**HIV Infection of Macrophages Is Restricted by Mannose Receptor**

**by**

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## Dedication

*To Sam and my family*

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**Abstract**

HIV infects several cell types in the body including $CD4^+$ T cells and macrophages. The viral accessory protein Vpr is highly conserved in primate lentiviruses and promotes infection of both T cells and macrophages *in vivo* by unknown mechanisms. Previous studies demonstrated that Vpr enhances expression of HIV Env in macrophages and that this effect accelerates viral spread in macrophage cultures. Interesting, Vpr did not significantly enhance HIV infection in T cell cultures. The discrepancy between Vpr's effect on T cell infection *in vivo* and *in vitro* was, until recently, unexplained. In a series of studies, we determined that Vpr and its cellular co-factor, DCAF1, are necessary to enhance infection of macrophages and co-cultured $CD4^+$ T cells. Remarkably, we found evidence that Vpr counteracted a macrophage-specific, intrinsic antiviral pathway that targeted Env protein and Env-containing virions to lysosomes. When infected macrophages were co-cultured with uninfected, autologous $CD4^+$ T cells we observed efficient spread via virological synapses, structures that form when Env on an infected macrophage binds CD4 on an uninfected T cell. By enhancing Env expression, Vpr enhanced formation of virological synapses and viral spread from macrophages to T cells. We found that the restriction of Env we observed in macrophages was mediated through detrimental interactions between mannose residues on Env and the macrophage mannose receptor (MR). Vpr counteracted this effect by reducing transcription of MR. Silencing

MR or genetically deleting mannose residues on Env rescued Env expression in HIV-1-infected macrophages lacking Vpr and increased spread from macrophages to T lymphocytes. Surprisingly, these experiments also demonstrated that disrupting interactions between Env and MR reduced initial infection of macrophages by cell-free virus, indicating that Env's interaction with MR promotes viral entry. Together these results reveal a Vpr-Nef-Env axis that hijacks a host defense mechanism to boost viral entry then disables MR to alleviate detrimental interactions that inhibit viral egress.

# Chapter 1

## Introduction

Human immunodeficiency virus (HIV-1) is a leading cause of morbidity and mortality around the globe. Due to increased access to antiretroviral therapy the number of deaths caused by HIV has halved from ~2 million/year in 2007 to ~1 million/year in 2017, but the population of persons living with HIV has swelled to 37 million (Frank et al., 2019). Persons receiving antiretroviral treatment still experience significant cognitive and cardiovascular symptoms due to infection (Montoya et al., 2017) and psychological symptoms due to social stigma (Lowther et al., 2014). We do not fully understand the physiology of these symptoms, highlighting the need to investigate understudied aspects of HIV pathogenesis. In this chapter I will provide an introduction to HIV biology with particular emphasis on infection of macrophages and the effects thereof. This will include a review of the innate defenses in macrophages that restrict infection and the viral proteins that counteract host defenses.

## Macrophages in health and disease

### *Distribution and variety of macrophages*

There are numerous types of macrophages in the human body that are specialized for their particular role and anatomical location. Some macrophages are derived from monocytes, which circulate in the blood and can differentiate into several different cell types including macrophages and dendritic cells (Jakubzick et al., 2017). Monocytes, in turn, are derived from hematopoietic progenitor cells in the bone marrow (Fogg et al., 2006). In recent years evidence has accumulated that another set of cells, collectively referred to as tissue resident macrophages, are derived from myeloid progenitors that migrate from the embryonic yolk sac within the first weeks of development (Perdiguero et al., 2015). These precede the development of monocytes derived from bone marrow and persist for the life of an organism (Hashimoto et al., 2013). The fraction of macrophages that are monocyte derived and the fraction that are embryonic varies across anatomical sites and is a matter of ongoing study. In the liver, macrophages known as Kupffer cells are primarily derived from yolk-sac myeloid progenitors (Ikarashi et al., 2013; Naito et al., 2004). By contrast in skin, Langerhans cells are thought to be derived from a mix of yolk sac progenitors and monocytes (Collin and Milne, 2016). The central nervous system is home to multiple types of macrophages that can be distinguished by surface receptors, location, and morphology. Of these, astrocytes are derived from neural stem cells (Yang et al., 2013) and microglia from myeloid progenitors (McKercher et al., 1996). In the lungs, alveolar macrophages develop from myeloid progenitors during embryogenesis (Guilliams et al., 2013) and are replaced by monocyte derived cells slowly or not at all (Murphy et al., 2008). These cells are primarily identified by surface markers, of which the

most useful for studies in humans are CD68 (Micklem et al., 1989), CD11b (Taylor et al., 2005), and CD14 (Becher et al., 1996; Matsuura et al., 1994) although immature monocytes also express CD14 (Goyert and Ferrero, 1987).

*Macrophage functions*

A wide array of receptors on the surface of macrophages allow macrophages to detect and respond to various stimuli [reviewed in (Taylor et al., 2005)]. A major target of macrophages are host cells displaying apoptotic markers, which are recognized by numerous receptors and phagocytosed (Arandjelovic and Ravichandran, 2015). This allows the clearance of dead and dying host cells and avoids activating an inflammatory response (Aderem and Underhill, 1999). The most common apoptotic marker is phosphotidylserine, a phospholipid that is restricted to the cytoplasmic side of the plasma membrane in healthy cells, but is transported to the extracellular side of the membrane during apoptosis and detected by the macrophage receptor TIM4 (Miyanishi et al., 2007).

Macrophages are among the first lines of defense in the innate immune system, primarily due to their ability to phagocytose pathogens. Phagocytosis can be mediated by a wide variety of receptors on the plasma membrane including pattern recognition receptors (PRRs), which directly bind pathogen-associated molecular patterns (PAMPs); complement receptors, which bind components of the complement system; and Fc receptors, which bind the constant regions of antibodies. Within these categories are numerous subtypes that collectively provide the capacity to phagocytose nearly any pathogen under the right circumstances including fungi (Erwig and Gow, 2016), parasites (Keen et al., 2007; Murta et al., 1999), *Listeria monocytogenes* (Schnitger et al., 2011),

influenza A virus (Benne et al., 1997), foot-and-mouth disease virus (McCullough et al., 1988), and *Staphylococcus aureus (Peiser et al., 2000)* among others. For those pathogens that do no express sufficient PAMPs to be phagocytosed directly, soluble factors including IgG (Indik et al., 1995), complement, and antimicrobial peptides (Wan et al., 2014) bind pathogens and significantly enhance phagocytosis.

