6-Vpr + –
n=20 n=17
G
DAPI
Gag
IFITM3
Merge
HIV-1 89.6wt
HIV-1 89.6Δvpr
20μm
H
Gag-positive MDM
IFITM3
****
Total Cell Fluorescence
89.6-Vpr + –
n=102 n=104
I
Gag-negative MDM
IFITM3
****
Total Cell Fluorescence
89.6-Vpr + –
n=24 n=21

Figure 3.3. Vpr counteracts the innate immune response to HIV infection.[4] (A) Selected biological processes associated with infection and inflammation (selected from GO terms represented in **Figure 3.2D**). Pathways were identified as associated with PU.1 regulated genes downmodulated in MDMs infected with 89.6^{wt} or $89.6^{\Delta vpr}$ virus as determined by expression of *gag* and *tat* transcripts (highlighted in **Figure 3.2C**). The -log10 FDR-adjusted p-values (q-values) are plotted for each gene ontology term. Blue bars represent terms associated with TLR signaling; Black bars represent related gene ontology terms that are similar, but not directly associated with TLR signaling. (B) Violin plots summarizing single-cell RNA transcripts expressed by primary human macrophages treated with the indicated virus and cultured for 10 days. *Gag+/tat+* are the subset of cells expressing HIV genes within in each culture. *SPI1* is the gene that codes for PU.1. *MRC1* is the gene that codes for mannose receptor. (C) Violin plots summarizing single-cell RNA transcripts expressed by primary human macrophages as in (B). False-discovery rate corrected two-sided Wilcoxon rank-sum p-values are shown above the conditions being compared. (D, G) Representative immunofluorescent images of MDMs from a single donor infected with either 89.6^{wt} or $89.6^{\Delta vpr}$ (MDMs from n=2 independent donors). HIV-infected cells identified by Gag staining. (E, H) Quantification of ISG15- or IFITM3-corrected total cell fluorescence in Gag+ cells, or (F, I) Gag- cells divided by the number of nuclei in the cell area. The number of cells quantified for each condition is indicated. Error bars represent standard error of the mean, n = the number of cells quantified. P values were determined using an unpaired two-sided t test. **, $p = 0.0096$; ****, $p < 0.0001$.

As expected, expression of genes involved in the TLR-mediated IFN-I response to infection, including interferon stimulated gene 15 (*ISG15),* which is upregulated in response to IFN-I and TLR-signaling [reviewed in (Perng and Lenschow, 2018; Schneider et al., 2014)], *LY96, and* interferon-inducible protein-6 (*IFI6)* (PU.1-IRF regulated genes) and *IFITM3* were very low in uninfected, unexposed cells (**Figure 3.3C**). In contrast, each of these genes was upregulated in cells from HIV-treated primary macrophage cultures (**Figure 3.3C**). As discussed above, Vpr counteracted upregulation of this antiviral response in infected cells (**Figure 3.2C, E** and **Figure 3.3C**). In addition, we were surprised to find many of these genes were upregulated in bystander cells as well (**Figure 3.3C**) and that Vpr limited their induction. For example, we found that *ISG15* was more highly expressed in $89.6^{\Delta vpr}$ bystander cells compared to 89.6^{wt}-exposed bystander cells. Similar observations were made for *LY96*, *IFI6*, and *IFITM3*. Interestingly, the protein

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product for *LY96* is myeloid differentiation 2 (MD2), which is required for TLR4 ligand-induced activation at the cell surface (Nagai et al., 2002; Shimazu et al., 1999). By comparison, other subsets of genes including *SPI1* and *MRC1* were primarily downmodulated in the infected subset and either not at all in the bystander cells or to a lesser extent than their infected counterparts (**Figure 3.3B**).

Next, we investigated whether we could observe a Vpr-dependent suppression of PU.1-regulated gene products involved in the TLR and IFN-I signaling pathways using confocal fluorescent microscopy, which allowed us to distinguish between infected and uninfected cells within the same culture of MDMs, analogous to our scRNA-seq experiments. Quantification of ISG15 and IFITM3 in Gag+ cells showed significant reduction of both PU.1 gene products in HIV-infected MDMs expressing Vpr relative to cells infected with a Vpr-*null* HIV (**Figure 3 E, H**). Strikingly, we also observed a similar pattern in virus-exposed but uninfected MDMs (**Figure 3F, I**), where uninfected cells exposed to wild type HIV had lower levels of PU.1 gene products compared to uninfected cells exposed to Vpr-*null* HIV. These results are consistent with the scRNA-seq data from **Figure 3C**, confirming Vpr-dependent downmodulation of PU.1 regulated genes and their protein products in wild type infected MDMs as well as bystander cells in the same culture.

*PU.1 protein levels decrease in the presence of Vpr***.**

Vpr acts as an adaptor protein that links host proteins to the Cul4A E3 ubiquitin ligase complex for ubiquitylation and degradation (McCall et al., 2008; Romani and Cohen, 2012). Thus, we hypothesized that Vpr downregulates PU.1-regulated genes in infected cells by targeting PU.1 for proteasomal degradation. To test our hypothesis, we

first investigated whether Vpr reduced PU.1 protein levels in infected MDMs. We treated macrophages with a VSV-G envelope-pseudotyped replication-defective clone of HIV NL4-3 that expresses GFP in the *env* reading frame (**Figure 3.4A**). We transduced MDMs with this virus containing all intact accessory proteins, or with additional mutations in the open reading frames of either *vpr, nef*, or both. We measured PU.1 levels by flow cytometry at 5-, 7-, and 10-days post infection. Notably, we found that Vpr expression resulted in lower PU.1 protein levels in infected (GFP^+) cells whether Nef was expressed or not (**Figure 3.4B, C**). These changes in PU.1 were consistently observed in MDMs from four independent donors (**Figure 3.4C**). Based on these results, we concluded that Vpr downmodulates PU.1 in HIV-infected primary human MDMs. Under the conditions of this assay, in which MDMs were treated with a replication-defective virus and analyzed at least five days post infection, we did not observe a significant Vpr-dependent downmodulation of PU.1 in uninfected (GFP^-) bystander cells (**Supplementary Figure 3.11A**).

To determine whether Vpr was sufficient for PU.1 downmodulation, we tested the ability of several 3xFLAG-tagged Vpr proteins derived from a panel of HIV molecular clones [89.6, NL4-3, AD8 and YU2-Vpr (**Figure 3.4D**)] to reduce exogenous PU.1 expression in transfected HEK 293T cells. Because the gene that encodes PU.1 (*SPI1*) contains PU.1 binding sites and is downmodulated by Vpr expression (**Figure 3.2B**), for these experiments PU.1 was expressed under the control of a heterologous promoter that allowed separation of transcriptional and post-transcriptional changes (**Figure 3.4E**). For comparison, we used the evolutionarily related protein, Vpx, from the $HIV2_{ROD}$ molecular clone. We found that all Vpr-expression constructs tested resulted in a notably lower level

of PU.1 protein compared to the level of PU.1 protein with HIV-2_{ROD}Vpx (**Figure 3.4F**). Vpr-dependent reduced levels of PU.1 were consistently observed in four independent experiments and were statistically significant (**Figure 3.4G**).

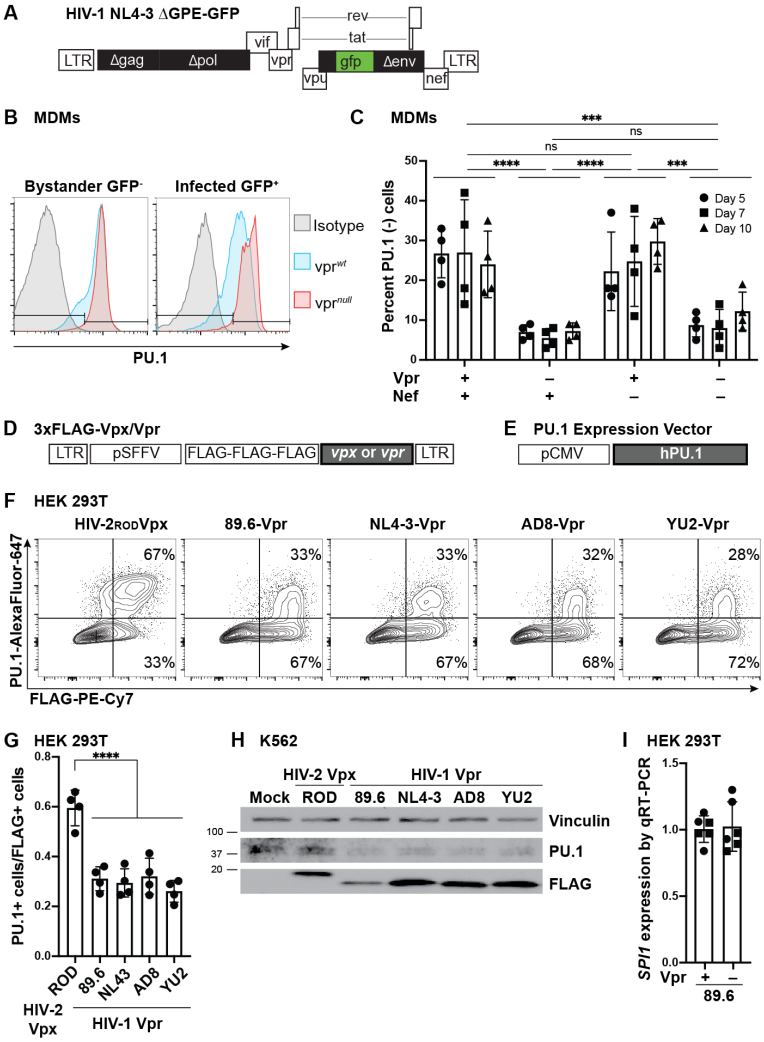

Figure 3.4. PU.1 levels decrease in the presence of Vpr.[5] (A) NL4-3 ΔGPE-GFP viral genome map. (B) Representative flow cytometry histogram of PU.1 expression in infected (GFP^+) or uninfected bystander (GFP^-) MDMs infected with NL4-3 ΔGPE-GFP with or without *vpr* and collected on day 7 post infection. (C) Summary graph showing the percentage of infected (GFP^+) cells that do not express PU.1 as determined by flow cytometry as depicted in **Figure 3.4B**. The mean +/- standard deviation from n=4 independent donors is shown for each time point. P values were determined using a two-sided, one-way analysis of variance (ANOVA) with Tukey's multiple

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comparisons test. ***, $p < 0.001$; ****, $p < 0.0001$. (D) Lentiviral map of vectors encoding 3xFLAG-encoding *vpr* or *vpx* genes. (E) PU.1 expression plasmid map for full-length, human PU.1. (F) Representative flow cytometric plots of HEK 293T cells transiently transfected with PU.1 and the indicated FLAG-tagged viral protein. (G) Summary graph showing $PU.1^+$ expression in transfected ($FLAG^+$) cells. Each point represents the mean of three technical replicates. The mean +/- standard deviation of four independent experiments is shown. P values were determined using one-way ANOVA compared to control; ****, $p < 0.0001$. (H) Immunoblot analysis of PU.1 in K562 cells transduced with the indicated lentivirus expressing 3xFLAG-tagged viral proteins. Results are representative of those from three independent experiments. (I) Summary graph of *SPI1* (the gene encoding PU.1) expression in HEK 293T cells co-transfected with PU.1 and an 89.6 expression vector with or without an intact *vpr* open reading frame. *SPI1* levels were assessed from purified RNA using RT quantitative real time PCR. Results represent the mean fold change compared with wild type +/- standard deviation for samples performed in triplicate for two independent experiments.

After determining that Vpr was sufficient to downmodulate PU.1 in HEK 293T cells, we confirmed these results in a second cell line, the lymphoblastic chronic myelogenous leukemia cell line K562, which endogenously expresses PU.1. For these experiments Vpr-expression was achieved using VSV-G envelope pseudotyped lentiviruses. Like the results obtained in HEK 293T cells, infections with viruses expressing all Vprs tested (89.6, NL4-3, AD8, YU2) had lower endogenous PU.1 levels compared to mock infection and HIV-2_{ROD}Vpx infection (**Figure 3.4H**).

Because the Vpr-dependent decrease of PU.1 occurred both for endogenous PU.1 expressed from its native promoter in K562 cells and in HEK 293T cells when PU.1 was expressed from a heterologous promoter, we hypothesized that Vpr was affecting PU.1 protein levels post-transcriptionally. To rule out transcriptional effects on the heterologous promoter, we quantified PU.1 mRNA in HEK 293T cells transfected with a PU.1 expression plasmid and HIV constructs derived from 89.6 that had or lacked the Vpr gene (**Supplementary Figure 3.12A**). As shown in **Figure 3.4I**, Vpr did not reduce expression of PU.1 mRNA when expressed from a heterologous promoter in transfected cells. Thus, we concluded that the mechanisms by which Vpr reduced PU.1 protein in HEK 293T cells

must be post-transcriptional. Moreover, in macrophages Vpr likely exerts negative effects directly on PU.1 protein and indirectly on PU.1 RNA through PU.1 binding sites in the *SPI1* promoter (Chen et al., 1995).

Prior reports indicated that PU.1 suppresses HIV gene expression in a Tat-reversible manner (Kao et al., 2022). If true, Vpr-targeting of PU.1 could explain the upregulation of HIV genes observed in Vpr-expressing HIV-infected macrophages (**Figure 3.1I**). To attempt to confirm these results, we co-transfected HEK 293T cells with a PU.1 expression plasmid (**Figure 3.4E**) and an 89.6-derived HIV genome that expresses GFP constitutively and mCherry upon HIV activation, allowing us to distinguish transfected cells through GFP expression and HIV-LTR activity through mCherry expression (**Supplementary Figure 3.12A**). To eliminate any complications from Vpr-mediated reduction of PU.1, we used an HIV genome that did not express *vpr*. Transfection of increasing amounts of PU.1 plasmid with the same amount of HIV in all conditions resulted in increasing amounts of PU.1 protein as measured by flow cytometry (**Supplementary Figure 3.12B, C**). However, we failed to confirm a PU.1-dependent suppression of HIV-LTR activity (**Supplementary Figure 3.12D**).

PU.1 downmodulation is a conserved activity of Vprs from HIV-2 and closely related SIV molecular clones.

Vpr is highly conserved in lentiviruses, including HIV-2 and all SIV strains (Collins and Collins, 2014; Sharp and Hahn, 2011). Interestingly, HIV-2 and certain SIVs contain both Vpr and Vpx, whereas HIV-1 and some SIVs contain only Vpr (**Figure 3.5A**). The extent to which these two proteins harbor overlapping functions has been the subject of

a number of research studies (Romani and Cohen, 2012; Sakai et al., 2016; Yurkovetskiy et al., 2018). Thus, we first examined whether Vprs from viruses containing both Vpr and Vpx would reduce PU.1 levels. To do this, we used the HIV-2_{ROD} molecular clone from which our Vpx control is derived. Transient transfection of HEK 293T cells with a PU.1 expression vector and either HIV-2_{ROD}Vpx, HIV-2_{ROD}Vpr, or HIV-$1_{89.6}$Vpr confirmed a significant decrease in PU.1 for cells expressing Vpr^{ROD} or $Vpr^{89.6}$ but not Vpx^{ROD} (**Figure 3.5B, C**). This indicates that Vpx and Vpr from the same molecular clone have divergent functions with respect to PU.1 downmodulation.

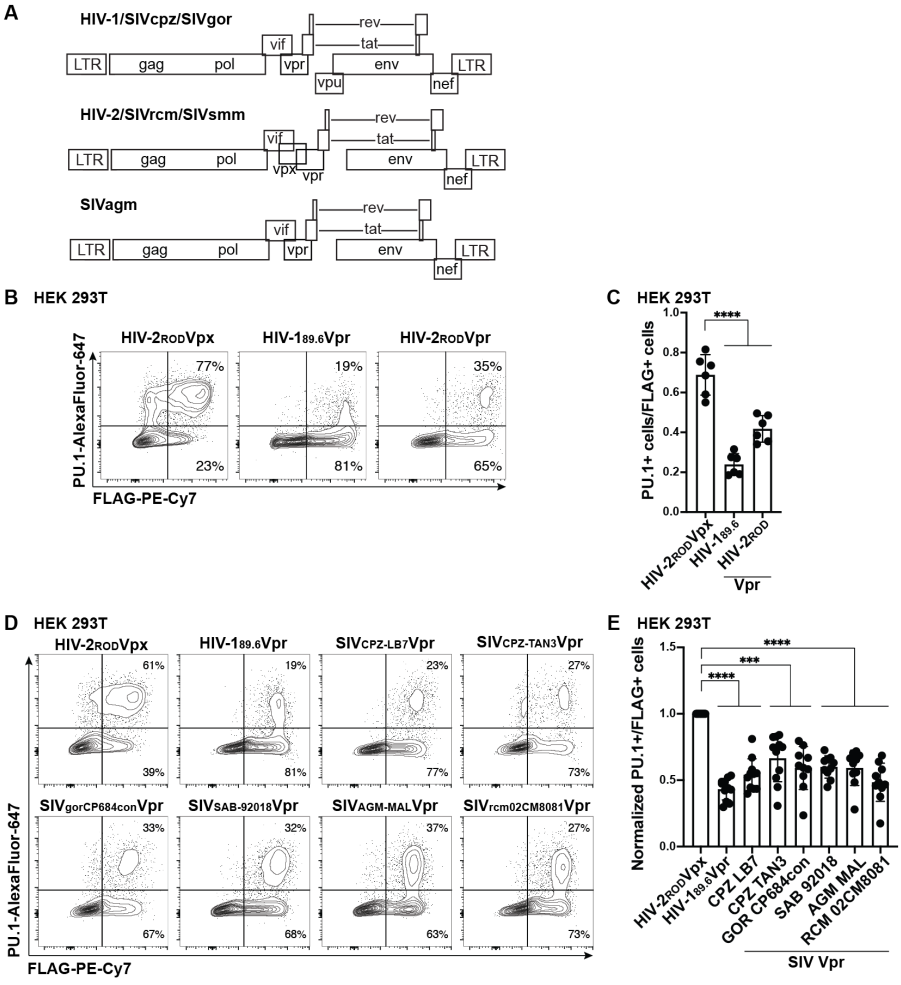

Figure 3.5. Vpr-mediated reduction of PU.1 is conserved in HIV-2 and SIV molecular clones.[6] (A) Genomic maps for HIV-1, HIV-2, and select SIV genomes. HIV-1, SIVcpz, and SIVgor genomes contain *vpr* and *vpu* genes but not *vpx*. HIV-2, SIVrcm, and SIVsmm contain *vpx* and *vpr*. SIVagm contains *vpr*. (B and D) Representative flow cytometric plots of HEK 293T cells transiently transfected with expression plasmids for PU.1 and the indicated FLAG-tagged viral protein. (C and E) Summary graph of data from B and D, respectively. The percentage of $PU.1^+$ cells per transfected ($FLAG^+$) cells is shown. Each point represents the average of three technical replicates. The mean +/- standard deviation is shown for n=6 (C) or n=11 (E) independent experiments, respectively. Part E was additionally normalized to $HIV\text{-}2_{ROD}Vpx$ for each experiment. ****, $p < 0.0001$ using two-sided one-way ANOVA compared to control with Tukey's multiple comparisons test.

We next tested the ability of Vpr from six isolates evolutionarily similar or dissimilar to HIV-1 to determine whether the PU.1 targeting function of Vpr is evolutionarily conserved. Vpr sequences from the indicated SIV molecular clones from separate clades, including two from chimpanzee and one from gorilla (the direct evolutionary relatives of HIV-1), were indeed able to mediate the degradation of PU.1 in HEK 293T cells (**Figures 3.5D, E**). Thus, Vpr-mediated reduction of PU.1 was consistently observed across all HIV-1, HIV-2, and SIV molecular clones tested, indicating a strong selective pressure for HIV-related viruses to downmodulate PU.1 in infected cells.

Both PU.1 and DCAF1 form a complex with Vpr.

To determine whether Vpr recruits PU.1 similarly to other host proteins for proteasomal degradation via the CRL4-DCAF1 ubiquitin ligase complex [reviewed in (Collins and Collins, 2014)], we first assessed whether Vpr and PU.1 formed a complex in transfected HEK 293T cells. We found that PU.1 efficiently co-precipitated with FLAG-tagged 89.6-Vpr. In addition, and as expected (McCall et al., 2008; Zhang et al., 2001), DCAF1 also co-precipitated with Vpr (**Figure 3.6A**).

[6] This figure was created by Maria C. Virgilio and Thomas Chen

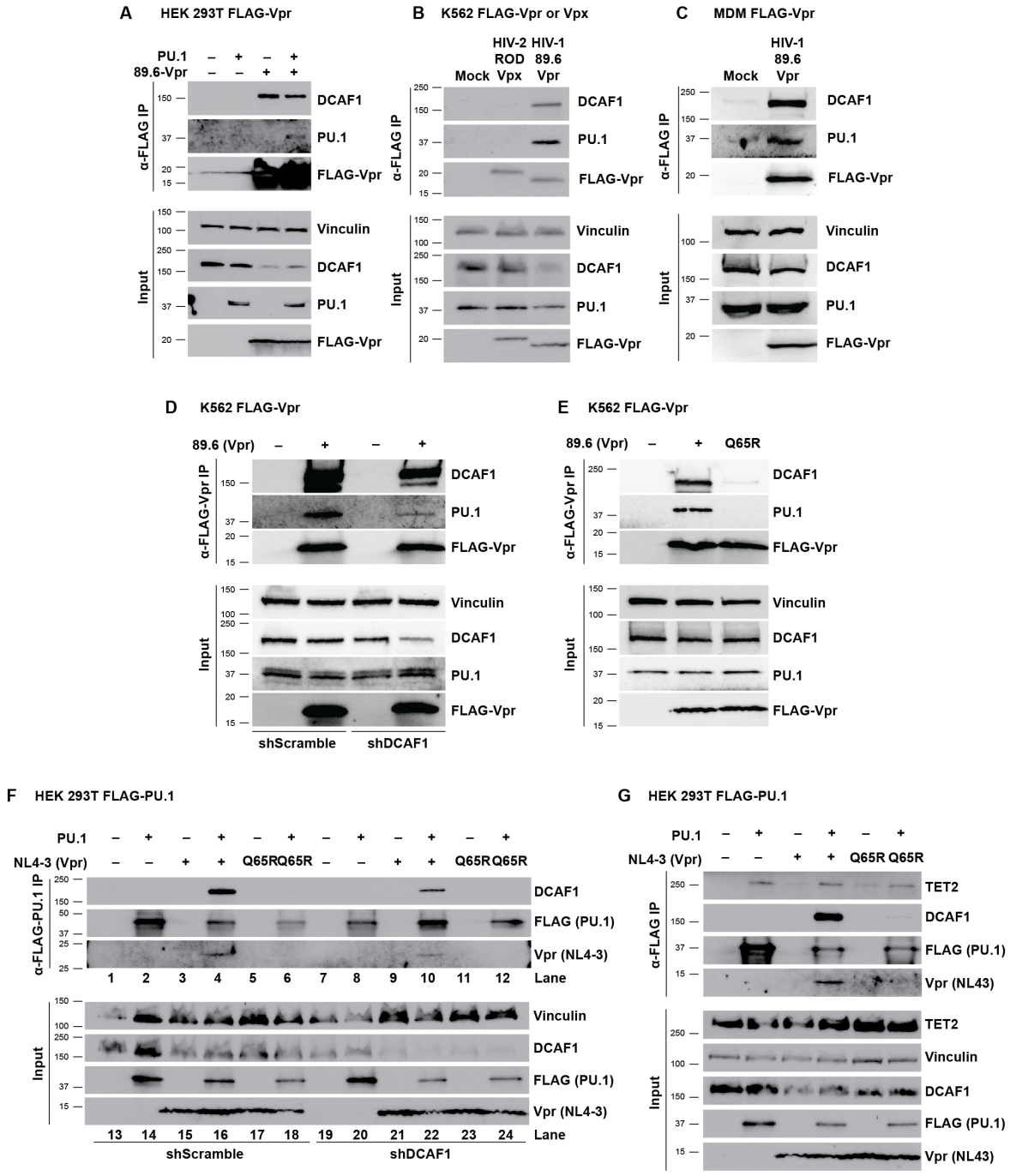

Figure 3.6. Vpr forms a DCAF-1-dependent complex with PU.1.[7] (A) Western blot analysis of lysates from HEK 293T cells co-transfected with the indicated expression constructs and immunoprecipitated with an antibody directed against the FLAG epitope. (B) Western blot analysis of lysates from K562 cells transduced with lentiviruses and treated as in part A. (C) Western blot analysis of lysates from MDMs transduced with lentivirus and treated as in part A. For parts A, B,

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and C, results are representative of three independent experiments. (D) Western blot analysis of lysates from K562 cells stably expressing either control, non-targeting shRNA (shScramble) or an shRNA targeting DCAF1 (shDCAF1), then transduced with FLAG-tagged 89.6-Vpr expression lentivirus and immunoprecipitated using an antibody directed against the FLAG epitope. (E) Western blot analysis of lysates from K562 cells transduced with FLAG-tagged 89.6-Vpr^{WT} or 89.6-Vpr^{Q65R} expressing lentiviruses and immunoprecipitated using an antibody directed against the FLAG epitope. (F) Western blot of lysates from HEK 293T cells stably transduced with virus expressing a non-targeting shRNA (shScramble) or one targeting DCAF1 (shDCAF1) and immunoprecipitated with an antibody directed against the FLAG epitope. (G) Western blot analysis of lysates from HEK 293T cells co-transfected with the indicated expression constructs and immunoprecipitated with an antibody directed against the FLAG epitope. Results are representative of three independent experiments.

We next examined whether Vpr co-precipitated with endogenous PU.1 in the K562 cell line (originally derived from a patient with CML) (Lozzio and Lozzio, 1975). To accomplish this, we stably expressed FLAG-tagged HIV-$1_{89.6}$Vpr or HIV-2_{ROD}Vpx in K562 cells using lentiviral vectors and repeated co-immunoprecipitation experiments. We found that both PU.1 and DCAF1 co-precipitated with Vpr to a greater extent than Vpx, again suggesting PU.1 binding is specific to Vpr and not to the similar accessory protein Vpx (**Figure 3.6B**). Finally, to determine if Vpr and DCAF1 interact with PU.1 in macrophages, we expressed FLAG-tagged HIV-$1_{89.6}$Vpr in MDMs using a lentiviral vector. As shown in **Figure 3.6C**, we determined that both endogenous PU.1 and DCAF1 interact with Vpr in this physiologically relevant target of HIV-1. Collectively, these data indicate that Vpr selectively forms complexes with both PU.1 and DCAF1 in cells endogenously expressing PU.1.

Interactions between PU.1 and Vpr require DCAF1.

To better understand the mechanism through which Vpr downmodulates PU.1, we assessed a potential requirement for the host protein DCAF1. DCAF1 (also known as VprBP (Zhang et al., 2001)) is the Vpr binding partner in the CRL4 ubiquitin ligase

complex that is necessary for proteasomal degradation of Vpr-recruited host proteins (Ahn et al., 2010; Lahouassa et al., 2016; McCall et al., 2008; Romani et al., 2015; Romani and Cohen, 2012). DCAF1 requirement was tested using a K562 cell line expressing a lentiviral vector containing either a non-targeting shRNA (shScramble) or a DCAF1-targeting shRNA cassette (shDCAF1). We found that the amount of PU.1 that co-precipitated with Vpr was decreased with DCAF1 silencing (**Figure 3.6D**), suggesting a role for DCAF1 in the interaction between Vpr and PU.1. Consistent with this, endogenously expressed PU.1 from K562 cells did not coprecipitate with a Vpr mutant (89.6-Vpr^{Q65R}) protein that is defective for DCAF1 interactions (DeHart et al., 2007; Zhao et al., 1994) (**Figure 3.6E**). These interesting results suggest the unexpected conclusion that PU.1, Vpr, and DCAF1 may form a trimeric complex and PU.1 binding in the complex is dependent on both Vpr and DCAF1, though Vpr can associate with DCAF1 independently of PU.1 (**Figures 3.6A, B**).

To validate this physical interaction, we performed the immunoprecipitation (IP) in reverse using HEK 293T cells that overexpressed FLAG-tagged-PU.1. Because these experiments used un-tagged Vpr, they were limited by the availability of antibodies, which do not recognize all Vpr allotypes equally. To overcome this limitation, we performed FLAG-tagged PU.1 pull-down experiments with NL4-3 Vpr, which was efficiently recognized by the available antibodies whereas 89.6 Vpr was not. As we observed in K562 cells using 89.6 FLAG-tagged Vpr, the amount of NL4-3 Vpr that co-precipitated with FLAG-tagged PU.1 in HEK 293T cells was decreased with DCAF1 silencing (**Figure 3.6F**). In addition, the Vpr mutant that is defective at interactions with DCAF-1 (NL4-3-

Vpr^{Q65R}) was also defective at co-precipitating PU.1 (**Figure 3.6F**), further confirming a role for DCAF1 in the interaction between Vpr and PU.1.

In comparison to single cell and flow cytometric approaches shown in **Figures 3.4 and 3.5**, which had the ability to differentiate infected from uninfected cells, western blot analysis of input lysates shown in **Figure 3.6** had a low sensitivity to detect Vpr-mediated PU.1 degradation. This was likely due to overexpression of tagged exogenous PU.1, and heterogeneous mixtures of transfected cells that were not optimized to ensure that Vpr was expressed in all PU.1 positive cells.

TET2, a PU.1 cofactor and Vpr target, coprecipitates with PU.1, Vpr, and DCAF1.

The PU.1-regulated antiviral factor, *IFITM3* (**Figure 3.2C, E**) is also controlled by TET2, another target of Vpr (Lv et al., 2018; Wang and Su, 2019). TET2 is a DNA dioxygenase that demethylates the *IFITM3* promoter during viral infection, inhibiting HIV Env trafficking and reducing viral spread (Compton et al., 2014; Lu et al., 2011). To prevent this, Vpr mediates the ubiquitylation and degradation of TET2, which in turn inhibits *IFITM3* expression. Interestingly, PU.1 and TET2 have been reported to form a complex to co-regulate myeloid specific genes (de la Rica et al., 2013; Fisher and Scott, 1998; Turkistany and Dekoter, 2011). We therefore hypothesized that TET2 could be co-recruited with PU.1 to DCAF1 by Vpr for degradation. Indeed, we found that TET2 co-precipitated with PU.1 in HEK 293T cells with Vpr and DCAF1 (**Figure 3.6G**).

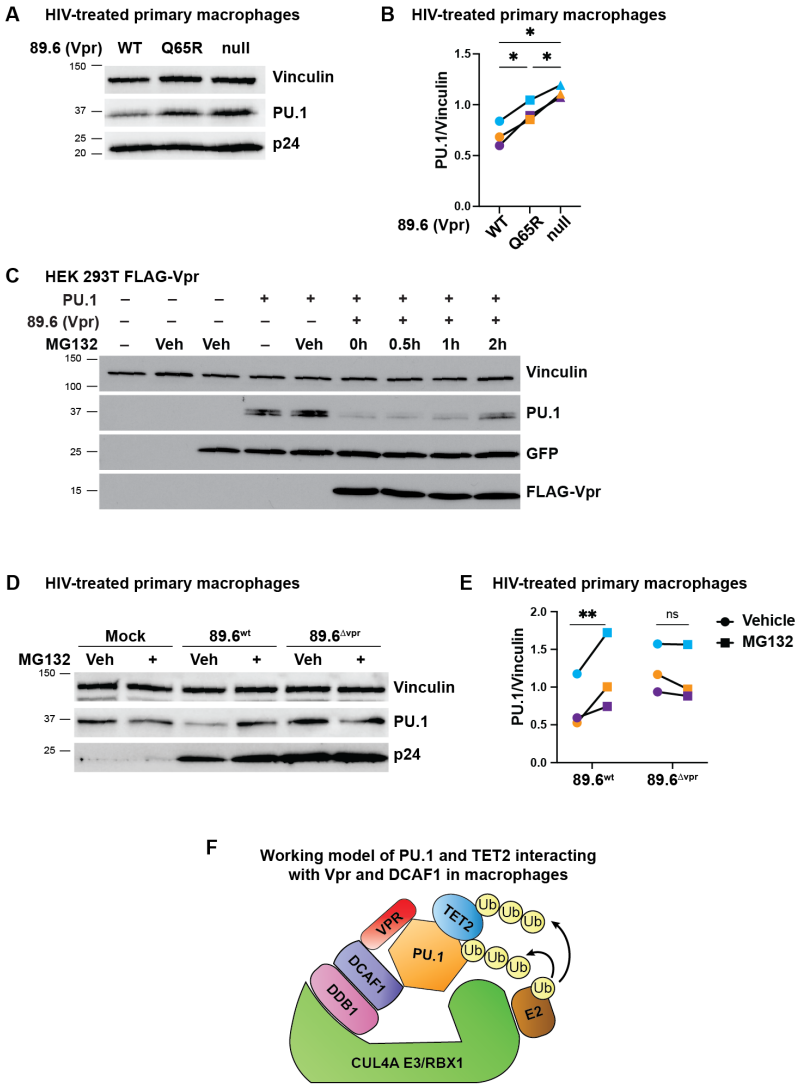

Figure 3.7. An intact DCAF-1 interaction domain is required for Vpr and DCAF1 to degrade PU.1.[8] (A) Immunoblot analysis of lysates from MDMs treated for five hours with the indicated virions. (B) Summary graph of PU.1 protein normalized to vinculin from MDMs incubated for five hours with the indicated viruses from (A). Each point and matched colour is representative of an independent donor. Statistical significance was determined using a mixed-effects analysis with Tukey's multiple comparisons test. *, $p < 0.05$. (C) Western blot analysis of lysates from HEK 293T cells transfected with the indicated expression construct and treatment as indicated with 10μM MG132 or vehicle (Veh) control (DMSO). A GFP-expressing plasmid was included where indicated as control for transfection efficiency, n = 2. (D) Immunoblot analysis of lysates from MDMs preincubated for two hours with vehicle (Veh) or MG132 as indicated and then treated for five hours with the indicated virus as in part (A). (E) Summary graph of PU.1 protein normalized to vinculin from MDMs treated for five hours with the indicated viruses from (D). Statistical significance was determined using a mixed-effects analysis with Šidák's multiple comparisons

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test. ** indicates p = 0.0073. Each point and matched colour is representative of an independent donor. (F) Working model of PU.1 and TET2 interacting with Vpr and DCAF1 in macrophages.