Phagocytosis destroys targeted pathogens and is the first step in the macrophage's role as a professional antigen presenting cell (APC). Macrophages, along with dendritic cells and B cells, degrade ingested proteins and present the resulting peptides on MHC class I and class II molecules to activate T cell responses. This process has been demonstrated in numerous types of macrophages including Kupffer cells (Winwood and Arthur, 1993), adipose tissue macrophages (Morris et al., 2013), and alveolar macrophages (Miyata and van Eeden, 2011). Presentation of exogenous antigens on MHC class I, a process called cross-presentation, only occurs in professional APCs and this process in macrophages is crucial for activating CD8+ cytotoxic T lymphocytes (Brode and Macary, 2004; Ramirez and Sigal, 2002; Tobian et al., 2004).

*Infection of macrophages by pathogens*

In addition to their role in clearing pathogens, macrophages can also be the target of infection. Due to their natural ability to phagocytose foreign bodies, they are particularly vulnerable to intracellular bacteria (Weiss and Schaible, 2015) including *Mycobacterium tuberculosis* (Schorey et al., 1997), *Legionella pneumophila* (Nash et al., 1984), and *Listeria monocytogenes* (Schnitger et al., 2011). Macrophages are also infected by a variety of viruses. Dengue virus (DENV) has been found in macrophages in the liver, lung,

and skin. Repeated infections of DENV in an individual are significantly more virulent than initial infection, and it is thought that this is due to highly-efficient, antibody-dependent infection of macrophages (Halstead, 1989). In monkeys, macrophages are infected by SIV (Simon et al., 1992), which leads to dissemination to the central nervous system of infected monkeys (Chakrabarti et al., 1991). Similar results have been observed during HIV infection in humans (Ho et al., 1986), and will be discussed in detail later in this chapter.

*Macrophage models*

Due to their significance in homeostasis, immunity, and infection by various pathogens, models that facilitate investigation of macrophages are in high demand. Mature macrophages harvested from human tissues are the most physiologically relevant but are limited by costs and ethical concerns. Lymphoid tissue collected from tonsillectomies are often used when 3 dimensional structures and other cell types are desired (Jayakumar et al., 2005). For models of isolated macrophages, alveolar macrophages obtained from bronchoalveolar lavage can also be used for *ex vivo* studies (Mautino et al., 1997; Wewers et al., 1984). Primary macrophages can also be generated from monocytes, of which tens of millions can be obtained from peripheral blood of an individual. These are stimulated *in vitro* using macrophage colony stimulating factor (M-CSF) often in conjunction with other cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4). Different stimulation conditions cause differential expression of key macrophage proteins including CD4, PRRs, and chemokines receptors (Lee et al., 1999). Additionally there is significant variability across

donors (Krapp et al., 2016), which can generate misleading results if a small number of donors are used.

A handful of cell line models exist and provide useful clues about macrophage biology. The most common is the THP-1 line, derived from a patient with monocytic leukemia (Tsuchiya et al., 1980). These monocyte-like cells can be induced to differentiate into macrophage-like cells with various combinations of phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS), and vitamin D3. These stimulated THP-1s replicate some but not all features of primary monocyte derived macrophages (Daigneault et al., 2010).

## Mannose receptor

### *Cell biology of mannose receptor*

Mannose receptor (MR) is a multidomain, multifunctional pattern recognition receptor, which is highly expressed on macrophages and a handful of other cell types, including dendritic cells and epithelial cells (Linehan et al., 1999; Martinez-Pomares, 2012). It bears significant similarity to three other proteins in the mannose receptor family, of which MR was the first characterized (East and Isacke, 2002). They are all type I transmembrane proteins composed of an N-terminal cysteine rich domain, a fibronectin type II domain, and 8-10 C type lectin domains before the transmembrane domain and cytoplasmic tail **(Figure 1.1)**. The cysteine rich domain binds sulfonated sugars such as 6-SO(4)-N-acetylglucosamine and multiple hormones (Leteux et al., 2000). The fibronectin type II domain binds collagen (Martinez-Pomares et al., 2006; Napper et al., 2006), which leads to endocytosis of collagen and is an important part of maintaining the

extracellular matrix (Madsen et al., 2011; Madsen et al., 2013). MR contains 8 C type (calcium dependent) lectin domains, which bind mannose and several other hexoses. When the domains are tested in isolation, lectin domain 4 has by far the highest affinity for mannose, but when multiple domains are tested together, a fragment containing domains 4 -8 has higher binding affinity still, indicating that binding is multivalent (Taylor et al., 1992; Taylor and Drickamer, 1993). That multiple MR domains can bind the same ligand simultaneously is also supported by the observation that MR has higher affinity for multiply branched polysaccharides than linear ones (Kery et al., 1992).

MR expression and function has been studied extensively in macrophages. It is estimated that at any given moment approximately 100,000 copies of MR are available for binding on the surface of a macrophage and that 5 times that number are in internal compartments (Stahl et al., 1980). Mannose-containing particles are internalized within 5 minutes of binding and this process was not inhibited by cycloheximide, which blocks translation of new proteins, indicating that newly synthesized MR is not required for MR activity (Stahl et al., 1980). This suggests that mannose receptor recycles to the cell membrane to bind and endocytose cargo repeatedly. The protein has been demonstrated to have a very long half-life of 33h (Lennartz et al., 1989), which means each MR molecule binds and endocytoses hundreds of ligands during its lifetime. Newly synthesized MR cannot bind mannose until 10-30 minutes post translation, indicating that a post translational maturation step, most likely in the Golgi, is required for activation (Pontow et al., 1996)

*Microbial interactions with mannose receptor*

MR's activity as a pattern recognition receptor that leads to endocytosis is an important part of macrophages' function as immune cells. MR binds polysaccharides and lipopolysaccharides found in capsules and cell walls of numerous species of bacteria, leading to endocytosis (Zamze et al., 2002). MR also binds mannoproteins, i.e. glycoproteins with mannose residues, on the fungal pathogen *Cryptococcus neoformans.* This leads to activation of $CD4^+$ T cells and mice that lack MR *(MRC1-/-)* are significantly less likely to survive infection by *C neoformans* (Dan et al., 2008).