Vpr downmodulates PU.1 via a pathway that depends on proteasome activity.

Having shown that Vpr requires DCAF1 to form a stable complex with PU.1, we next asked whether interactions with DCAF1 were also important for promoting PU.1 degradation. Compared to the flow cytometric approaches shown in **Figures 3.4** and **3.5**, detection of degradation by western blot is more challenging. The model systems described in **Figure 3.7** did not reliably show PU.1 degradation, most likely because PU.1 was overexpressed and/or Vpr was not expressed in a sufficient number of PU.1 expressing cells. Therefore, to further study PU.1 degradation, we employed another approach in which virion-associated Vpr is delivered to primary MDMs, resulting in Vpr-dependent proteasomal degradation of targets within five hours of viral treatment (Mashiba et al., 2014). Using this approach, we observed Vpr-dependent degradation of PU.1 treated with wild type HIV-1 but not an HIV harboring a Vpr mutant defective at interacting with DCAF1 (89.6-Vpr^{Q65R}, **Figure 3.7A, B**). These findings support the conclusion that interactions between PU.1, DCAF1, and Vpr are needed for efficient PU.1 degradation. Additionally, this experiment suggests that it is possible for virion-associated Vpr to act on uninfected bystander macrophages, potentially explaining some of the results from **Figure 3.3** showing that Vpr can suppress innate immune responses in uninfected bystander cells.

Because the Vpr/DCAF1 complex promotes ubiquitylation and proteasomal degradation of Vpr-bound host proteins, we investigated whether inhibition of the proteosome could restore PU.1 levels. As shown in **Figure 3.7C**, where we optimized PU.1 expression in HEK 293T cells expressing Vpr, PU.1 levels were restored with

MG132, a specific inhibitor of the proteasome. Furthermore, MG132 treatment of MDMs transduced with 89.6^{wt} (as for **Figure 3.7A**) prevented virion-associated Vpr-mediated degradation of endogenous PU.1 (**Figure 3.7D, E**).

The results from the immunoprecipitation and degradation assays indicate a requirement for DCAF1 for Vpr to bind PU.1 and promote its degradation, suggesting all three molecules interact as modeled in **Figure 3.7F**. While other explanations remain possible, our data suggest PU.1 binding in the complex is dependent on both Vpr and DCAF1. Vpr can associate with DCAF1 independently of PU.1 and PU.1 can associate with TET2 independently of Vpr.

Reducing PU.1 enhances HIV-1 Env production in MDMs

Based on scRNA-seq data (**Figure 3.1**), Vpr reduces expression of several antiviral factors regulated by the transcription factor, PU.1 (**Figure 3.2**). Two of these genes, *MRC1* and *IFITM3*, inhibit the spread of HIV in MDMs by targeting HIV-1 Env (Lubow et al., 2020; Wang and Su, 2019). Therefore, we hypothesized that reduction of PU.1 in MDMs would restore Env production in Vpr-*null*-HIV-infected primary macrophages. To test this hypothesis, we modified the 89.6 genome to remove *vpr* and replace it with either an shScramble sequence, or one of three different shRNAs targeting the PU.1 gene, *SPI1* (**Figure 3.8A**). All three PU.1-targeting cassettes were independently validated in K562 cells to confirm their ability to reduce endogenous PU.1 expression (**Figure 3.8B**). Consistent with our hypothesis, MDMs from two independent donors infected with all three shSPI1-containing viruses showed a marked increase in Env compared to MDMs infected with virus expressing shScramble (**Figure 3.8C**).

Overall, our findings support a model in which Vpr dramatically alters the transcriptional landscape in macrophages by targeting myeloid-specific transcription factors that are required for the expression of key antiviral restriction factors, including those that target HIV-1 Env. (**Figure 3.8D**).

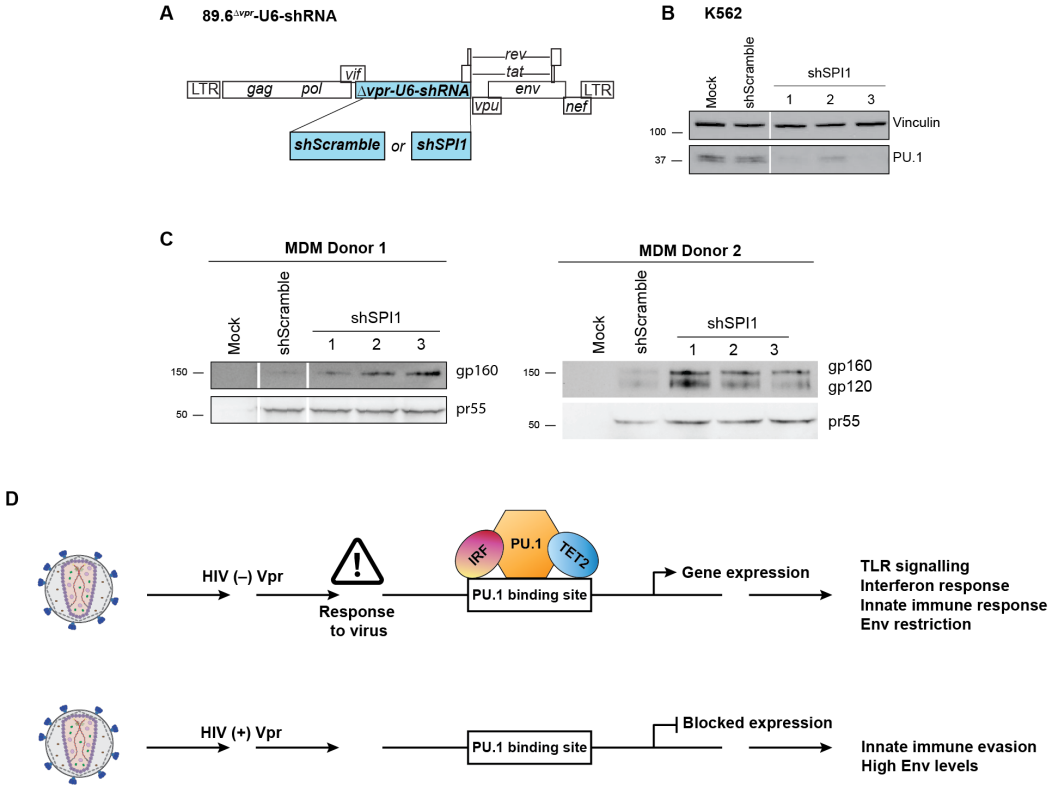

Figure 3.8. Reducing PU.1 increases Env output in HIV-1 infected MDMs.[9] (A) Genome map for full-length 89.6^{wt} HIV-1 modified to replace the *vpr* ORF with a U6-promoter followed by either a non-targeting shRNA control (shScramble) or an shRNA targeting the PU.1 transcripts (shSPI1). (B) Immunoblot from K562 cells stably expressing the shRNAs from (A), n = 2. (C) Immunoblot analysis from two independent MDM donors. Lysates collected from MDMs infected with the virus from (A) expressing the indicated shRNA. White lines indicate the location where the digital image of the stained membrane was cropped to remove irrelevant samples. (D) Proposed model of the PU.1-mediated antiviral response disrupted by HIV-Vpr. HIV (-) Vpr indicates an infection of primary macrophages with HIV that does not express Vpr. PU.1 protein is maintained and available to regulate anti-viral response genes with co-factors such as IRF or TET2. HIV (+) Vpr indicates an infection with HIV that expresses Vpr. PU.1 protein is less available to regulate anti-viral response genes, contributing to innate immune evasion.

[9] This figure was created by Maria C. Virgilio

Discussion

Although HIV accessory proteins have been widely studied, the critical function that drives evolutionary conservation of Vpr remains largely enigmatic. While Vpr significantly enhances infection of macrophage-containing cultures, primarily by enhancing cell-to-cell transmission, it does not substantially affect HIV infection in cultures of $CD4^+$ T cells that lack macrophages (Balliet et al., 1994; Collins et al., 2015; Connor et al., 1995; Dedera et al., 1989; Eckstein et al., 2001; Lubow et al., 2020; Mashiba et al., 2014). While a number of Vpr targets implicated in post-replication DNA repair have been identified, there is a lack of compelling explanations for the dramatic selective effects of Vpr on infection and spread in macrophage-containing cultures. In this work, we identified a macrophage-specific target of Vpr, the myeloid transcription factor PU.1 and its associated co-factors that Vpr targets, averting antiviral effects (**Figure 3.8D**). Using single-cell RNA sequencing of MDMs treated with replication-competent virus with and without the gene for Vpr we could distinguish effects of Vpr on cells harboring bona fide infections as well as bystander cells.

In infected cells, we found that Vpr reduces the transcription of hundreds of genes regulated through PU.1 and its cofactors. Several PU.1 regulated genes we identified as being impacted by Vpr are involved in TLR signaling. TLRs are highly conserved PRRs that help cells identify PAMPs and respond quickly to infection. Activation of TLRs through binding of a PAMP, initiates a cascade of intracellular signaling that results in the release of inflammatory cytokines and upregulation of type I interferon response genes (Beutler, 2009; Kumar et al., 2009; Schneider et al., 2014). The products of these genes have

antiviral effects on HIV and include *ISG15*, *STMN1, IFI6, LY96*, *TREM2*, *FABP4*, *IFITM3,* and *MRC1*. *STMN1* is thought to play a role in the establishment of HIV latency, and its depletion leads to higher expression of HIV-1 (Deletsu et al., 2021). *MRC1* and *IFITM3* interrupt Env trafficking, reducing viral spread. *IFI6*, *LY96, and ISG15* are members of the type I IFN response to infection (Del Cornò et al., 2016; Park et al., 2013; Perng and Lenschow, 2018), and *ISG15* also inhibits HIV spread by disrupting Gag polymerization (Okumura et al., 2006).

Unexpectedly, we found that bystander cells within HIV infected cultures also responded to HIV infection by upregulating a subset of these antiviral genes, including *ISG15*, *LY96*, *IFI6*, and *IFITM3.* Moreover, we found that upregulation of these factors was reduced in bystander cells from Vpr-positive versus Vpr-negative HIV-infected primary macrophage cultures. Because Vpr is efficiently packaged into the virus particle through specific interactions with p6Gag (Lu et al., 1995), the Vpr phenotype we observed in bystander cells could be due to a low and/or transient presence of Vpr in MDMs that have taken up viral particles but remained uninfected. This could occur if a subset of Vpr-containing viral particles were defective and/or if innate immune responses blocked completion of reverse transcription and/or integration. We did not consistently detect effects of Vpr in uninfected bystander cells exposed to VSV-G pseudotyped replication-defective virus five days post-infection, but this is not surprising given the likely turnover of the viral protein and the lack of continuous exposure to virions by MDMs treated at a single time point with replication-defective viruses. In contrast, we did find that MDMs exposed to wild type HIV-1 had low PU.1 levels at short time points (five hours) following exposure to virus. Thus, the Vpr-dependent bystander phenotype we identified in MDMs

continuously exposed to wild type replication-competent HIV is most likely due to Vpr delivered by wild type viral particles. However alternative and additional explanations are possible. For example, it is possible that there is differential antiviral cytokine production by wild type versus Vpr-mutant infected macrophages that acts on bystander cells. Regardless of the precise mechanism, our results indicate that Vpr can exert systemic antiviral affects that favor virus infection and spread.

A prior study reported scRNA-seq analysis of THP-1 cells transduced with a replication-defective HIV (Lim et al., 2022). The authors identified a population of cells with low HIV gene expression they felt was due to the presence of unintegrated pre-integration complexes (PIC) and noted transcriptomic changes within this population of cells compared to the fully infected population. Our study differed from this prior report in that we utilized wild type HIV infected primary macrophages and characterized Vpr-dependent transcriptomic changes comparing fully infected and uninfected (HIV-RNA-negative), bystander cells. We did not identify a similar population of cells that expressed low levels of HIV gene products. As their data was not made publicly available, we were unable to make a direct comparison between the data sets.

We have previously shown that mannose receptor is a host restriction factor that reduces HIV spread in macrophages by binding to mannose residues on Env and directing Env to the lysosome for degradation (Lubow et al., 2020). However, our previous data lacked a mechanism for Vpr-mediated transcriptional reduction of *MRC1* in macrophages. The data here confirm findings from other groups that PU.1 regulates *MRC1* expression (Caldwell et al., 2000; Kao et al., 2022), and we provide new evidence

that Vpr reduces the transcriptional expression of *MRC1* in macrophages via PU.1 degradation.

In agreement with other studies (Lv et al., 2018; Wang and Su, 2019), our unbiased survey of Vpr's effect on expression of the host transcriptome showed that Vpr reduced *IFITM3* gene expression substantially. IFITM proteins are broad antiviral factors that inhibit viral entry and exit for HIV-1, SIV, MLV, VSV, EBOV, WNV, among other viruses (Tartour et al., 2017). Of the IFITM proteins, IFITM3 is well documented as an HIV-1 antagonist. Like mannose receptor, IFITM3 interacts with Env in infected cells, inhibiting Env processing and virion incorporation and strongly inhibiting cell-to-cell spread. Interruption of Env processing by IFITM3 has been demonstrated for several HIV-1 molecular clones (Yu et al., 2015) many of which were included within our own study (AD8, YU2, and NL4-3, HIV-2_{ROD}, SIVagm, and SIVcpz). *IFITM3* expression is regulated by the DNA dioxygenase, TET2 (Wang and Su, 2019). During HIV-1 infection of macrophages, in the absence of Vpr, TET2 demethylates the *IFITM3* promoter, relieving suppression, contributing to the antiviral response. When Vpr is present, *IFITM3* expression is reduced after Vpr recruitment of TET2 to DCAF1 for polyubiquitylation and degradation of TET2 (Lv et al., 2018). TET2 is ubiquitously expressed in the nuclei of all cells (Lorsbach et al., 2003); thus it was unclear how Vpr targeting of TET2 could result in a macrophage-specific Env phenotype. In monocytes, TET2 interacts with PU.1, leading to interaction with genetic targets (de la Rica et al., 2013). Therefore, we hypothesized that Vpr exerts a macrophage-specific effect on the antiviral response by targeting PU.1 and by extension, PU.1-associated proteins. Consistent with this hypothesis, we demonstrated TET2 immunoprecipitating with PU.1, Vpr, and DCAF1 in

HEK 293T cells, however confirmation of this interaction in MDMs has not yet been achieved. Altogether, this work provides evidence that Vpr can reprogram cellular transcription in macrophages by targeting myeloid-specific transcription factors.

A role for PU.1 in Vpr-dependent counteraction of Env restriction was confirmed by replacing the *vpr* ORF with shRNA cassettes targeting *SPI1*/PU.1. This approach allowed us to measure the impact of reducing PU.1 exclusively within HIV-infected macrophages rather than knockdown within the entire culture. This approach was necessary because PU.1 is the master transcriptional regulator necessary for macrophage differentiation and silencing PU.1 prior to infection results in a change in the cellular phenotype that causes resistance to HIV infection (Hrecka et al., 2011; Laguette et al., 2011). While this strategy successfully confirmed that PU.1 knockdown increases Env expression, more research is needed to determine whether other Vpr-dependent transcriptional changes, such as those impacting the PU.1 and interferon-induced gene products ISG15, IFITM3 and IFI6, are mediated through Vpr-dependent degradation of PU.1 alone or whether additional Vpr-dependent pathways play a role.

Vpr-mediated reduction of PU.1 occurred in all cell types tested in our study including MDM, K562, and HEK 293T cells. The Vpr-dependent reduced PU.1 levels were observed regardless of whether PU.1 was expressed from its native promoter or a heterologous promoter. Reversal of PU.1 downmodulation with proteasome inhibitors in both HEK 293T cells and MDMs support the conclusion that Vpr directly reduces PU.1 protein by promoting its degradation. Vpr-mediated degradation of PU.1 was consistent for all HIV-2, SIV, and HIV-1 Group M isolates tested - Group M being largely responsible for the global HIV pandemic. In macrophages, *SPI1* mRNA levels encoding PU.1 protein

were also lower in the presence of Vpr, indicating that downmodulation of PU.1 can occur at both the transcriptional and the post-transcriptional level in macrophages.

It is important to note that we failed to confirm prior observations that PU.1 suppresses HIV-LTR activity in a Tat-reversible manner (Kao et al., 2022). While it is possible that expression of Tat by our construct prevented our ability to detect an effect of PU.1 on the HIV-1 LTR, these results nevertheless indicate that downmodulation of PU.1 by Vpr is unlikely to impact HIV-1 gene expression in infected macrophages that also express Tat. Thus, in primary HIV infected macrophages, Vpr mainly targets PU.1 to counteract its anti-viral defense response rather than to counteract an inhibitory effect on HIV gene expression. However, it is possible that the higher expression of HIV genes we observed in Vpr-containing cells may result from Vpr counteracting a PU.1-regulated factor that inhibits HIV gene transcription.

Vpr is highly conserved amongst primate lentiviruses and promotes infection of nondividing cells, especially macrophages (Mashiba et al., 2014). In addition, a requirement for Vpr to achieve maximal replication and persistence in vivo was first discovered using an SIV molecular clone in rhesus monkeys (Lang et al., 1993). We therefore speculated that the ability of Vpr to degrade PU.1 is an important evolutionary function of Vpr. HIV-2 differs from HIV-1 in that it contains Vpx, an accessory protein that shares a common genetic ancestor with Vpr (Sharp and Hahn, 2011) but is lacking from all HIV-1 genomes. Thus, we tested the relative ability of Vpx and Vpr from the same molecular clone to promote PU.1 degradation (Sharp and Hahn, 2011). We identified a unique function of HIV-2 Vpr to degrade PU.1 that was not shared by Vpx. Similarly, both SIVcpz and SIVgor, the evolutionary precursors to HIV-1, and Vpr proteins from more

evolutionarily distant viruses consistently lowered PU.1 levels when expressed in the same cell. However, the greatest decrease in PU.1 levels were achieved with HIV-1-derived molecular clones. We therefore speculate that the strength of Vpr-mediated degradation of PU.1 plays an important role in driving spread in human pandemic strains of HIV-1, and PU.1 may be a critical restriction factor, limiting spread between species and within populations. However, more extensive studies comparing the relationship between Vpr and PU.1 across SIV, HIV-2, and HIV-1 isolates is necessary and part of our ongoing efforts.

The relationship between DCAF1 and Vpr is well documented (McCall et al., 2008; Romani and Cohen, 2012; Zhang et al., 2001). We confirmed the binding of Vpr to DCAF1 and identified PU.1 as a new Vpr-binding factor. We demonstrated that all three components coprecipitate under many different cellular conditions regardless of whether we first precipitated using Vpr or PU.1. To our surprise, mutation of the glutamine residue at position 65 of Vpr (Vpr^{Q65R}) not only resulted in the loss of DCAF1 association but also disrupted formation of the Vpr-PU.1 complex. Additionally, when we reduced DCAF1 or used Vpr^{Q65R}, the amount of both Vpr and DCAF1 precipitating with PU.1 was reduced. These results indicate that interactions amongst all three proteins are necessary for stable complex formation, although further studies are necessary to understand the detailed protein-protein interactions. Based on these findings, we propose a model in which interactions amongst PU.1, Vpr, and DCAF1 promote the ubiquitylation of PU.1 via the associated CUL4A ubiquitin ligase complex with resultant proteasomal degradation. Consistent with this model, PU.1 was not degraded in the presence of proteasome inhibitors or by a Vpr mutant defective at interacting with DCAF1 in primary MDMs. Thus,

our working model is that PU.1 is poly-ubiquitylated following its interaction with Vpr and DCAF1. However complete confirmation of this model has not yet been achieved because we have not yet directly detected ubiquitylated intermediates of PU.1 in Vpr-expressing cells.

Although the role of Vpr in HIV infection has remained largely undefined, we provide evidence that the primary selective pressure for Vpr in lentiviruses is to disrupt the macrophage innate antiviral response to infection, which is achieved by reducing PU.1 levels in infected cells. The ability of Vpr to degrade PU.1 is highly conserved among all HIV-1, HIV-2, and SIV isolates tested, and degradation of PU.1 relies on both Vpr and DCAF1. Reducing PU.1 in HIV-infected macrophages lacking Vpr rescued macrophage-dependent restriction of HIV-1 Env, helping to explain the requirement for Vpr in macrophage spreading infections. We are continuing to investigate the transcriptional consequences of PU.1 degradation in macrophages. In addition to TET2, PU.1 associates with other transcription factors, potentially piggybacking other secondary targets to the DCAF1-Cul4A E3 ubiquitin ligase complex via Vpr. Because PU.1 is necessary to maintain macrophage function, future studies should address the greater impact of Vpr-mediated reduction of PU.1 on the infected macrophage.

Materials and Methods

Ethics statement

Anonymized leukocytes isolated by apheresis were obtained from New York Blood Center after obtaining informed consent. Studies using these cells were determined to be exempt from human studies requirements by the University of Michigan Institutional Review Board because the project involves only biological specimens that cannot be linked to a specific individual by the investigator(s) directly or indirectly through a coding system.

Cell culture and preparation of human MDMs

All cell cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. HEK 293Ts (CRL-3216) and K562 (CCL-243) cells were obtained from ATCC and independently authenticated by STR profiling. HEK 293Ts were maintained in DMEM medium (Gibco) supplemented with 100 U/mL penicillin, 100µg/mL streptomycin, 2mM glutamine (Pen-Strep-Glutamine, Invitrogen), and 10% fetal bovine serum (Invitrogen). K562 cells were maintained in IMDM (Gibco) and supplemented as HEK293Ts. To generate monocyte-derived macrophages (MDMs), peripheral blood mononuclear cells were purified by Ficoll density. $CD14^+$ monocytes were positively selected using a $CD14^+$ sort kit following manufacturer instructions (cat# 17858, StemCell Technologies, Vancouver, Canada). $CD14^+$ monocytes were cultured for seven days in R10 [RPMI-1640 with 10% certified endotoxin-low fetal bovine serum (ThermoFisher), penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (292 µg/mL)] supplemented with carrier-free M-CSF and GM-CSF (both at 50ng/mL, R&D Systems, Minneapolis, Minnesota).

Monocytes were plated at 0.5 x 10^6 cells/well in a 24-well dish, or 1 x 10^6 for lentiviral transduction with puromycin selection. After seven days the MDMs were treated with virus and maintained in conditioned R10 as described below.

Viruses, viral vectors, and expression plasmids

The molecular clone p89.6 (cat# 3552, AIDS Reagent Program from Dr. Ronald G. Collman) was obtained from the AIDS Reagent Program. The *vpr*-null version was generated as previously described (Mashiba et al., 2014). The human, full length PU.1 (hPU.1) expression vector was a gift of Dr. Gregory M. K. Poon and generated as previously described (Munde et al., 2014). A triple N-FLAG-tagged version of PU.1 was generated by using PCR amplification of hPU.1 to add a KpnI site to the 5' end and EcoRI to the 3' end (5' GTAGGTACCGCCACCATGGAAGGGTT primer and 3' GTAGAATTCCACCACACTGGACTAGTG primer). The new product replaced an existing gene when inserted into pcDNA3.1 containing a triple N-FLAG-tag between KpnI and EcoRI (Addgene plasmid #67788). The GFP transfection control plasmid, pcDNA3-EGFP, was a gift from Doug Golenbock (Addgene plasmid # 13031; http://n2t.net/addgene:13031; RRID: Addgene_13031). The pNL4-3 ΔGPE-GFP plasmid used for single round infection of macrophages was previously described (McNamara et al., 2012), as were the *vpr*-null, *nef*-null, and *vpr-nef* null versions (Lubow et al., 2020). pUC19 (Norrander et al., 1983) was used as control DNA to adjust transfection samples to the same final DNA concentration. The p89.6-ΔGPEN-mCherry-pSFFV-EGFP single-round infection plasmid was previously described (Carter et al., 2010) and further modified by replacing *gag* and *pol* with mCherry. Deletion of *vpr* was achieved using Q5

Site-Directed Mutagenesis kit (cat# E0554, New England Biolabs) where the majority of the *vpr* coding sequence was deleted using PCR exclusion (forward - CAGAATTGGGTGTCGACATAG, reverse - TCACAGCTTCATTCTTAAGC). Primers were designed using the NEB Base Changer website (https://nebasechanger.neb.com/) and used following the manufacturer's instructions. pSIV3+ *vpr*-null used in MDM lentiviral transductions to allow Vpx-mediated degradation of SAMHD1 was generated as previously described (Lubow et al., 2020).

Triple FLAG-tagged Vpr and Vpx lentiviral expression vectors for HIV-2_{ROD}Vpx (Addgene plasmid #115816), $SIV_{SAB\text{-}92018}$Vpr (Addgene plasmid #115822), $SIV_{AGM\text{-}MAL}$Vpr (Addgene plasmid #115828), $SIV_{CPZ\text{-}TAN3}$Vpr (Addgene plasmid #115833), $SIV_{CPZ\text{-}LB7}$Vpr (Addgene plasmid #115834), $SIV_{gorCP684con}$Vpr (Addgene plasmid #115835), and $SIV_{rcm02CM8081}$Vpr (Addgene plasmid #115838) were a gift from Jeremy Luban (Yurkovetskiy et al., 2018). Similar vectors for HIV-2_{ROD}Vpr, HIV-$1_{89.6}$Vpr, HIV-$1_{89.6}Vpr^{Q65R}$, HIV-$1_{NL4\text{-}3}$Vpr, HIV-1_{AD8}Vpr, HIV-1_{YU2}Vpr were generated by synthesizing the gene as a gBlock (IDT, Coralville, Iowa, USA) between NotI and either EcoRI or AflIII in the same lentiviral expression plasmid. Untagged expression vectors for HIV-$1_{NL4\text{-}3}$Vpr and HIV-$1_{NL4\text{-}3}Vpr^{Q65R}$ were generated by synthesizing the genes as a gBlocks (IDT, Coralville, Iowa, USA) and inserting them between SbfI and NotI in LeGO-IV, a gift from Boris Fehse (Addgene plasmid #27360) (Weber et al., 2008).

The short hairpin RNAs targeting *DCAF1* (target sequence: CCTCCCATTCTTCTGCCTTTA) and *SPI1* (target sequence 1: GCCCTATGACACGGATCTATA, target sequence 2: CGGATCTATACCAACGCCAAA, and target sequence 3: CCGTATGTAAATCAGATCTCC) were designed using Genetic

Perturbation Platform (Broad institute) and cloned into pLKO.1 – TRC cloning vector, a gift from David Root (Addgene plasmid # 10878 ; http://n2t.net/addgene:10878; RRID:Addgene_10878) (Moffat et al., 2006). The control shRNA, scramble shRNA was a gift from David Sabatini (Addgene plasmid #1864; http://n2t.net/addgene:1864; RRID:Addgene_1864) (Sarbassov et al., 2005). For shRNA expression from full-length HIV-1-89.6 virus, the *vpr*-ORF was first disrupted by the insertion of a U6-promoter followed by multiple unique restriction enzyme sequences generated by synthesizing the segment as a gBlock (IDT, Coralville, Iowa, USA). The segment was inserted between XcmI and SalI without disrupting the *vif* or *tat* ORFs. The same shRNAs as above were then cloned into HIV-1-89.6 after the U6-promoter.

Co-transfections

Co-transfections of 3xFLAG-Vpx/Vpr and hPU.1 were performed in HEK 293T cells. Cells were plated at 1.6 x 10^5 per well in a 12-well dish. 24hrs after plating, 1ng of hPU.1, 500ng of 3xFLAG-Vpx/Vpr, and pUC19 to a total of 1µg of DNA per well were combined with 4µg of PEI, mixed and added to each well. 48hrs later, cells were harvested for flow cytometry or immunoblotting. For co-immunoprecipitations, transfection experiments were scaled to achieve 60 x 10^6 cells per condition. Co-transfections with p89.6-ΔGPERN-mCherry-pSFFV-EGFP and hPU.1 were performed as described above and with DNA amounts described in the legend.

Transduction of MDM, K562, and HEK 293T

All transductions were performed via spin inoculation at 1050 x g for 2 hr at 25°C with equal virus amounts determined by Gag p24 mass in medium containing 4μg/mL polybrene (Sigma). MDMs were inoculated with 10μg p24 mass equivalents of NL4-3 DGPE-GFP virus or 20μg p24 mass equivalents of 3xFLAG-89.6-Vpr. K562 and HEK 293Ts were spin inoculated with 10μg p24 mass equivalents of shScramble and shDCAF1 viruses. K562 cells were inoculated with varying amounts of 3xFLAG-tagged Vpr/Vpx expression viruses to achieve equal FLAG expression. After infection, viral medium was removed and replaced with fresh medium.

Short hairpin RNA-mediated silencing in MDMs was achieved through spinoculation of freshly isolated primary monocytes with VSV-G-pseudotyped SIV3+ *vpr*-null virus at 1000 x g for 1.5 hr with 4μg/mL polybrene to allow Vpx-mediated degradation of SAMHD1. Cells were then spinoculated with 10μg p24 mass equivalents of VSV-G-pseudotyped pLKO.1 containing shScramble or shSPI1 lentiviruses at 1000 x g for 1.5 hr. After virus removal, monocytes were cultured as described above for seven days with R10 containing M-CSF and GM-CSF. At day five, transduced cells were treated with 2.5μg/mL of puromycin for two days. Thereafter, cells were cultured for an additional 10 days in R10 before harvesting.

HIV infection of MDM

Prior to infection, half of the medium was removed from each well of MDMs and saved to make diluted conditioned media post-infection. MDM were infected with 5μg, 10μg, and 20μg (scRNA-seq) or 20μg and 50μg (immunofluorescence) equivalents of

Gag p24 mass diluted in R10 for 6 hr at 37°C. After the 6 hr infection, media was removed and replaced with conditioned media diluted 1:2 in R10. Half-media changes were performed every four days. For assessment of virion-associated impact on PU.1 (including MG132 treatment), MDMs were infected with 200-300µg of virus in R10 of either 89.6^{wt}, 89.6$^{\Delta vpr}$, or 89.6$^{\Delta vpr\text{-}Q65R}$ for 5hrs.

Single-cell RNA sequencing

At 10 days post-infection, uninfected, 89.6^{WT}, and 89.6$^{\Delta vpr}$ infected MDMs were lifted using enzyme free cell dissociation buffer (ThermoFisher). Replicate samples were fixed with paraformaldehyde and stained for Gag to assess viral spread by flow cytometry. The resulting flow cytometry data was used to select 89.6^{WT} and 89.6$^{\Delta vpr}$ conditions with similar percentages of infected cells. Wells of the selected conditions were harvested, counted, and prepared according to manufacturer instructions for 10X Chromium Next GEM Single Cell 3' v3 Gene Expression (10X Genomics).