MR can also be hijacked by pathogens to evade immunity and enhance pathogenesis. Mannose-capped lipoarabinomannans produced by *Mycobacterium tuberculosis* bind MR and inhibit production of IL-12, TNF-α, and TGF-β (Knutson et al., 1998; Nigou et al., 2001). MR is responsible for binding and internalization of *Streptococcus pneumoniae* by macrophages, which results in intracellular survival and reduced inflammation (Subramanian, Neill et al. 2019). MR also mediates entry of *S. pneumoniae* to olfactory ensheathing cells (Macedo-Ramos et al., 2011) and Schwann cells (Macedo-Ramos et al., 2014), which provide a path through which the bacterium accesses the central nervous system. Several viruses also use MR to enhance entry. Most notably, MR increases infection of macrophages by DENV and increases inflammation (Miller et al., 2008). Treatment of MDM with vitamin D3 lowers MR expression, which reduces DENV infection and release of inflammatory cytokines (Alzate et al., 2017).

HIV has previously been shown to interact with MR, but the mechanisms by which this occurs and the implications for viral replication are not clear. One group found that

MR binds HIV on the plasma membrane of macrophages and transfers virions to CD4+ T cells, a process called trans-infection (Nguyen and Hildreth, 2003). They did not investigate direct infection of macrophages. Another group observed that HIV infection decreases phagocytic function in alveolar macrophages (Koziel et al., 1993), which correlates with the virus's effect on MR expression (Koziel et al., 1998). Consistent with this finding, the highly related simian immunodeficiency virus (SIV) reduces MR staining of glial cells in the brains of infected macaques (Holder et al., 2014). Although a reduction in MR expression by HIV infection has been observed repeatedly and is likely clinically relevant, the mechanism for this action has not been established.

## Human Immunodeficiency Virus

### *Basic biology*

Human immunodeficiency virus type I (referred to as simply HIV throughout this dissertation) is a lentivirus of the retroviridea family (Levy, 2013). The viral particle contains two copies of a positive strand RNA genome, a capsid composed of the viral protein Gag, and a lipid membrane derived from the producer cell. The viral protein Env is anchored in the membrane and is responsible for binding the main HIV receptor CD4 and a co-receptor, either CCR5 or CXCR4 (**Figure 1.2**). Env structure and function is described in greater detail below. HIV can only infect cells that express CD4 and CCR5 or CXCR4, which to the best of our knowledge limits the virus's tropism to immune cells, namely $CD4^+$ T cells (Stein et al., 1987), macrophages (Gartner et al., 1986), and hematopoietic stem and progenitor cells (Alexaki and Wigdahl, 2008; Carter et al., 2010).

After entry to the cell, a double-stranded cDNA copy of the viral genome is reverse transcribed by the virally encoded enzyme reverse transcriptase. The cDNA genome is imported into the nucleus where it is integrated into the host genome by the viral protein integrase. Whereas most retroviruses wait for the nuclear membrane to be dissolved during mitosis, the nuclear import capability of HIV enables it to infect non-dividing cells and categorizes it specifically as a lentivirus (Narayan and Clements, 1989). The integrated viral genome can continue the replication cycle by making new copies of viral proteins and RNA genomes or it can enter a latent state, in which transcription of the viral genome is suppressed. The latent state can persist for years or even decades before being reactivated, at which point the genome can produce fully functional virus.

When the HIV genome is active, host transcriptional and translational machinery are induced to generate numerous viral proteins and genomes. Host RNA polymerase II transcribes full length RNA copies of the viral genome, most of which are spliced by host machinery to generate mRNAs specific for a single viral protein. In later stages of infection full length viral genomes are exported, which serve multiple critical functions. The full length viral RNA can be translated to produce Gag or Gag-Pol, two polyproteins that are cleaved to form most of the virus's structural proteins including the capsid, reverse transcriptase, and integrase. The full length genome also contains a packaging sequence that causes it to bind the capsid and be packaged into new virions.

In addition to structural and regulatory proteins that are essential to the replication cycle, HIV also encodes accessory proteins that do not actively participate in replication processes, but which alter the host cell environment to maximize virion production and persistence. Vif, Vpu, and Nef counteract numerous innate and adaptive defenses. They

enhance infection in cell lines, primary CD4+ T cells and primary macrophages (Balliet et al., 1994; Collins et al., 1998; Sheehy et al., 2002). By contrast the viral accessory protein Vpr primarily enhances infection of primary macrophages (Balliet et al., 1994; Mashiba et al., 2014). The functions of Vpr and Nef are discussed in greater detail below.

*Evidence for HIV infection of macrophages*

HIV has been detected in macrophages isolated from infected humans at many anatomical sites, including lymph nodes (Embretson et al., 1993) brain (Koenig et al., 1986), urethra (Ganor et al., 2019), and liver (Kandathil et al., 2018). Infection of macrophage cultures is less productive than infection of CD4+ T cells when measured by Gag p24 ELISA of the supernatant (Ochsenbauer et al., 2012), which has led some to speculate that macrophage infection is of limited importance in a natural infection. However, in infected persons viral production by macrophages maintains high viremia even after CD4+ T cell counts fall (Orenstein et al., 1997). Also, studies in transgenic mice that produce human macrophages but not human T cells replicate significant aspects of HIV infection in humans. The myeloid/macrophage-only-mouse can sustain infection for at least 10 weeks, at which point HIV was detected in numerous tissues throughout the body (Honeycutt et al., 2016). A follow up study using the same mouse model found that anti-retroviral treatment led to a rapid decrease in viremia. After treatment interruption, viral rebound was observed in 3 of 9 mice, indicating that macrophages can act as a long lived reservoir (Honeycutt et al., 2017). An important caveat of this study is that the transgenic mouse produces human macrophages derived from monocytes, but its tissue resident macrophages are murine only and therefore cannot be infected by HIV. Because

tissue resident macrophages are long-lived and self-renewing, an *in vivo* model that lacks these cells may not fully recapitulate the long term role of macrophages in HIV infection.

Whereas most HIV infected T cells undergo apoptosis within days of infection, HIV infected macrophages survive for weeks or months (Aquaro et al., 2002). This is likely because they are less susceptible to the cytopathic effects of the virus (Gendelman et al., 1988; Orenstein et al., 1988). The number of alveolar macrophages in humans is virtually unaffected by HIV infection (Koziel et al., 1998), but defects in alveolar macrophage function have been observed, namely reduced phagocytosis of *Candida albicans* (Crowe et al., 1994) and *Plasmodium falciparum* (Keen et al., 2007). HIV infected macrophages are thought to be the primary driver of HIV-associated cardiomyopathy and atherosclerosis (Crowe et al., 2010) and HIV-associated neurocognitive disorders (Gannon et al., 2011).

In addition to direct effects on macrophages, HIV infection of macrophages contributes to overall pathogenesis by increasing viral loads in infected individuals. Because macrophages are not killed even by long term infection, macrophages are the dominant source of viremia in late stages of AIDS (Orenstein et al., 1997). Replication competent virus has been recovered from monocytes and macrophages of people receiving antiretroviral therapy (Lambotte et al., 2000), suggesting that they may contribute to viral rebound after therapy is withdrawn. In *ex vivo* experiments using explants of human lymphoid tissue, infection of macrophages boosts virion production significantly, despite comprising a small fraction of infected cells (Eckstein et al., 2001). This suggests that macrophages amplify infection in other cell types.

There is significant evidence that HIV spreads via cell-to-cell contact and that this method is particularly important in macrophages. Direct cell-to-cell spread is mediated through a virological synapse, a connection that is formed when viral Env on an HIV infected cell binds CD4 on a neighboring uninfected target (Jolly et al., 2004). This connection allows multiple viral particles to be transmitted to a single target cell, greatly enhancing the likelihood that the virus establishes a successful infection (Del Portillo et al., 2011). Transmission via synapses is resistant to neutralization by antibodies (Schiffner et al., 2013), meaning it may pose an additional barrier to developing a useful HIV vaccine. Finally, this efficient, robust method of transmission has been demonstrated from infected macrophages to uninfected T cells (Groot et al., 2008), which may explain how the presence of macrophages boosts viral burden in lymphoid tissues containing T cells (Eckstein et al., 2001).

*The role of macrophages in HIV transmission*

Whether and to what degree macrophages contribute to HIV transmission is a matter of much debate. Early studies of tropism found that most HIV isolates from early stages of infection did not infect T cell lines and were thus thought to be “macrophage tropic” (Schuitemaker et al., 1991; van't Wout et al., 1994). More recent studies found that this observation, though correct, was misleading, because most T cell lines do not express CCR5, the co-receptor used by most viral isolates (Joseph et al., 2014). Viruses isolated within the first few weeks of transmission, called transmitted/founder or T/F viruses, can infect primary $CD4^+$ T cells and macrophages (Ochsenbauer et al., 2012). This study found that over 4-14 days T/F viruses replicated to higher titers in T cells than

in macrophages, suggesting that T/F viruses have a greater capacity for infection of T cells, which express high levels of CD4 (Joseph et al., 2014).

There is significant *in vivo* evidence that indicates macrophages may facilitate transmission. Macrophages are present in semen, urethra, foreskin, vaginal mucosa, rectal mucosa, and cervical mucosa (Iijima et al., 2008), making them a potential source or target of HIV in a sexual transmission event. In infected male humans HIV has been found in $CD4^+$ T cells and macrophages in semen (Quayle et al., 1997) and in the urethra (Ganor et al., 2019). In infected macaques, SHIV (an SIV – HIV hybrid) was found in macrophages in the testes and epididymis (Shehu-Xhilaga et al., 2007). In infection simulation experiments using explants of human cervical tissue, HIV has been found in macrophages and T cells (Cummins et al., 2007; Greenhead et al., 2000). In both of these studies, infected macrophages were more abundant than infected T cells, although the strain used (BaL) is notably macrophage tropic. One study (Greenhead et al., 2000) attempted the same experiment with two lab adapted, T cell tropic clones (IIIB and RF) and saw very little infection of any cell type. Similar studies using HIV clones isolated early in infection would be highly informative.

**The HIV structural protein Env**

The HIV gene *env* produces a large glycoprotein responsible for mediating binding and entry of the virion to target cells. Env is synthesized as a 160kDa polyprotein which is processed into two components, gp120 and gp41 via the host protease furin (Checkley et al., 2011). Entry to a target cell requires the presence of two host proteins, the primary receptor CD4 and one of two potential co-receptors CCR5 or CXCR4. The receptor CD4

is bound first, which induces a conformational change in gp120 that exposes the co-receptor binding site. Binding by CCR5 or CXCR4 induces another conformational change that exposes the fusion peptide, a hydrophobic region of gp41 that inserts into the membrane of the target cell and promotes fusion (Bosch et al., 1989).

*Env biosynthesis*

Env is a large and structurally complex protein which must be produced in the secretory pathway, where it undergoes numerous post-translational modifications (**Figure 1.4**). It is a type 1 transmembrane protein, meaning it has an N terminus facing the lumen/extracellular space, a single transmembrane domain, and a C terminus in the cytosol. A signal peptide in Env causes it to be translated directly into the lumen of the rough ER where, simultaneously with translation, it is glycosylated at numerous asparagine residues (Leonard et al., 1990). At each of these residues the enzyme complex oligosaccharyltransferase attaches a glycan tree (Aebi, 2013) that is identical for all asparagine-linked (N-linked) glycosylation (**Figure 1.4A**). The glycan tree, composed of 3 glucoses, 9 mannoses and 2 N-acetylglucosamines ($Glc_3Man_9GlcNAc_2$) is trimmed by removing the glucoses after translation is complete but before the protein transits to the Golgi. At this point all branches of the glycan tree terminate in mannose (**Figure 1.4B**). This form is called oligomannose, $Man_{5\text{-}9}GlcNAc_2$, or high-mannose (Doores et al., 2010).