Single-cell data analysis

10X filtered expression matrices were generated from CellRanger version 3.0.0 (10X Genomics). We analyzed all single-cell gene expression data using the standard LIGER (Welch et al., 2019) (https://github.com/welch-lab/liger) data integration pipeline. All WT and Vpr-null infected MDM raw data expression matrices from each donor were combined before merging the data. We used a value of k = 20 during joint matrix factorization, resolution of 0.05 for Louvain clustering, and nearest neighbor = 30 with a minimum distance = 0.3 for UMAP visualization. We identified infected cells by sub-

setting cells with a non-zero expression value for both *gag* and *tat* transcripts. We determined differential gene expression between WT and Vpr-null infected MDMs using the two-sided Wilcoxin rank-sum test. Volcano plots of differentially expressed genes were generated using ggplot2 (Wickham, 2009) (https://ggplot2.tidyverse.org/). Downregulated genes in the presence of Vpr with a log2FC > 1 and adjusted p-value > 0.05 were used as input for the HOMER (Heinz et al., 2010) (http://homer.ucsd.edu/homer/motif/) 'findMotifs' function using the human reference set. PU.1-motif associated genes were identified using the 'find' function in 'findMotifs' from HOMER. Gene Ontology analysis for biological processes for PU.1 motif-containing genes was determined using Gorilla (Eden et al., 2009, 2007) (http://cbl-gorilla.cs.technion.ac.il/) with PU.1 regulated genes as target genes and all expressed genes in our dataset as background. Biological processes were plotted using REVIGO (Supek et al., 2011) (http://revigo.irb.hr/). The -500bp sequence for MRC1 used for PU.1 motif scanning was obtained from UCSC Genome Browser (Kent et al., 2002) (https://genome.ucsc.edu/) using Human reference genome GRCh38/hg38. The PU.1 binding motif probability matrix was obtained from HOMER and used with FIMO (Grant et al., 2011) (https://meme-suite.org/meme/doc/fimo.html) to scan the *MRC1* input sequence. Violin plots were generated by importing our LIGER generated dataset into Seurat (Hao et al., 2021) (https://satijalab.org/seurat/) and running the VlnPlot function. All single-cell data analysis and plots were done using RStudio (RStudio Team, 2020) (http://www.rstudio.com/) except for HOMER-predicted motifs.

Virus production

Virus stocks were produced by transfected HEK 293T cells (ATCC, Manassas, Virginia) with viral DNA and polyethylenimine (PEI) (Polysciences, Warrington, PA) as previously described (Lubow et al., 2020). For replication defective constructs, cells were plated 24hrs before transfection with a DNA ratio of 1:1:1 with pCMV-HIV-1 (Gasmi et al., 1999), pHCMV-V (VSV-G expression plasmid) (ATCC 75497), and lentiviral expression plasmid. Viral supernatant was collected two days post-transfection and stored at -80°C. For infectious virus, pCMV-HIV and pHCMV-V were omitted.

Virion quantification

Supernatants containing viral particles were lysed in lysis buffer (0.05% Tween 20, 0.5% Triton X, 0.5% casein in PBS). Gag p24 antibody (1µg/mL, clone 183-H12-5C, cat# 1519 AIDS Reagent Program from Dr. Bruce Cheseboro and Dr. Hardy Chen) was bound to Nunc MaxiSorp plates (cat# 12-565-135, ThermoFisher) at 4℃ overnight. Lysed samples were captured at 4℃ overnight and then incubated with biotinylated antibody to Gag p24 (1:4000, clone 31-90-25, cat# HB-9725, ATCC) for 1 hr. Clone 31-90-25 was biotinylated with the EZ-Link Micro Sulfo-NHS-Biotinylation Kit (cat# PI-21925 ThermoFisher). Clones 31-90-25 and 182-H12-5C were purified using Protein G columns (cat# 45-000-054, GE Healthcare) following the manufacturer's instructions. Samples were detected using streptavidin-HRP for 30min (1:10000, Fitzgerald, Acton, Massachusetts) and 3,3',5,5'-Tetramethylbenzidine substrate (cat# T8665-IL Sigma). Reactions were quenched with 0.5M H_2SO_4. Absorbance was measured at 450nm with

a reference wavelength of 650nm. CAp24 concentrations were measured by comparison to recombinant CAp24 standards (cat# 00177 V, ViroGen, Watertown, Massachusetts).

Immunoblots

For western blots, cells were lysed in Blue Loading Buffer (cat# 7722, Cell Signaling Technology), sonicated with a Misonix sonicator (Qsonica, LLC. Newtown, CT), boiled for 10min at 95°C before loading, and analyzed by SDS-PAGE immunoblot.

For coimmunoprecipitation, cells were lysed in Pierce IP Lysis Buffer (Thermo Fisher Scientific) and 1x Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were incubated with Anti-FLAG M2 Magnetic Beads (MilliporeSigma, Darmstadt, Germany) according to the manufacturer's instructions. Proteins were eluted using 3xFLAG peptide (MilliporeSigma, Darmstadt, Germany) and analyzed by SDS-PAGE immunoblot. FLAG-tagged proteins were visualized using Pierce ECL (Thermo Scientific) after treatment with an HRP-conjugated primary antibody directed against the FLAG epitope (Millipore Sigma). HRP-conjugated secondary antibodies against murine and rabbit antibodies to other targets (see below) plus ECL Prime reagent (Cytiva Amersham) were used to visualize all other proteins.

Antibodies

Antibodies to Vinculin (1:1000, cat# V9131, Millipore Sigma), DCAF1 (1:1000, cat# 11612-1AP, Proteintech), PU.1 (1:100, cat# 2266S, Cell Signalling Technology), FLAG (1:1000, cat# F1804, Millipore Sigma), TET2 (1:250, cat#MABE462, EMD Millipore), Vpr (1:500, AIDS Reagent Program cat# ARP-11836 from Dr. Jeffrey Kopp), pr55 and p24

(1:1000, AIDS Reagent Program cat# ARP-3957), gp120 (1:1000, AIDS Reagent Program cat# 288), and GFP (1:1000, cat# ab13970, Abcam) were used for immunoblot analysis. Secondary HRP conjugated antibodies against murine (1:10000, rat anti-mouse IgG1, eBioscience), rabbit (1:5000, goat anti-rabbit IgG, cat# 65-6120, Invitrogen), sheep (1:20000, rabbit anti-sheep IgG, Dako), and human (1:10000, goat anti-human IgG, cat# 62-8420, ThermoFisher) were also used. Antibodies to PU.1 (1:100, clone 7C6B05, BioLegend), FLAG (1:3000, cat# 637324, BioLegend) were used for flow cytometry. Antibodies to ISG15 (cat# 15981-1-AP, Proteintech), IFITM3 (cat# 11714-1-AP, Proteintech), and AlexaFluor 647 (A21244, Fisher Scientific) secondary antibody were used for immunofluorescence. Dilutions listed below. CAp24 (1:400, clone KC57-PE cat# 6604667, Beckman Coulter) was used for both flow cytometry and immunofluorescence.

Immunofluorescence

MDMs were generated as described above in µ-slide glass-bottomed cell chambers (Ibidi, Gräfelfing Germany) and infected as described above. Cells were fixed by adding 4% paraformaldehyde (PFA) and permeabilized by adding 0.1% TritonX-100 in PBS. Cells were then blocked by incubating with 5% goat serum (Millipore Sigma) and 1% bovine serum albumin (BSA) in PBS for 30mins at room temperature. Primary antibodies against ISG15 (Proteintech) or IFITM3 (Proteintech) were diluted 1:450 or 1:400 respectively in 1% BSA in PBS and were incubated with cells for 90min at room temperature. Goat anti-rabbit AlexaFluor 647 secondary antibody (Fisher Scientific) was diluted 1:200 in 1% BSA and incubated with the cells for 30min at room temperature, protected from light. Cells were incubated with Anti-PE conjugated Gag (1:400) antibody

for 30min at room temperature. Cells were washed three times with PBS after each step. Nuclei were stained by diluting a 1 mg/mL stock of DAPI (ThermoFisher Scientific) 1:1,000 in PBS and incubating with the cells for 5 minutes at room temperature. Cells were imaged with a Nikon N-SIM + A1R confocal microscope. Identical laser and gain settings were used across all images for each individual replicate of the experiment. Images were processed using NIS viewer imaging software and corrected total cell fluorescence (CTCF) was calculated using Image J software (Schindelin et al., 2012). CTCF = Integrated Density – (area of selected cell x mean fluorescence of background readings). Total corrected fluorescence per cell was divided by the number of nuclei to normalize cell volume to account for multinucleated syncytia.

Quantitative RT PCR

HEK 293T cells sorted as described in 'Flow Cytometry' below were counted using the Countess II Cell Counter (Thermo Fisher Scientific), and cell samples were diluted such that all conditions contained the same cell numbers as input. RNA was isolated using the Zymo DirectZol RNA MiniPrep Plus extraction kit with an on-column DNaseI digestion. RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Quantitative PCR was performed using SYBR green qPCR Master Mix (Applied Biosystems) on a QuantStudio 3 Real-Time PCR System (Applied Biosystems) with ReadyMade PrimeTime primers for *SPI1* (cat# Hs.PT.58.19735554, Integrated DNA Technologies Inc, USA) and RT2 qPCR Primer Assay for Human GAPDH (cat# PPH00150F-200, Qiagen). Expression was quantified using ABI Sequence Detection software compared to serial dilutions of an SPI1 or

GAPDH synthetic sequence gBlock (Integrated DNA Technologies Inc, USA). Measured values for *SPI1* were normalized to measured values of *GAPDH*.

Flow cytometry

For cells requiring intracellular staining using antibodies directed against HIV Gag p24, FLAG-Vpx and -Vpr, and PU.1, paraformaldehyde-fixed cells were permeabilized with 0.1% Triton-X100 in PBS for 2 min followed by incubation with antibody for 30 min at room temperature. In all experiments, cells were gated sequentially by forward scatter vs. side scatter for cells and then by forward scatter area vs. height to exclude doublets. The gating strategy is shown in **Supplementary Figure 3.11B**. All transduced MDMs and transiently transfected HEK 293T cells were assessed for protein expression on the Cytek Aurora. GFP+ HEK 293T cells in the MG132 treatment experiments and for qPCR were sorted on the Sony SH800 cell sorter into R10. Untreated, GFP- cells were also sorted. All flow cytometry data was analyzed using FlowJo v10 software (BD Life Sciences).

Proteasome inhibition

Lyophilized MG132 was purchased from MilliporeSigma (cat# M8699) and dissolved in DMSO. For HEK 293Ts, MG132 was added to cellular medium to achieve a final concentration of 10μM at varying timepoints. Cells were harvested from replicate wells and all drug treatment timepoints were collected at once. DMSO-only control treatment wells (Vehicle) were treated with the same volume of DMSO as contained in the MG132 treatment conditions. For MDMs, cells were pre-treated with 2.5μM MG132 for 2hrs prior to infection, then maintained in 2.5μM MG132 throughout the infection.

Statistical Analysis

All non-single cell statistical analyses were performed using GraphPad Prism v10 Software (Boston, MA) as described in figure legends for each experiment.

Data Availability

CellRanger version 3.0.0-processed data generated in the manuscript have been deposited in GEO under accession code GSE220574 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE220574]. Raw sequencing data have been deposited in NCBI dbGAP database under accession code phs002915.v2.p1. The raw data are available under restricted access due to data privacy concerns and can be obtained by requesting access from NCBI. TF motif data and analysis from HOMER (Heinz et al., 2010) (http://homer.ucsd.edu/homer/) are described above.

Code Availability

We analyzed all single-cell gene expression data using the standard LIGER (Welch et al., 2019) (https://github.com/welch-lab/liger) multiple single-cell RNA-seq data integration pipeline. TF binding motif data was generated using the HOMER (Heinz et al., 2010) (http://homer.ucsd.edu/homer/motif/) 'findMotifs' function using the human reference set. PU.1-motif associated genes were identified using the 'find' function in 'findMotifs' from HOMER.

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Appendix

Population	Virus-treated	*gag+/tat+*	*gag-/tat-*
Cluster 0	47880	14641	33239
Cluster 1	1539	214	1325
WT-vpr	13639	6156	7483
Vpr-null	35780	8699	27081

Supplementary Table 3.1. MDM infection status by population measured with Gag.[10] The number of MDMs treated with the indicated virus (89.6^{wt} or $89.6^{\Delta vpr}$) included in our scRNA-seq analysis (**Figure 3.1E**) are indicated. Cells are listed as either virus treated, *gag+/tat+* or *gag-/tat-* within each cluster, or within infection exposure (WT = 89.6^{wt} and ΔVpr = $89.6^{\Delta vpr}$) across all three donors.

[10] This table was created by Maria C. Virgilio

			Percent Infected	
Donor	Experiment Type	Molecule	89.6 WT vpr	89.6 vpr-null
1	Flow Cytometry	Protein	65%	50%
1	scRNA-seq	mRNA	71%	48%
2	Flow Cytometry	Protein	45%	31%
2	scRNA-seq	mRNA	53%	28%
3	Flow Cytometry	Protein	52%	35%
3	scRNA-seq	mRNA	N/A	(a) 42%
3	scRNA-seq	mRNA	N/A	(b) 54%

Supplementary Table 3.2. MDM infection rates measured with Gag.[11] Percent infection of 89.6^{wt} and $89.6^{\Delta vpr}$ infected MDMs from each of three donors was determined by quantifying the percent Gag^{+} cells by either flow cytometry (protein) or gene expression levels from scRNA-seq data (mRNA), as indicated by the experiment type and molecule, over the total number of cells analyzed.

[11] This table was created by Maria C. Virgilio

ETS Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	ETV4	0.0000	1270
	ETV1	0.0000	1228
	ETS1	0.0000	987
	Fli1	0.0000	1229
	Elk4	0.0000	1038
	Elf4	0.0000	930
	Elk1	0.0000	1013
	ERG	0.0000	1135
	GABPA	0.0000	995
	ELF1	0.0000	925
	EHF	0.0000	876
	ETS	0.0000	674
	ELF5	0.0000	557
	Etv2	0.0000	768
	EWS	0.0001	327
	PU.1	0.0005	316
	ELF3	0.0011	417
	SpiB	0.0038	180
	EWS:FLI1-fusion	0.0063	534
	ETS:RUNX	0.0082	121
	SPDEF	0.0121	624

Homeobox Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	Hoxa13	0.0043	647
	HOXB13	0.0183	292
	Hoxd13	0.0183	1
	Nkx3.1	0.0255	746
	Hoxa9	0.0355	796

bZIP Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	CRE	0.0000	385
	Atf1	0.0043	434
	CREB5	0.0632	203

NRF Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	NRF1	0.0000	544
	NRF	0.0003	1

CCAAT Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	NFY	0.0000	782

ETS/IRF Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	PU.1-IRF	0.0004	670

IRF Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	IRF8	0.0006	209
	IRF2	0.0049	75
	IRF1	0.0453	68
	T1ISRE	0.0504	8

ZF Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	Sp5	0.0000	1786
	Sp2	0.0000	2047
	KLF1	0.0000	1607
	Sp1	0.0000	1125
	KLF5	0.0000	1787
	KLF3	0.0000	1089
	YY1	0.0000	203
	KLF6	0.0000	1497
	KLF14	0.0000	2044
	Klf9	0.0001	780
	Maz	0.0012	1644
	Klf4	0.0056	531
	GFY	0.0183	200
	ZNF143|ST	0.0567	275

bHLH Family Transcription Factors

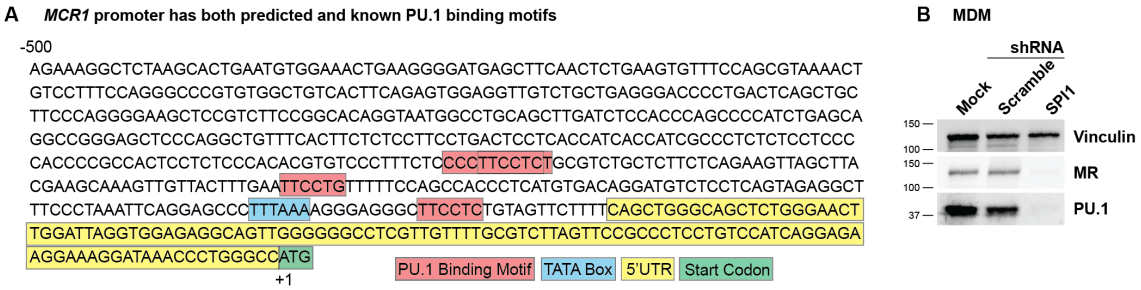
Motif	TF Name	q-value	# Targets w/ Sequence
	E-box	0.0000	212
	Usf2	0.0000	290
	MITF	0.0000	505
	TFE3	0.0000	160
	CLOCK	0.0000	402
	USF1	0.0002	371
	c-Myc	0.0006	495
	bHLHE41	0.0036	888
	bHLHE40	0.0041	280
	BMAL1	0.0043	679
	n-Myc	0.0150	432
	Max	0.0228	347
	NPAS	0.0254	612
	HIF2a	0.0636	246