As most glycoproteins transit through the Golgi, the terminal mannose residues are removed by mannosidases (Kornfeld and Kornfeld, 1985). These are replaced by many possible sugar conformations composed of fucose, galactose, sialic acid, and n-acetylglucosamine (Stanley et al., 2017). Collectively these are known as “complex

glycans" and they do not terminate in mannose residues (**Figure 1.4C**). Because terminal mannose is removed from healthy host proteins, it serves as a useful PAMP.

HIV Env does not complete this process like most eukaryotic proteins. Due to the unusually high number of glycosylation sites on Env and the fact that it forms a trimer in the cis-Golgi, some of the glycan trees cannot be accessed by mannosidases (Doores et al., 2010). These sites retain high mannose glycans rather than complex glycans. Studies of Env in which N-linked glycosylation sites are removed one at a time found that the loss of some sites leads to >25% loss of oligomannose, presumably by increasing accessibility of neighboring glycans (Coss et al., 2016). The final step of its synthesis, cleavage by host protease furin to produce gp120 and gp41, occurs in the trans-Golgi and is required for Env function (McCune et al., 1988).

Due to its distinctly non-eukaryotic glycan structures and position as the only viral protein outside the viral membrane, Env is the target of numerous innate and adaptive immune mechanisms. Env is the most common target of antibodies produced in infected humans (Kwong and Mascola, 2012) and has therefore been the target of most efforts to generate an antibody based vaccine (Haynes and Mascola, 2017). That Env can be targeted by innate mechanisms was first determined by studies that demonstrated that interferon-$\alpha$ (IFN$\alpha$) treatment reduces infectivity of virions by inhibiting Env assembly (Hansen et al., 1992). Since then, two interferon-inducible innate immune factors have been shown to restrict Env. Guanylate binding protein 5 (GBP5) is expressed in the Golgi following interferon treatment and prevents proper Env processing (Hotter et al., 2017). Interferon-inducible transmembrane protein 3 (IFITM3) binds Env and reduces infectivity of virions (Wang and Su, 2019; Wang et al., 2017).

**The HIV accessory protein Vpr**

The HIV gene *vpr* produces a 96 amino acid 14kDa protein (Wong-Staal et al., 1987) that has many known biochemical functions, but its role in the viral replication cycle has been a subject of much debate. Evolutionary analysis provides evidence that it is crucial for infection *in vivo*. Vpr is conserved in all primate lentiviruses (Tristem et al., 1998) and individuals infected with Vpr mutants are extremely rare. The first known case was an occupational transmission of HIV HxB2, which contains a truncation in Vpr at amino acid 78. This mutation reverted to wildtype in vivo (Beaumont et al., 2001). The second and third were a mother-child pair. The mother was infected via blood transfusion and the child via breastfeeding. Both displayed no loss of $CD4^+$ T cells or any other symptoms for at least 13 years (Wang et al., 1996). The final known infection by a Vpr mutant was via a needlestick containing ΔVpr NL4-3. After 10 years of close observation the infected person's viral load has usually been clinically undetectable (<20 copies per mL) and $CD4^+$ T cell count has been unaffected (Ali et al., 2018). Combined these results indicate that Vpr is a critical factor in HIV pathogenesis and transmission.

Because Vpr is packaged in the HIV virion (Cohen et al., 1990) it has long been thought that it plays a role in early infection events. Packaging of Vpr is dependent on the p6 component of the Gag polyprotein, and Vpr is packaged at a nearly 1:1 molar ratio with Gag (Paxton et al., 1993). A later study found that a conserved L-X-S-L-F-G motif in p6 Gag is necessary and sufficient to package Vpr and that the interaction between these proteins is direct (Bachand et al., 1999). Vpr localizes to the nucleus/nuclear envelope in PBMCs (Lu et al., 1993) and macrophages (Jacquot et al., 2007), suggesting it may play a role in integration, although later studies have not observed this (Mashiba et al., 2014;

Wang and Su, 2019). Early studies of Vpr's biochemistry identified a host protein, originally named Vpr binding protein or VprBP (Zhao et al., 1994), that immunoprecipitates with Vpr, but the purpose of this interaction was unknown. This protein, which was later renamed DCAF1, is required for Vpr to mediate many of its functions (Belzile et al., 2007).

*Vpr-mediated cell cycle arrest*

The most easily observable and most studied function of Vpr is that it arrests the cell cycle at the transition from G2 to M phase (Jowett et al., 1995). This arrest at G2 phase increases activity of the HIV LTR and virion production (Goh et al., 1998), but it also induces apoptosis (Stewart et al., 1997). These opposing effects may be why Vpr does not have a clear positive or negative impact on infection of cycling CD4+ T cells *in vitro* (Balliet et al., 1994).

Various models of Vpr cell cycle arrest have been proposed (**Figure 1.5**). There is broad agreement that the proximal cause of Vpr mediated arrest is hyperphosphorylation of the host protein cdc2, a regulator of the DNA damage checkpoint, which prevents cdc2 from becoming activated (He et al., 1995). Activity of cdc2 is controlled by at least two inputs, the phosphatase cdc25 and the kinase Wee1, and Vpr has been demonstrated to affect both. One group demonstrated that Wee1 is necessary for Vpr-mediated cell cycle arrest by silencing Wee1 in HeLa cells (Yuan et al., 2003), which prevented Vpr's action. Another group later demonstrated that Vpr enhances Wee1 activity by directly binding to the kinase domain of Wee1 (Kamata et al., 2008), although they found several Vpr

mutants that bind and activate Wee1 but do not induce cell cycle arrest, indicating that binding Wee1 is not sufficient for cell cycle arrest.

It is very likely that Vpr also arrests the cell cycle via the other regulator of cdc2 activity, cdc25. During the normal cell cycle, tyrosine residues on cdc2 are de-phosphorylated by the phosphatase cdc25. Vpr has been shown to inhibit cdc25 which leads to hyperphosphorylation of cdc2 (Bartz et al., 1996). cdc25 is under the control of at least three upstream kinases, chk1, which is in turn controlled by ATM, chk2, which is controlled by ATR, and Srk1, which is activated by various stress responses (López-Avilés et al., 2005). Of these, only ATM is definitively not involved in Vpr-mediated arrest (Bartz et al., 1996). One study found that Srk1 is directly bound by Vpr, which increases Srk1-mediated phosphorylation of cdc25 and leads to a reduction in cdc25 activity (Huard et al., 2008).

There is evidence from several studies indicating that Vpr can also act via ATR (ATM and Rad3 related protein), a host protein that arrests the cell cycle in response to DNA damage (Paulsen and Cimprich, 2007). Inhibition of ATR by RNAi or overexpression of a dominant-negative mutant prevents Vpr-mediated G2 arrest (Roshal et al., 2003). The mechanism by which Vpr activates ATR has not been fully elucidated, but several studies have demonstrated potential pathways. One found that in infected T cells, Vpr induces formation of replication protein A foci, which are known to activate ATR (Zimmerman et al., 2006). Another demonstrated that Vpr's interaction with UNG2 promotes excision of uracil from viral cDNA (Norman et al., 2011). Abasic sites left behind when UNG2 excises uracil have been demonstrated to activate ATR in cancerous cells (Buisson et al., 2017). It is possible that these are steps of the same pathway, i.e. that

UNG2's activity leads to the formation of replication protein A foci or vice versa, but this has not yet been tested.

Although there is disagreement on how Vpr initiates the signal that leads to cell cycle arrest, it is universally agreed that it relies on DCAF1 to do so. The determinants within DCAF1 and Vpr necessary for Vpr to induce G2 arrest are well established (Gérard et al., 2014; Hrecka et al., 2007; Le Rouzic et al., 2007; Wen et al., 2007). Interestingly, one group identified mutations near the C terminus of Vpr that abrogate cell cycle arrest but do not affect DCAF1 binding, indicating that binding to DCAF1 is not sufficient to arrest the cell cycle (Belzile et al., 2007).

Following a 2014 paper by Laguette at al., there has been considerable attention given to a model in which Vpr and DCAF1 act via components of the SLX complex (SLXcom), a multi-protein complex that resolves Holliday junctions following DNA repair by homologous recombination. SLX4 functions as a scaffold protein that recruits and assembles other subunits including the endonucleases MUS81 and EME1. SLX4 expression is required for Vpr to induce G2 arrest in HeLa cells and MEFs (Laguette et al., 2014). This study also demonstrated that Vpr directly interacts with SLX4 and DCAF1, which led to decreased steady state levels of MUS81 and EME1 and activation of SLXcom. They also demonstrated that this activation causes HIV reverse transcripts to co-immunoprecipitate with SLX4 and prevents induction of an interferon response. The authors proposed that evading IFN may be the true purpose of SLXcom activation by Vpr and that G2 arrest may be a side effect. The details of this mechanism have been debated. A study in our laboratory confirmed that Vpr decreased steady state levels of MUS81 protein and *IFNA1* mRNA in primary MDM and $CD4^+$ T cells (Mashiba et al.,

2014). A study by another group found that activation of SLX4 is not broadly conserved across isolates of HIV-1 and HIV-2 and that SLX4 is not required to mediate G2 arrest in U2OS and 293T cells (Fregoso and Emerman, 2016). A third group found that neither SLX4 nor DCAF1 binding was required for Vpr-mediated downregulation of MUS81 and EME1 and that downregulation of MUS81 and EME1 was not sufficient to arrest the cell cycle at G2 (Zhou et al., 2016). The differences between these findings and those of Laguette et al. may be due to differences in viral strains and cell lines used. Neither Fregoso and Emerman nor Zhou et al. investigated Vpr-mediated reduction of IFN, which may require SLX4. Follow up studies focused on the mechanism of IFN evasion in primary cells would be highly informative.

*Vpr role in viral replication*

The first investigations of Vpr's activity in infected cell cultures found that it did not have significant effects in CD4+ T cells (Balliet et al., 1994; Eckstein et al., 2001). Recently there is evidence that Vpr enhances T cell infection under certain conditions. Vpr causes ubiquitination and subsequent proteasomal degradation of helicase like transcription factor (HLTF), a multi-domain, multi-functional protein that activates post replication DNA repair (Hrecka et al., 2016; Lahouassa et al., 2016). Vpr-mediated degradation of HLTF enhances HIV replication in CD4+ T cells, but this effect is only apparent in competition assays (Yan et al., 2019). Also silencing HLTF did not fully restore replication by the Vpr mutant, indicating that Vpr has additional functions.

Vpr has a much stronger effect in macrophages, which has been documented by numerous laboratories over decades (Balliet et al., 1994; Connor et al., 1995; Eckstein et

al., 2001; Hattori et al., 1990; Mashiba et al., 2014; Westervelt et al., 1992). Vpr enhances infection of human lymphoid tissue, which contains macrophages and T cells in a three dimensional environment, but only when an M-tropic strain is used (Eckstein et al., 2001). An early model proposed that Vpr enhanced nuclear import of the viral cDNA genomes, but the evidence has been mixed. Assays using H9 cells demonstrated that Vpr enhanced the appearance of 2-LTR circles, a form of double stranded viral cDNA that only forms in the nucleus (Popov et al., 1998). Production of 2-LTR circles by Vpr-null HIV was rescued by addition of cytosol from HeLa cells, indicating that the nuclear import function of Vpr can be performed by unidentified host factors in some cell types. A study in our laboratory (Mashiba et al., 2014) indicated that Vpr-null HIV 89.6 did not display a defect in the first round of infection of monocyte derived macrophages (MDM), indicating that Vpr was not required for nuclear import under these conditions. That Vpr is not necessary for nuclear import is also supported by an earlier finding that providing Vpr in trans, i.e. packaged in the virion but not encoded in the genome, does not fully rescue ΔVpr virus; therefore Vpr's main functions occur after integration (Connor et al., 1995).