THAP Family Transcription Factors

Motif	TF Name	q-value	# Targets w/ Sequence
	Ronin	0.0227	185

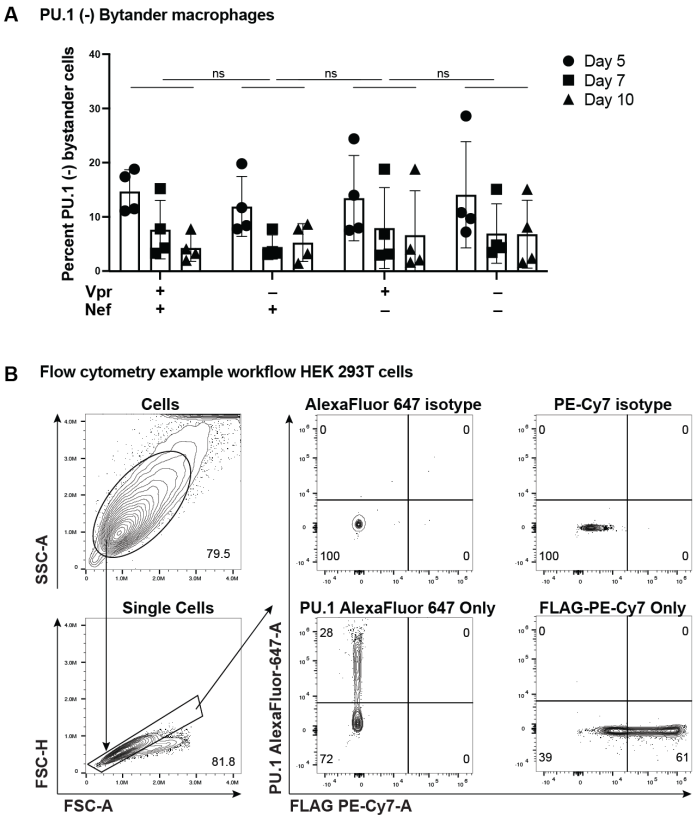
Supplementary Figure 3.9. Transcription factors and their predicted binding motifs in downregulated genes in MDMs expressing Vpr.[12] Motifs, transcription factor names, significance (FDR q-value), and number of target genes identified by HOMER analysis of genes downregulated in the presence of Vpr (**Figure 3.1E**) from several transcription factor families.

[12] This figure was created by Maria C. Virgilio



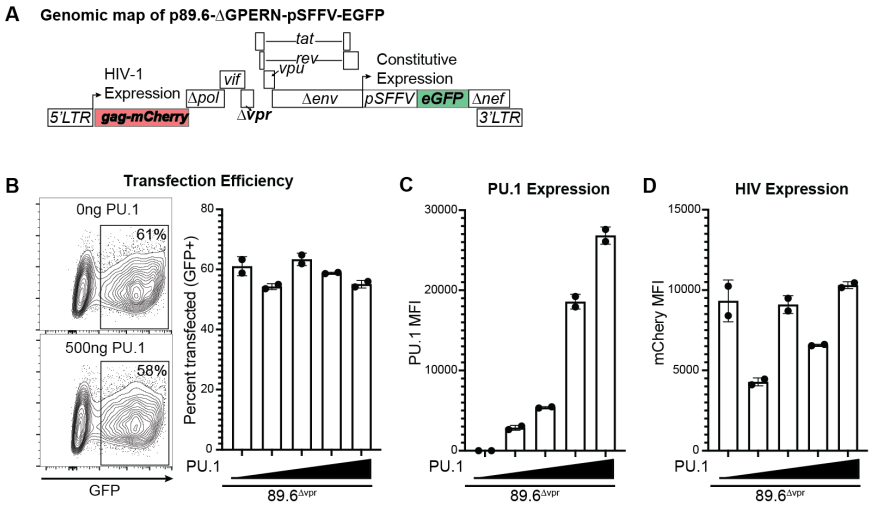
Supplementary Figure 3.10. The *MRC1* promoter contains multiple PU.1 binding motifs.[13] (A) The first 500 bp upstream of the start codon in *MRC1*, the gene that codes for mannose receptor. Previously reported PU.1 binding motifs are outlined in red. The double-box PU.1 motif was identified through inputting the HOMER generated PU.1 motif parameters into FIMO (Find Individual Motif Occurrences). The TATA box is outlined in blue, the 5'UTR in yellow, and the start codon in green. (B) Immunoblot analysis from MDMs stably expressing the indicated shRNAs, n = 2. Source data are provided as a Source Data file.

[13] This figure was created by Maria C. Virgilio



Supplementary Figure 3.11. PU.1 is not significantly reduced in bystander MDMs at 5-, 7-, and 10-days post infection with replication defective (non-spreading) HIV constructs.[14] (A) Summary graph showing the percentage of infected (GFP^-) cells that do not express PU.1 as determined by flow cytometry as depicted in **Figure 3.4B**. The mean +/- standard deviation from n=4 independent donors is shown for each time point. P values were determined using an analysis of variance (ANOVA) with Tukey's multiple comparisons test; ns = not significant. (B) Gating strategy used for flow cytometry in **Figures 3.4** and **3.5**, and **Supplementary Figure 3.12**. Source data are provided as a Source Data file.

[14] This figure was created by Maria C. Virgilio



Supplementary Figure 3.12. PU.1 does not alter HIV-LTR activity.[15] (A) Genomic map for 89.6-derived HIV-1 fluorescent reporter virus (89.6-ΔGPERN-pSFFV-EGFP). (B) Representative flow plots and bar graph assessing transfection rate via GFP expression in HEK 293T cells transfected with the indicated amount of PU.1 construct from **Figure 3.2E** plus 500ng of 89.6-ΔGPERN-pSFFV-EGFP from (A). (C) Summary graph of PU.1 expression in GFP^+ cells from (B). PU.1 levels were assessed using intracellular staining as described in Methods and measured by flow cytometry. (D) Summary graph of HIV-LTR activity as assessed by mCherry MFI from the same cells as in (C), measured flow cytometrically. All conditions were transfected with 500ng of HIV expression plasmid plus 0, 1, 10, 100, or 500ng of PU.1 plasmid. MFI = mean fluorescence intensity. Source data are provided as a Source Data file.

[15] This figure was created by Maria C. Virgilio

CHAPTER 4

Discussion

Summary of results

Vpr is an enigmatic HIV-1 accessory protein and virulence factor. Although it is highly conserved across lentiviruses, it does not provide a substantial benefit to $CD4^+$ T lymphocyte infection *in vitro* and can even be harmful due to cytotoxicity. Instead, many studies have confirmed Vpr is an essential protein necessary for efficient spread in macrophages and from macrophages to $CD4^+$ T lymphocytes. Several studies have (1) confirmed the necessity of Vpr to induce maximal infection and spread in tissues and cultures where both macrophages and $CD4^+$ T lymphocytes reside, and (2) confirmed that Vpr facilitates efficient spread by dampening the immune response to infection. Yet how Vpr exerts a macrophage specific effect remains incompletely understood and is highly contested in the literature.

Previous studies from our lab have demonstrated Vpr counteracts a macrophage specific restriction factor that would otherwise reduce HIV Env protein, increasing virion production and spread, primarily through virological synapses between infected macrophages and T cells or through syncytia formation.

In Chapter 2 we identified a macrophage specific restriction factor previously alluded to as the macrophage mannose receptor (MR). Unlike T lymphocytes,

macrophages express high amounts of MR (Stahl et al., 1980), and MR can bind to Env (Fanibunda et al., 2011; Lai et al., 2009; Trujillo et al., 2007). We found Env was significantly decreased by 89.6 vpr-null but not wild-type 89.6. Under conditions where infection rates were matched by Gag pr55 between 89.6-WT and 89.6ΔVpr, all three forms of Env (gp160, gp120, gp41) were significantly more abundant in Vpr-containing infections.

Previous reports indicated that Nef might also decrease expression of MR, therefore we similarly asked whether Nef could also downmodulate MR (Vigerust et al., 2005). These experiments were challenging because we were reluctant to knock out both *nef* and *vpr* in our spreading virus, because viruses lacking accessory proteins, particularly Vpr and Nef*,* spread very inefficiently. Instead, we used a replication-defective virus with mutations in *vpr*, *nef*, both, or neither to measure MR by flow cytometry. MR was dramatically suppressed when both Vpr and Nef were present; however the reduction was additive. Neither Vpr nor Nef could achieve the same level of reduction alone as they could together. Several complimentary experiments also confirmed our finding that both Nef and Vpr reduce MR and rescue Env levels. Therefore, we concluded both were needed for maximal reduction of MR and that MR is likely an important restriction factor to HIV, because the two different accessory proteins Nef and Vpr converge on reducing MR in infected macrophages.

HIV Env has a mannose patch which is a dense, mannose-containing structure, and the mannose patch is present on all Env proteins that require Vpr for stability in macrophages (Collins et al., 2015; Mashiba et al., 2014). We confirmed the binding relationship between MR and mannose residues on Env by several different methods.

The Env from the macrophage-tropic strain YU-2, which was isolated from the CNS of an AIDS patient (Li et al., 1991), lacks a mannose patch and could therefore be theoretically naturally resistant to MR. We observed no defect in spread between YU-2 WT or *vpr*-null in MDMs, and the *vpr*-null mutant only showed a minor defect in Env. Similarly, deleting the mannose patch in 89.6 Env rendered the Env less dependent on Vpr. Conversely, infection of MDMs by WT 89.6 could be inhibited through exogenous addition of D-mannose, which could competitively inhibit 89.6 Env from binding to surface MR. The effect was decreased infection in MDMs, suggesting MR is helpful for HIV on entry, presumably by binding Env and facilitating interaction between Env and surface receptors. Our finding that MR boosts HIV infection on entry is supported by previous findings that MR on the plasma membrane of macrophages binds HIV and passes the virus to T cells (Nguyen and Hildreth, 2003). MR typically binds pathogens at the cell surface and internalizes them through endocytosis, so it is a little surprising that MR enhances infection. More work needs to be done to address this finding.

Finally, to confirm MR was responsible for restricting Env, we performed a series of experiments using MDMs in which MR had been silenced. We observed that both Env expression and spread from MDMs to autologous $CD4^+$ T cells were no longer dependent on Vpr. The canonical method Vpr uses for modulating the cellular environment is by acting as an adaptor protein between host proteins and DCAF1, part of the Cul4A-E3 ubiquitin ligase complex (Hakata et al., 2014; Romani and Cohen, 2012; Yan et al., 2018). The result is poly-ubiquitylation and proteasomal degradation of the target of Vpr. When investigating the effect of Vpr on UNG2, a target of Vpr, compared to MR (Schröfelbauer et al., 2005), Vpr did not degrade MR by direct ubiquitylation using DCAF1 like it did for

UNG2. Instead, we found mRNA quantities of the MR gene *MRC1* were reduced in the presence of Vpr. Even though Vpr did not directly bind MR for recruitment to DCAF1, puzzlingly Vpr's ability to reduce MR expression still relied upon DCAF1. Reliance on DCAF1 was confirmed in experiments using the Vpr-Q65R mutant defective at DCAF1 interactions (Mashiba et al., 2014; McCall et al., 2008). Vpr-Q65R was unable to reduce MR to the same extent as Vpr-WT, and Env levels were reduced comparatively as with infections using Vpr-null virus. This indicated that MR was not a direct target of Vpr, but instead Vpr must be targeting an unknown intermediary with the reduced MR protein outcome of decreased expression.

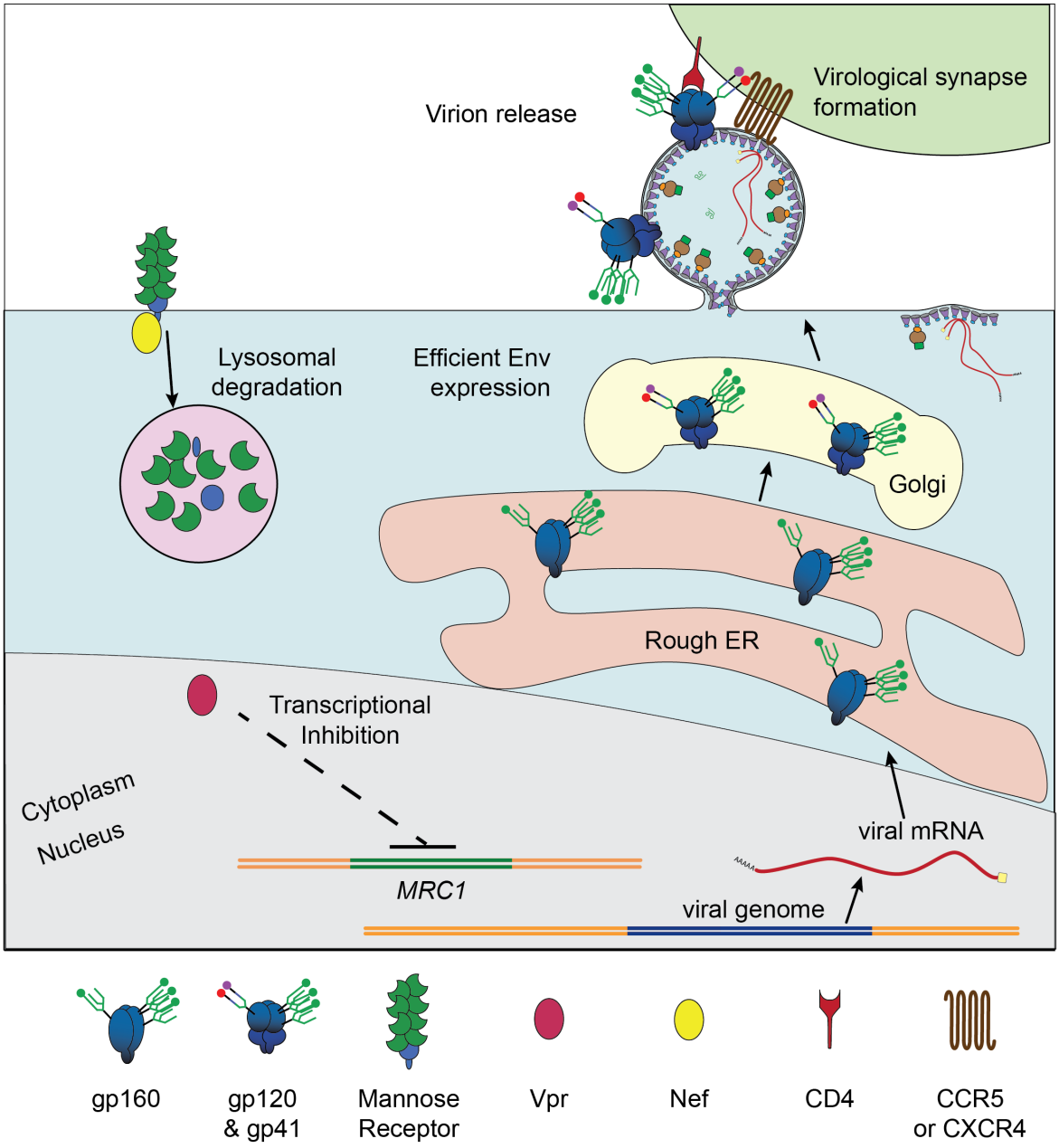


Figure 4.1. Model of Vpr- and Nef-mediated reduction of MR in HIV-infected macrophages.[1] Graphical depiction of MR reduction in HIV-infected macrophages. Nef associates with the cytoplasmic tail of MR at the cell surface and mediates trafficking of MR to the lysosome. Vpr reduces the transcription of the MR gene, *MRC1*. In the absence of MR, HIV Env traffics to the cell surface where new virions bud, and virological synapses form through Env binding to HIV-receptors on nearby uninfected cells.

[1] This figure was modified by Maria C. Virgilio from an earlier version made by Jay Lubow.

Chapter 3 details our efforts to understand the transcriptional impact of Vpr in HIV-infected macrophages. Our initial idea was that Vpr might target a transcription factor responsible for regulating MR expression. To test this hypothesis, we infected MDMs with 89.6 replication-competent virus having or lacking Vpr. After allowing the virus to spread naturally, we performed single-cell gene expression analysis. The benefit of this technique rather than bulk analysis of the cells was our ability to track natural HIV spread within each culture condition. We were able to use the detection of *gag* and *tat* transcripts to computationally segregate cells based on whether they expressed HIV transcripts (bona fide infections) or had yet to be infected (bystanders) based on no HIV transcription. Infected cells were also collected at different stages of infection – some freshly infected and others that had sustained infection for longer.

Other studies have suggested Vpr boosts HIV gene expression in macrophages (Zhang and Bieniasz, 2020). Indeed, when we compared the gene expression profiles between WT-Vpr and Vpr-null infected cells, the cells expressing *vpr* also expressed high levels of other HIV genes. HIV expression was also at the expense of thousands of host genes. To tease apart the relationship between Vpr and the thousands of genes suppressed in a Vpr-dependent manner, we looked for transcription factor (TF) binding sites in the promoter regions of suppressed genes. Although there were several TF-binding motifs capable of driving gene expression, many of these TFs are not expressed in macrophages or were not expressed at high levels (Aggarwal et al., 2012; Ohler and Wassarman, 2010). One of the exceptions was the myeloid master regulator PU.1. PU.1 is highly expressed in macrophages and it is a lineage-defining TF; high PU.1 expression is required for macrophage formation and maintenance (Dakic et al., 2005; Hohaus et al.,

1995). What was even more intriguing was that a co-TF binding motif also appears in many gene promoters. This was the PU.1-IRF motif.

PU.1 is well known to co-regulate gene expression with the coordination of other transcription factors, particularly members of the interferon regulatory factor (IRF) family (Gupta et al., 2009; Marecki and Fenton, 2000; Pongubala et al., 1992, 1993). As antigen presenting cells, macrophages must be able to sense pathogens, respond to their presence, and coordinate with the rest of the immune system to launch a response. PU.1 acts in concert with IRF proteins to regulate the expression of hundreds of important immune regulatory genes, particularly those involved in type I interferon responses and in pathogen sensing such as toll-like receptors (Marecki and Fenton, 2000). Unsurprisingly, the genes suppressed in the presence of Vpr and regulated by PU.1/PU.1-IRF accounted for approximately one third of the genes directly suppressed by Vpr. However, some of the PU.1 regulated genes are themselves transcription factors or capable of driving gene regulatory circuits that can contribute to feedback loops, further regulating gene expression profiles. This might explain the change in expression of at least a significant portion of the remaining two-thirds of the genes.

Remarkably, one of the genes we identified in our transcriptomic data as both suppressed by Vpr and regulated by PU.1 was mannose receptor. MR expression was reduced by about a single Log2-fold change, which was comparable to the suppression quantified by RT-qPCR (real time quantitative PCR) in HIV-infected MDMs with and without *vpr* from the data presented in Chapter 2. There are three PU.1 binding sites in the rat *MRC1* promoter, and one of these is shared by the mouse gene (Egan et al., 1999; Eichbaum et al., 1997). To confirm the regulation of *MRC1* by PU.1, we computationally

scanned the *MRC1* promoter for PU.1 binding motifs and found the conserved motif from mouse and rat in addition to two more. We achieved further confirmation of the PU.1 regulation of MR by knocking down PU.1 in MDMs, which reduced the amount of both PU.1 and MR. Therefore, we were confident we found the protein that Vpr targets in macrophages, regulating *MRC1* expression and preventing MR from limiting viral spread.

Upon further investigation of PU.1-regulated genes, we found clear patterns for the biological functions of PU.1-regulated genes suppressed by Vpr. Many of the functions both suppressed by Vpr and regulated by PU.1 are involved in intrinsic and innate immune functions such as toll-like receptor signaling, activation of the innate immune response, PRR function, and regulation of cytokine signaling. Some of the genes associated with these functions are associated with the type I interferon response and are interferon-stimulated genes. Some genes of interest stood out such as *LY96*, whose product is MD2, which senses pathogens and pathogen-associated products such as LPS, TLR4, interferon alpha inducible protein 6 (*IFI6*), interferon-stimulated gene 15 (*ISG15*), and interferon-induced transmembrane protein 3 (*IFITM3*). These were significant findings for many reasons. *IFITM3* is regulated by the DNA demethylase TET2. TET2 is a target of Vpr and its degradation prevents the expression of *IFITM3,* which would otherwise behave similarly to MR by binding Env and trafficking it to the lysosome, inhibiting spread (Lv et al., 2018; Wang and Su, 2019). *IFI6* and *ISG15* are both interferon-regulated genes and ISG15 inhibits HIV spread through targeting of Gag (Okumura et al., 2006).

Our finding that PU.1 is a target of Vpr and regulates the type I IFN response in macrophages puts important previous work from our lab into perspective. We previously showed that the effect of Vpr on virological synapse formation could be abrogated by

treatment with exogenous IFNα treatment (Collins et al., 2015). This is consistent with previous reports that Vpr prevents induction of type I interferons (IFNα, IFNβ) and MxA (an interferon stimulated gene) (Laguette et al., 2014). The connection to interferons may also explain the bystander effect we observed. Due to the single-cell nature of our transcriptional experiments, we could not only examine the transcription changes induced by Vpr within infected MDMs, but also how nearby uninfected, virus-exposed bystander cells behaved. We also observed that many interferon stimulated genes were upregulated in bystander cells in Vpr-null cultures compared to WT-Vpr and even uninfected, unexposed MDMs. The transcriptomic results were further confirmed through immunofluorescence confocal microscopy. IFITM3 and ISG15 proteins were suppressed in Gag+ cells and their bystander neighbors compared to Vpr-null treated MDMs. This suggests PU.1 plays a role in paracrine signaling to nearby immune cells, warning of infection, which is counteracted by Vpr.

Several lines of evidence suggest HIV-1 Vpr mediates the degradation of PU.1 protein in infected macrophages rather than directly altering expression. Neither PU.1 (measured by *SPI1*) or MR (measured by *MRC1*) RNA was significantly altered in bystander cells in our single-cell data. PU.1 protein measured by flow cytometry in both bystander and infected MDMs confirmed this. We used a non-spreading version of NL4-3 with GFP in the *env* reading frame. MDMs were infected with virus with all accessory proteins or lacking Vpr, Nef, or both. PU.1 protein was reduced in all viruses tested if they expressed Vpr and was unaffected in the bystander cells at the timepoints tested (5-10 days post infection). We wondered whether Nef might similarly affect PU.1 protein as it does for MR, but we found no evidence for this. Because PU.1 can self-regulate its

expression (Chen et al., 1995), we observed in our scRNA-seq data that PU.1 expression was lower in WT-Vpr cells than Vpr-null infected cells, but when we directly tested the impact of Vpr on PU.1 when expressed off a heterologous promoter, we observed no mRNA expression difference of PU.1 in the presence of Vpr in this system.

Vpr is a highly conserved accessory protein found in all lentiviruses (Tristem et al., 1998, 1992). It is also the only HIV-1 accessory protein that is packaged into virions. The only other accessory protein that resembles Vpr is the genetically related Vpx protein. Vpr and Vpx share a common genetic ancestor of unknown origin (Tristem et al., 1992). Though their functions have diverged over time, they share some behavioral similarities. Both interact with Gag and are packaged into virions, and both mediate the degradation of host restriction factors by associating with DCAF1 as part of the DDB1-Cul4-E3-Ub complex (Hrecka et al., 2011, 2007). Therefore, when attempting to address how well-conserved the ability of Vpr to degrade PU.1 in macrophages is, we used Vpx as our viral accessory control. Regardless of whether PU.1 was expressed from a heterologous promoter in HEK 293T cells, or endogenously in K562 cells, all HIV-1 *vpr* alleles tested were able to reduce PU.1 protein but not Vpx. This was true for the dual-tropic Vpr from 89.6, T-tropic from NL4-3, M-tropic from AD8, and the microglia isolate from YU2.

While Vpr from various HIV-1 sources was able to consistently reduce PU.1 levels, we did not know if the ability to degrade PU.1 was as conserved as Vpr itself. We therefore tested the ability of Vprs from both an HIV-2 molecular clone and several SIV molecular clones from various non-human primate sources. Unlike HIV-1, HIV-2 expresses both Vpr and Vpx, allowing us to directly test the function of these two intimately related accessory proteins from the same molecular source. Even from the same molecular clone, Vpr and

Vpx had divergent functions related to PU.1. Like 89.6 Vpr, HIV-2 Vpr was able to reduce PU.1 but not Vpx. Similarly, many of the Vprs from SIV molecular clones were also able to reduce PU.1. Those with the strongest effect on PU.1 came from SIVcpz and SIVgor, raising questions as to whether the ability of Vpr to reduce PU.1 was advantageous during zoonotic transmission from our non-human primate relatives into humans.

DCAF1 was originally identified through its association with Vpr and was appropriately named Vpr binding protein (VprBP) (Belzile et al., 2007; Zhao et al., 1994). Vpr mediates the recruitment of several host proteins to DCAF1 that would otherwise not associate with DCAF1 or would do so loosely. Vpr adapts many proteins to DCAF1, which results in not only the inhibition of the antiviral response as has been discussed but also cell cycle arrest phenotypes (Belzile et al., 2007; Hrecka et al., 2007; Jowett et al., 1995). Because we observed that the Vpr-mediated reduction of MR was DCAF1-dependent, we tested whether Vpr was recruiting PU.1 to DCAF1. We immunoprecipitated Vpr in HEK 293T cells over-expressing exogenous PU.1, in K562 cells endogenously expressing PU.1, or MDMs endogenously expressing PU.1. Regardless of the cell type or source of PU.1 Vpr precipitated with both PU.1 and DCAF1. In HEK 293T cells when PU.1 was not expressed, Vpr still precipitated DCAF1 as expected. In line with our other Vpx findings, Vpx did not precipitate with PU.1 in K562 cells. We confirmed the specificity of the complex by knocking down DCAF1 in K562 cells, which reduced the ability of PU.1 to associate with Vpr. Similarly, precipitating with a Vpr mutant defective in DCAF1 binding (Vpr-Q65R) completely abrogated the binding of both DCAF1 and PU.1 with Vpr, suggesting all three proteins must be present for complex formation. Performing the reverse IP in HEK 293T cells, where we could over express a FLAG-tagged PU.1 protein,

we also confirmed that all three proteins must be present for complex formation, because both knocking down DCAF1 and using Vpr-Q65R disrupted precipitation of both DCAF1 and Vpr with PU.1. Unfortunately, due to cell number constraints, we were unable to perform immunoprecipitations with Vpr-WT and Q65R or shRNA conditions in MDMs due to the low infection rate in each condition and the great number of cells required for each condition.

Using reverse immunoprecipitation where we precipitated with PU.1, we were able to investigate the association of PU.1 with TET2, to test our hypothesis that TET2 co-regulates IFITM3. TET2 immunoprecipitated with PU.1 in all conditions, regardless of whether Vpr was present in the cells. TET2 precipitated with PU.1, Vpr-WT, and DCAF1 but not with Vpr-Q65R, suggesting PU.1 is an important part of the Vpr-dependent degradation of TET2 in regulating *IFITM3* expression.

Taking advantage of the ability of Vpr to be packaged into virions at approximately 1:1 with p6 Gag (Paxton et al., 1993), we tested whether virion-associated Vpr was sufficient to degrade PU.1 in primary macrophages. Exposing MDMs to large titers of 89.6 with WT Vpr rapidly degraded PU.1 within five hours of exposure. The same virus with Vpr-Q65R did not degrade PU.1 as efficiently, and Vpr-null 89.6 virus did not degrade PU.1 at all. This indicates Vpr packaged into virions and released upon cellular entry can rapidly (in less than 5hrs) degrade PU.1 to quickly suppress the immune response. In contrast, newly synthesized Vpr would require a minimum of 24 hrs in macrophages to allow time for viral lifecycle events necessary to produce new viral protein products (including Vpr) such as reverse transcription of the viral genome, integration, expression, and protein synthesis. Treatment of these same macrophages with a proteasome inhibitor

prevented PU.1 degradation, confirming DCAF1 and the proteasome are involved in Vpr-mediated degradation of PU.1.

If Vpr specifically targets PU.1 in macrophages, dampening the immune response and enhancing viral spread, then reducing PU.1 in macrophages in the absence of Vpr should recapitulate at least some of Vpr's function in macrophages. To answer this question several important limitations needed to be addressed. First, as was discussed in Chapter 2, MR is a PU.1-regulated gene that supports viral entry while inhibiting viral egress once infection has been established. Therefore, to measure the effect of reducing PU.1 on spread, would necessarily also reduce MR before infection began, limiting entry. Secondly, PU.1 is an essential TF necessary for macrophage differentiation. Therefore, reducing PU.1 would likely have the side effect of de-differentiating the MDMs back into monocytes. Indeed, while attempting this experiment, we could see physiologic evidence that reducing PU.1 in MDMs did cause morphological changes consistent with de-differentiation and reversion of adherence to the culture dish. To avoid both pitfalls, we designed a modified 89.6 virus. In place of the *vpr* open reading frame we inserted an shRNA cassette targeting a scramble sequence or the gene encoding PU.1, *SPI1*. Using this virus would allow us to test whether reducing PU.1 only in infected cells could recapitulate the effect of Vpr on PU.1, enabling recovery of the inhibition of HIV Env without the complications of reducing MR before infection or changing the phenotype of the cells. Although the modified 89.6 viruses did not spread as well as WT 89.6, they were able to infect MDMs. With all three shRNAs targeting SPI1, we observed a noticeable increase in Env protein compared to the control shRNA. This was strong confirmation that

PU.1 is directly responsible for mediating inhibition of Env trafficking, viral spread, and the immune response to HIV.

In sum, Chapter 3 describes a novel target of Vpr; this target (PU.1) would otherwise orchestrate the innate immune response to HIV infection and severely limit the ease of spread. These results also underscore the importance of macrophages in HIV pathogenesis, antiviral immunity, and the conservation of accessory proteins like Vpr.

Working models, limitations, and future directions

The results presented in this dissertation support a model in which Vpr simultaneously suppresses several arms of the innate immune response to infection by singularly targeting PU.1. **Figure 4.1** briefly summarizes our findings discussed in Chapter 2, and **Figure 4.2** summarizes findings primarily discussed in Chapter 3. We presented several lines of evidence that Vpr reduces at least two host restriction factors (MR and IFITM3) capable of directly misdirecting Env to the lysosome and away from the cell surface, preventing spread. Several PRRs are also dysregulated through the PU.1-DCAF1-Vpr axis including MR and at least TLR4, though other TLRs could be affected. The upregulation of interferon-stimulated factors is stunted, as evidenced by a reduced transcription and protein output for several factors including *ISG15*, *IFITM3*, *IFI6*, among others. Not only does Vpr reduce the signaling capacity of infected cells, the effects of Vpr also reach bystander cells, blunting a systemic response to infection. Vpr is an extremely important accessory protein for all the immunodeficiency viruses discussed. While we answered many outstanding gaps in knowledge regarding Vpr's role in HIV pathogenesis, we did not address all aspects of Vpr's relationship with PU.1 and DCAF1,

nor did we discuss all the implications of our findings. Sections of this discussion will focus on some outstanding questions and avenues of future studies I find compelling.