*Vpr-mediated degradation of host proteins*

In addition to the host proteins implicated in Vpr-mediated cell cycle arrest described above, Vpr alters the expression of numerous other host proteins (Greenwood et al., 2019). Several of these are direct targets, which Vpr ubiquitylates using its cellular cofactor DCAF1. DCAF1's normal function in the cell is to direct the activity of the DCAF1-DDB-Cullin4 E3 ligase complex, which ubiquitylates host proteins, usually to cause their degradation, but occasionally to regulate their activity (Nakagawa et al., 2015). Vpr

simultaneously binds DCAF1 and its protein targets, which changes the ligase complex's substrate specificity.

It is well established that Vpr degrades UNG2 and SMUG1, two uracil deglycosylases (Schrofelbauer et al., 2007; Schrofelbauer et al., 2005), although the purpose of this is unclear. Degradation is mediated by Vpr binding directly to both UNG2 and DCAF1 (Ahn et al., 2010; Wu et al., 2016). Different studies of UNG2 have determined that its effect on viral replication can be negative, positive, or neutral. One study found that in addition to depleting UNG2 in infected cells, Vpr recruits UNG2 to virions, which reduces the mutation rate of reverse transcription (Chen et al., 2004). This study also demonstrated that a mutation in Vpr (W54R) that prevents binding to UNG2 increased mutation of HIV genomes in MDM. A different study found that UNG2 caused the degradation of uracilated viral cDNA, indicating that UNG2 is a viral restriction factor, although in their cell line model Vpr did not counteract the restriction (Weil et al., 2013). A third study found that UNG2 had no effect, positive or negative, on viral infection of several cell lines and MDM (Kaiser and Emerman, 2006).

More recently, Vpr has been implicated in the regulation of TET2, a member of the TET family that regulates 5' methylation of cytosine. In the absence of HIV and Vpr, DCAF1 monoubiquitylates TET2, which increases its affinity for chromatin (Nakagawa et al., 2015) and therefore its activity. A follow up study found that Vpr induces polyubiquitylation and subsequent degradation of TET2. Because TET2 inhibits transcription of the *IL6* gene, Vpr's anti-TET2 activity increased IL-6 expression (Lv et al., 2018). A later paper by the same group found that Vpr mediated degradation of TET2

prevented induction of interferon inducible trans-membrane protein 3 (IFITM3) which interferes with Env processing (Wang and Su, 2019).

*Vpr and the interferon response*

Through mechanisms that are incompletely understood, HIV induces a relatively weak interferon response, and Vpr contributes to this evasion. Vpr reduces transcription of IFNα (Mashiba et al., 2014), IFNβ and *Mxa,* an antiviral interferon stimulated gene, by activating SLXcom which leads to degradation of incompletely transcribed viral cDNA genomes (Laguette et al., 2014). It has also been demonstrated that Vpr acts via a different pathway to specifically prevent induction of IFNβ (Doehle et al., 2009). In 293T cells, this is achieved by Vpr-mediated degradation of interferon regulatory factor 3 (IRF3), in which the HIV accessory protein Vif also plays a role (Okumura et al., 2008). Vpr has also been demonstrated to act further upstream by dysregulating TANK-binding kinase, which prevents activation of IRF3 (Harman et al., 2015). Given that Vpr alters expression of numerous host proteins (Greenwood et al., 2019), it is possible that Vpr affects interferon signaling by multiple mechanisms.

The understanding of Vpr-mediated evasion of innate immunity was advanced significantly by an earlier study in our laboratory (Mashiba et al., 2014) that demonstrated that Vpr counteracts a restriction factor in macrophages. Crucially this study found that Vpr does not boost first round infection of MDM, indicating that the enhancement of nuclear import/integration is not important in these cells. Recently a study by another group confirmed that at very early time points (2 days post infection) Vpr does not affect infection of MDM (Wang and Su, 2019). Mashiba et al. found that Vpr enhances infection

frequency starting on day 4 and continuing for up to 20 days. This study confirmed prior observations that Vpr enhances virion production in MDM as measured by Gag p24 concentrations in the culture supernatant (Balliet et al., 1994; Vodicka et al., 1998). Crucially this effect is eliminated if the virus lacks Env (Mashiba et al., 2014), suggesting that Env is the proximal target of a restriction pathway. Western blot from infected MDM revealed that in the absence of Vpr, all three forms of Env (gp160, gp120, and gp41) are degraded, indicating the factor can act early in the secretory pathway. Finally this study found that degradation of Env could be inhibited by treatment with ammonium chloride, indicating that degradation occurred in the lysosome. Although this study provided important details about the action of the proposed restriction factor, the identity of the factor remained unknown.

**The HIV accessory protein Nef**

Like Vpr, Nef is not strictly required to complete the viral replication cycle but it significantly enhances replication *in vivo* and in many cell culture models. Nef is myristoylated (Allan et al., 1985), which causes it to concentrate on the cytosol facing side of the plasma and organellular membranes. This localization allows Nef to bind the cytoplasmic tails of host defense factors and traffic them to lysosomes (Collins and Collins, 2014). Nef uses this mechanism to downmodulate the expression of MHC class I on the surface of infected cells (Schwartz et al., 1996), which prevents recognition and killing by HIV-specific CD8+ T cells (Collins et al., 1998). The molecular mechanism of this process has been well established. Nef and the host trafficking complex AP-1 simultaneously bind MHC-I at the Y320 site of its cytoplasmic tail, which has been

demonstrated genetically (Wonderlich et al., 2008) and is evident in a crystal structure of the three-way interaction (Jia et al., 2012). Normally MHC-I moves from the trans Golgi to the plasma membrane, but Nef-mediated recruitment of AP-1 redirects it to Rab7+ endosomes (Roeth et al., 2004) and eventually lysosomes, where it is degraded (Schaefer et al., 2008).

Another major target of Nef is the HIV receptor CD4, which is removed from the cell surface to prevent superinfection and facilitate viral egress. Nef causes CD4 to be endocytosed from the cell surface and this process is dependent on a dileucine motif in CD4's cytoplasmic tail (Aiken et al., 1994). Nef forms an interaction between the cytoplasmic tail of CD4 and the AP-2 complex, which induces clathrin-mediated endocytosis (Chaudhuri et al., 2007). In addition to downmodulating the main HIV receptor, Nef also downmodulates the co-receptors CCR5 (Michel et al., 2005) and CXCR4 (Venzke et al., 2006), although the mechanisms of action have not been defined.