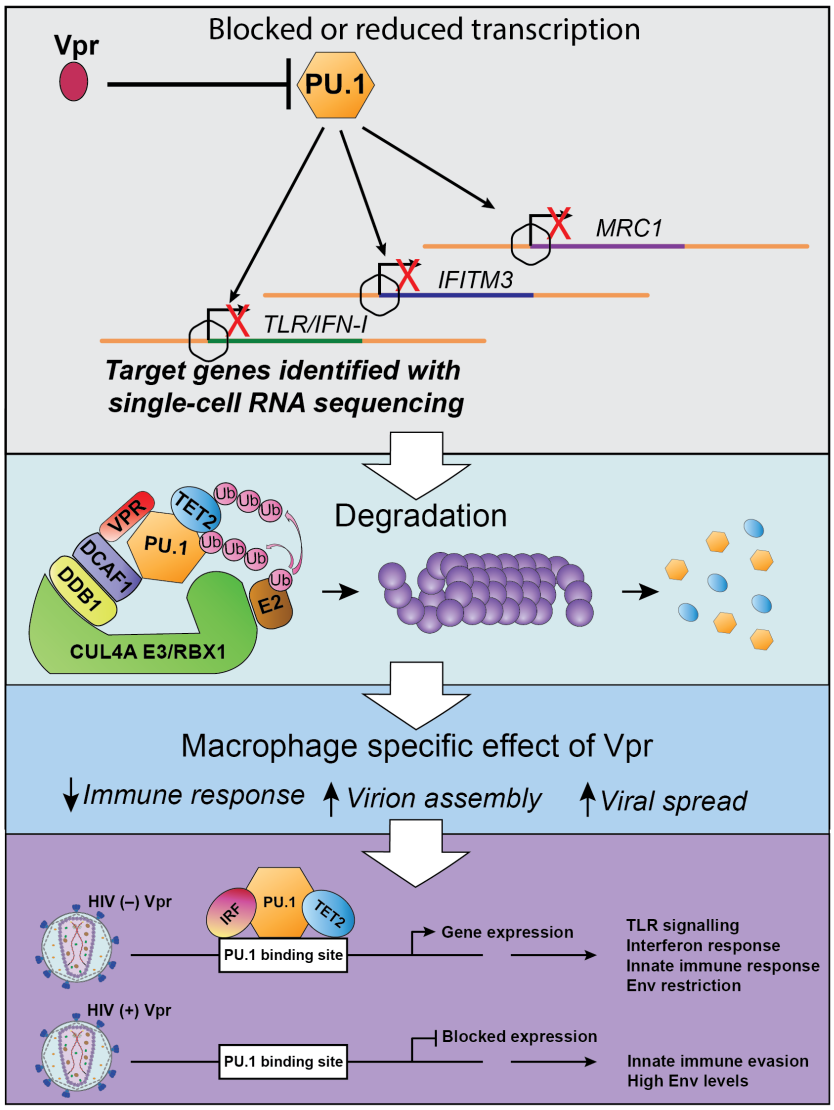


Figure 4.2. Model of Vpr-mediated reprogramming of HIV-infected macrophages.[2] Graphical depiction of HIV Vpr targeting the myeloid transcription factor PU.1, preventing PU.1 from upregulating target genes involved in the anti-viral response. Vpr recruits PU.1 to DCAF1, leading to poly-ubiquitylation and degradation of PU.1. The overall effect is suppression of the immune response, allowing virion assembly and enhancing viral spread. In summary, in the absence of Vpr, PU.1 and its co-factors respond to HIV infection by regulating genes involved in several immune processes, restricting Env. In the presence of Vpr, PU.1 is unavailable to upregulate target genes, leading to innate immune evasion without Env restriction.

[2] This figure was made by Maria C. Virgilio

Molecular structure of the Vpr-PU.1-DCAF1 complex

A remaining mechanistic question is exactly how Vpr, PU.1 and DCAF1 interact on an amino acid residue level. Vpr directly recruits host proteins to DCAF1 for degradation (Ahn et al., 2010; Laguette et al., 2014; Lv et al., 2018; Romani and Cohen, 2012). Perhaps the best characterized target of HIV is UNG2. Several groups and we have demonstrated Vpr acts as an adapter to recruit UNG2 to DCAF1 for polyubiquitylation and degradation. The standard interaction between Vpr-DCAF1 with a host protein is such that Vpr alone acts as intermediary between host protein and DCAF1, which is also the model for UNG2, such that they stack together as part of the Cul4A complex (model shown in **Figure 1.5**). In fact a high resolution crystal structure has definitively demonstrated the model protein-protein interaction depicted in **Figure 1.5** (Wu et al., 2016). Our model is slightly different. Based on immunoprecipitation experiments, we determined the most likely model of interaction requires all three proteins for PU.1 to interact with DCAF1 and Vpr such that PU.1 interacts with both Vpr and DCAF1 rather than Vpr only while Vpr interfaces with DCAF1. We did, however, confirm Vpr can associate with DCAF1 in the absence of PU.1 (Romani and Cohen, 2012).

We hypothesize that Vpr has many amino acid residues/motifs that can interact with different cellular targets. From our understanding of PU.1 and UNG2, it appears that that is possible. Therefore, future studies should focus on increasing the resolution of interactions among components of the PU.1-Vpr-DCAF1 complex. Likely single amino acid mutations will be necessary in both PU.1 and Vpr to determine which residue(s) in each protein coordinate contact. As a member of the ETS family of transcription factors, PU.1 has several domains including transactivation, PEST, and ETS DNA binding domains (Gupta et al., 2009). Which PU.1 domain interacts with Vpr is unknown, however

PU.1 requires phosphorylation of the PEST domain for activation in macrophages and the PEST domain is involved in protein-protein interactions (Marecki and Fenton, 2000). In preliminary experiments we discovered Vpr does not degrade the activated form of PU.1 as easily nor does it specifically interact with it. PU.1 must undergo phosphorylation for activation (Pongubala et al., 1993). Therefore, future studies might focus on residues associated with phosphorylation and activation of PU.1 such as serine, threonine, or tyrosine residues, particularly in the PEST domain for interactions with Vpr.

The relationship between PU.1 and TET2 as part of the Vpr-DCAF1 complex also lacks resolution. TET2 is monoubiquitylated by the DCAF1-Cul4A E3 ligase under normal conditions, promoting binding to chromatin (Nakagawa et al., 2015). During HIV infection of macrophages, Vpr targets TET2 for polyubiquitylation and degradation using the DCAF1-Cul4A UB ligase complex (Lv et al., 2018). What is not understood is why a broadly expressed transcription factor, which presumably is also expressed in $CD4^+$ T cells, would have a measurable effect on HIV infection of macrophages. Though there are many possible explanations, one that is supported by our results is that TET2 relies on the availability and coordination of other transcription factors for direction to promoters for reversing methylation. A previous study published findings that PU.1 directs TET2 to promoters in osteoclasts, which are bone macrophages (de la Rica et al., 2013). Because PU.1 often associates with other TFs to regulate expression, we wondered whether PU.1 is capable of associating with TET2, and specifically if we could provide some evidence that PU.1 directs TET2 to IFITM3 to relieve suppression. While we have not directly demonstrated this, we have shown TET2 strongly immunoprecipitates with PU.1, and we know from our scRNA-seq data that PU.1 regulates the expression of *IFITM3* (**Figures**

3.2 and **3.3**). Together, the data point toward a model in which the most likely explanation for the relationship between IFITM3, TET2, and PU.1 is that PU.1 directs TET2 to IFITM3, contributing to host defenses; however other explanations are possible and more mechanistic studies are needed (**Figure 4.2**).

One important limitation of the work presented here is our inability to demonstrate direct polyubiquitylation of PU.1 in complex with Vpr and DCAF1.We also could not detect polyubiquitylation of TET2 in the presence of Vpr as has been previously published (Lv et al., 2018). Attempts to IP using tagged PU.1, Vpr, and ubiquitin (Ub) in HEK 293T cells and show polyubiquitination of PU.1 in the presence of Vpr failed. Attempts to also treat cells overexpressing Vpr, PU.1, and Ub with MG132 to inhibit the proteasome were also unsuccessful for unknown reasons. In some cases, we could not confirm that MG132 treatments were successful. The sensitivity of untagged proteins was somewhat unreliable, among many other technical issues. In our attempts to confirm that TET2 could precipitate with either Vpr or PU.1 but be polyubiquitylated in the presence of Vpr also did not yield any interpretable results. TET2 did not appear to precipitate with Vpr in the absence of PU.1. Precipitation of TET2 was only achievable in our transfection system when immunoprecipitating with PU.1 to detect PU.1-associated proteins. While we successfully demonstrated MG132 could block Vpr-mediated reduction of PU.1 in HIV-infected macrophages, direct evidence of both PU.1 and TET2 polyubiquitylation in the presence of Vpr within our experimental system has yet to be demonstrated.

Evolutionary history of Vpr and PU.1 for zoonotic transmission

The degree to which Vpr influences PU.1 varies with the Vprs we tested. One of the limitations in our study was we did not test the efficacy of Vprs from SIV isolates against PU.1 from the SIV host species. Although hematopoiesis is highly conserved within vertebrates, and the lineage-defining transcription factors tend to also be highly conserved (Orkin and Zon, 2008), there are always exceptions and the possibility of minor variations in genetic or amino acid sequence. Future studies should test the ability of Vpr to degrade PU.1 against the native host PU.1 protein. It is possible that in a species-equivalent system, SIV Vprs may reduce PU.1 equivalently to what we observe with HIV-1 and human PU.1. It also entirely possible that Vpr does not always target PU.1 equivalently with other accessory proteins such as Nef and Vpu, which undertake different roles in different SIV lineages (Collins and Collins, 2014).

As was discussed in the previous section, understanding the protein-protein interactions among PU.1-Vpr-DCAF1 from the same species will be important, e.g., whether those of SIV have a similar relationship as human PU.1 with the Vprs tested, and why. Some of the Vpr alleles from SIV origins did not downmodulate human PU.1 and were not included in the data presented in Chapter 3. Whether they would have done so with their host species PU.1 is unknown. It is curious that some of the close relatives of HIV-1 and HIV-2 and some more distant SIV ancestors could downmodulate human PU.1. Some non-human primates seem to have a high prevalence of SIV in their native population and tolerate high viral titers without showing signs of disease (Sharp and Hahn, 2011). Although some of the reasons why have been identified, perhaps additional

explanations could be that their Vprs are not toxic to transcription factors like PU.1 (Paiardini et al., 2009).

In Chapter 3, we expressed Vpr from two different SIVcpz isolates that varied in their ability to degrade PU.1. The SIVcpzPtt, which is where HIV-1 Group M comes from, was better able to degrade PU.1 than the allele from SIVcpzPts which originated from a group of chimpanzees living separately from the Ptt group (Vanden haesevelde et al., 1996). That SIVcpzPtt has not crossed into humans might indicate the virus lacks sufficient ability to counteract host restriction factors and SIVcpzPtt maintained such an ability. This hypothesis is supported by the data in Chapter 3, **Figure 3.5**, where we found that a Vpr allele originating from an SIVcpzPtt isolate was able to reduce PU.1 protein in HEK 293T cells more than the isolate from SIVcpzPtt. Whether Vpr's ability to interrupt PU.1 activity contributed to zoonotic transmission from chimpanzees to humans is an interesting idea that should be explored.

Finally, the effect of Vpr on PU.1 is also dependent on PU.1's functionality and relationship with other partners in signaling. The TLR4 gene in sooty mangabeys contains a frameshift mutation resulting in a truncated tail domain (Palesch et al., 2018). The truncation influences signaling from stimulants such as LPS and also SIV, which might explain why sooty mangabeys can tolerate high viral titers without showing signs of disease. The old world monkeys with this mutation have a reduced inflammatory response to the virus (reduced IL-6 and TNFα production). The same mutation is not found in humans, chimps, or gorillas. If signaling through TLR4 is important for PU.1-mediated response to HIV, then it seems plausible to think that a truncated version of TLR4 might not signal as strongly to PU.1, IRF, and other transcription factors, blunting

the response to infection. Perhaps the Vprs produced by the SIVs from hosts with a truncated TLR4 do not need to degrade PU.1 as much. It would be interesting to test whether a similar truncation in human PU.1 could render Vpr unnecessary in HIV infection of macrophages.

What is driving the bystander effect?

The bystander effect is particularly important when considering the current model of HIV pathogenesis. Macrophages are present at mucosal surfaces at sites of HIV exposure such as the genitals (Anderson et al., 2011; Ganor et al., 2019) and pass HIV to T cells through virological synapses, a process that can be inhibited by IFNα (Collins et al., 2015). A hypothesis is that the ability of Vpr to inhibit the IFN-I in more than just infected cells is potentially a very important aspect of HIV pathogenesis. Vpr may be able to dampen the immune system enough to prevent further spread to uninfected cells without having to infect them. What exactly is responsible for this bystander effect is still unknown. We also demonstrated virion associated Vpr could degrade PU.1 within 5 hrs of infection, i.e., in a time frame before HIV genome integration, transcription, and translation to produce new Vpr. It is possible some of the bystander effect we observe is caused by Vpr introduced to cells through virion fusion without us being able to detect new Gag synthesis. Implementing spatial transcriptomic approaches (**Figure 1.9**) would allow us to observe both RNAs and proteins of interest within this system. Macrophages are easily cultured on glass slides as adherent cells and could be processed for spatial transcriptomics, with protein staining for Vpr and/or pr55 Gag in bystander cells. We would

then be able to locate infected cells and examine the transcriptomic profile in bystander cells at different distances to the source of virus or paracrine signals.

Consequences of Vpr-PU.1 in HIV-infected HSPCs

PU.1 is an important, lineage defining transcription factor, and expression of PU.1 is tightly regulated within the hematopoietic compartment. High levels of PU.1 push cells toward macrophage differentiation, whereas lower levels of PU.1 push cells towards B cell development (DeKoter and Singh, 2000). PU.1 is also needed for T lymphocyte development. In the absence of PU.1, hematopoietic cells progress towards other lineages including megakaryocytes and erythrocytes (Iwasaki et al., 2003). HSCs give rise to all hematopoietic lineages for the life of the individual, including lymphoid cells (such as T and B cells) and myeloid lineage cells (such as monocytes, macrophages, and dendritic cells). HSCs possess enormous proliferative potential, are extremely long lived, and are also permissive to HIV infection. Our lab has isolated and sequenced several proviral sequences from HSPCs from patients (Carter et al., 2010; McNamara et al., 2012; Sebastian et al., 2017; Zaikos et al., 2018). Given that PU.1 is a lineage-determining factor expressed very early in hematopoiesis to drive differentiation of multiple hematopoietic lineages and these same cells could become infected with HIV producing Vpr, this begs the question of whether Vpr from HIV-infected HSCs can alter their differentiation trajectory toward lineages with a lower PU.1 requirement or toward lineages totally non-reliant on PU.1. If so, daughter cells derived from HIV-infected progenitors would mostly be in the erythrocyte/megakaryocyte lineage or the B/T lymphocyte lineage and pushed away specifically from the myeloid lineage. Answering

this question would be challenging and would likely involve several forms of evidence. One possible option would be to trace replication defective proviral sequences from HSC sources with both the promoter and *vpr* ORF intact and match the sequence to daughter cells from the same donor. This is an imperfect solution, because the dynamics of hematopoiesis and the immune system are multifaceted. Alternatively, tracing the differentiation trajectory of HSPCs infected with *vpr* expressing virus and uninfected bystander cells from the same culture using single-cell gene expression analysis would help to clarify whether the presence of Vpr in infected stem cells can alter the trajectory compared to uninfected sister cells.

Finally, lowered levels of PU.1 are commonly associated with development of acute myeloid leukemia (Rosenbauer et al., 2006, 2004; Will et al., 2015). Given the findings presented in this dissertation, it is curious what the long-term effect of PU.1 reduction in HIV infected macrophages is for PLWH. Macrophages survive active HIV infection for long periods of time, and from our knockdown experiments discussed in Chapter 3 and the literature, we know that broadly reducing PU.1 leads to de-differentiation of macrophages into monocyte-like phenotypes. Therefore, it is curious why we don't observe more cases of myeloid leukemias in HIV+ persons. Perhaps in AIDS patients, the person does not live long enough to develop hematopoietic cancers. It is also possible that PLWH who are treated with ART do not have a large enough myeloid reservoir to support oncogenesis, or perhaps the effect of Vpr on PU.1 is not oncogenic in nature. Whether there is an increased incidence of PU.1-reltated cancer in PLWH would be an interesting epidemiological study.

Conclusions

In summary, the work presented in this dissertation expands our understanding of the role of Vpr in HIV infection of macrophages. We identified the myeloid transcription factor, PU.1, as a target of Vpr. This finding allowed us to piece together many unresolved mysteries of Vpr function in suppressing the immune response and aiding viral spread specifically from macrophages. PU.1 orchestrates the maintenance of macrophage function and the macrophage immune response. This is achieved through regulation of several HIV restriction factors targeting HIV Env and the expression of interferon-stimulated genes. The effect of Vpr on infected macrophages also influences the innate immune function of nearby uninfected cells. We identified MR as a macrophage-specific restriction factor counteracted by Vpr and regulated by PU.1. MR is an HIV restriction factor mediating the degradation of Env, which ultimately limits viral spread. Both Vpr and Nef coordinate to reduce MR levels through independent mechanisms. Unexpectedly, MR acts both as an entry factor and a restriction factor, which HIV uses to establish infection but then counteracts to permit egress, respectively. By targeting PU.1, Vpr boosts Env production and trafficking, and simultaneously suppresses the innate immune response. Ultimately, Vpr significantly enhances viral spread from macrophages to CD4+ T cells.

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