Finally, Nef also causes mannose receptor (MR) to be removed from the cell surface in macrophages and transfected 293T cells (Vigerust et al., 2005). The cytoplasmic tail of MR, which includes a SDXXL$\phi$ motif similar to the SQXXLL motif in the tail of CD4, was sufficient to induce endocytosis of a chimeric surface protein (Vigerust et al., 2005). Interestingly CD4 was degraded following its endocytosis but MR was not, suggesting MR does not enter the same endocytic pathway or it is resistant to lysosomal degradation, presumably because it repeatedly traffics cargo to lysosomes as part of its normal host functions (Stahl et al., 1980). The implications of Nef-mediated MR removal from the cell surface for HIV replication and pathogenesis has not yet been established.

## Summary of dissertation

Previous work in our laboratory provided significant evidence that HIV Vpr counteracts a macrophage-specific restriction factor that targets viral Env. Our goal has been to characterize the effects of Vpr on viral spread from macrophages and to identify this restriction factor. Chapter 2 presents the results of a study that investigated viral spread from HIV infected macrophages to uninfected, autologous CD4+ T cells. We found that this form of spread to T cells is far more efficient than direct infection by cell-free virus and that this process was boosted significantly by Vpr. Chapter 3 presents the results of a study that identified the restriction factor as mannose receptor (MR). We present evidence that HIV Vpr reduces transcription of the gene that produces MR and confirm an earlier finding that Nef dysregulates MR trafficking. The combined effect of Vpr and Nef reduces MR expression dramatically, which rescues Env expression, virion release, and spread from macrophages to T cells. Chapter 4 provides a discussion of the context and implications of these findings, including proposals for future experiments that would illuminate the mechanisms by which Vpr and Nef antagonize MR and how this activity enhances HIV pathogenesis.

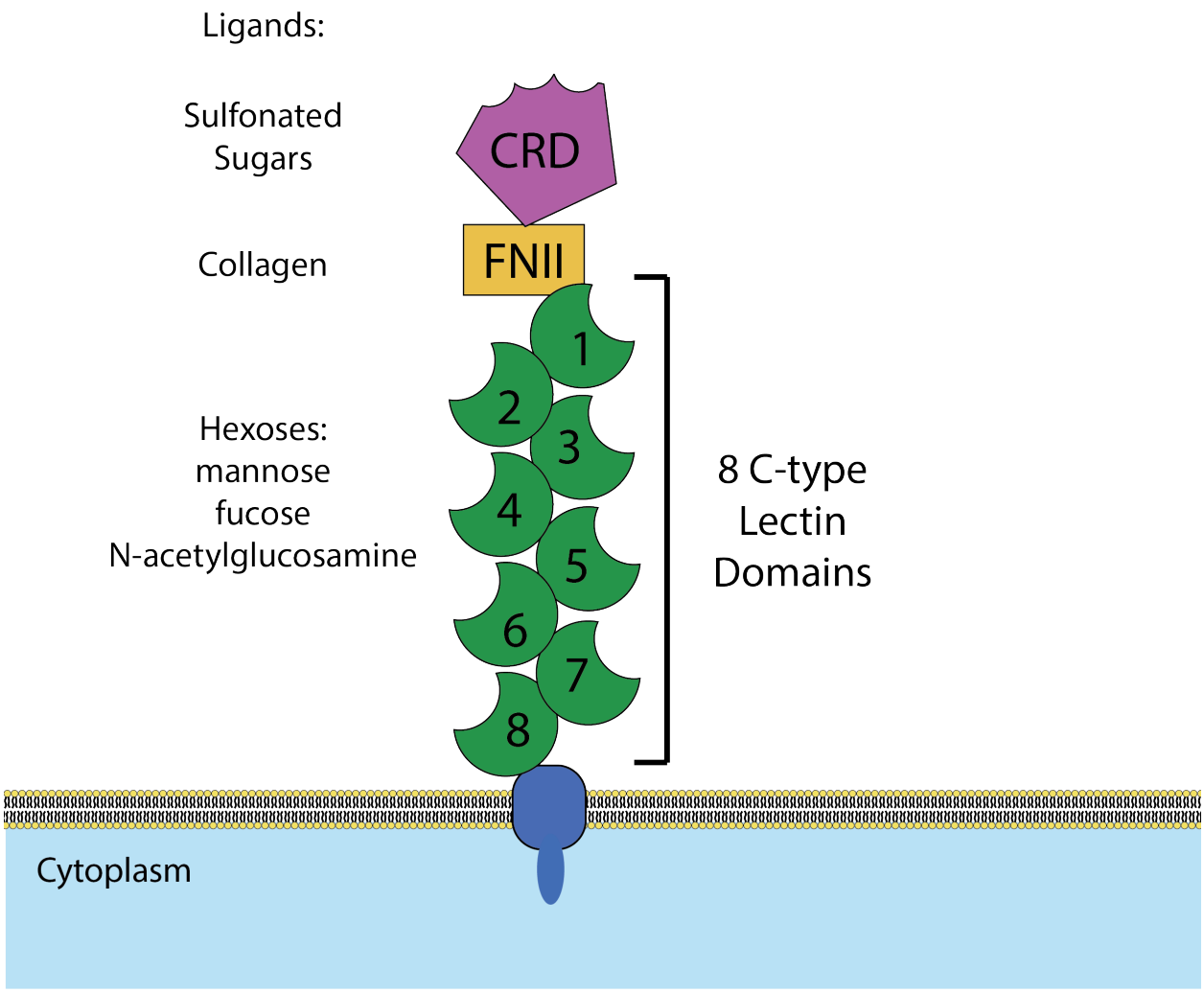


**Figure 1.1 Structure of mannose receptor (MR)**[1] Graphical depiction of the domains of MR and the ligands bound by each domain. CRD – cysteine rich domain, which binds sulfonated sugars. FNII – Fibronectin type II domain, which binds collagen. C-type lectin domains – Calcium-dependent domains that bind mannose, fucose, and N-acetylglucosamine.

[1] This figure was created by Jay Lubow

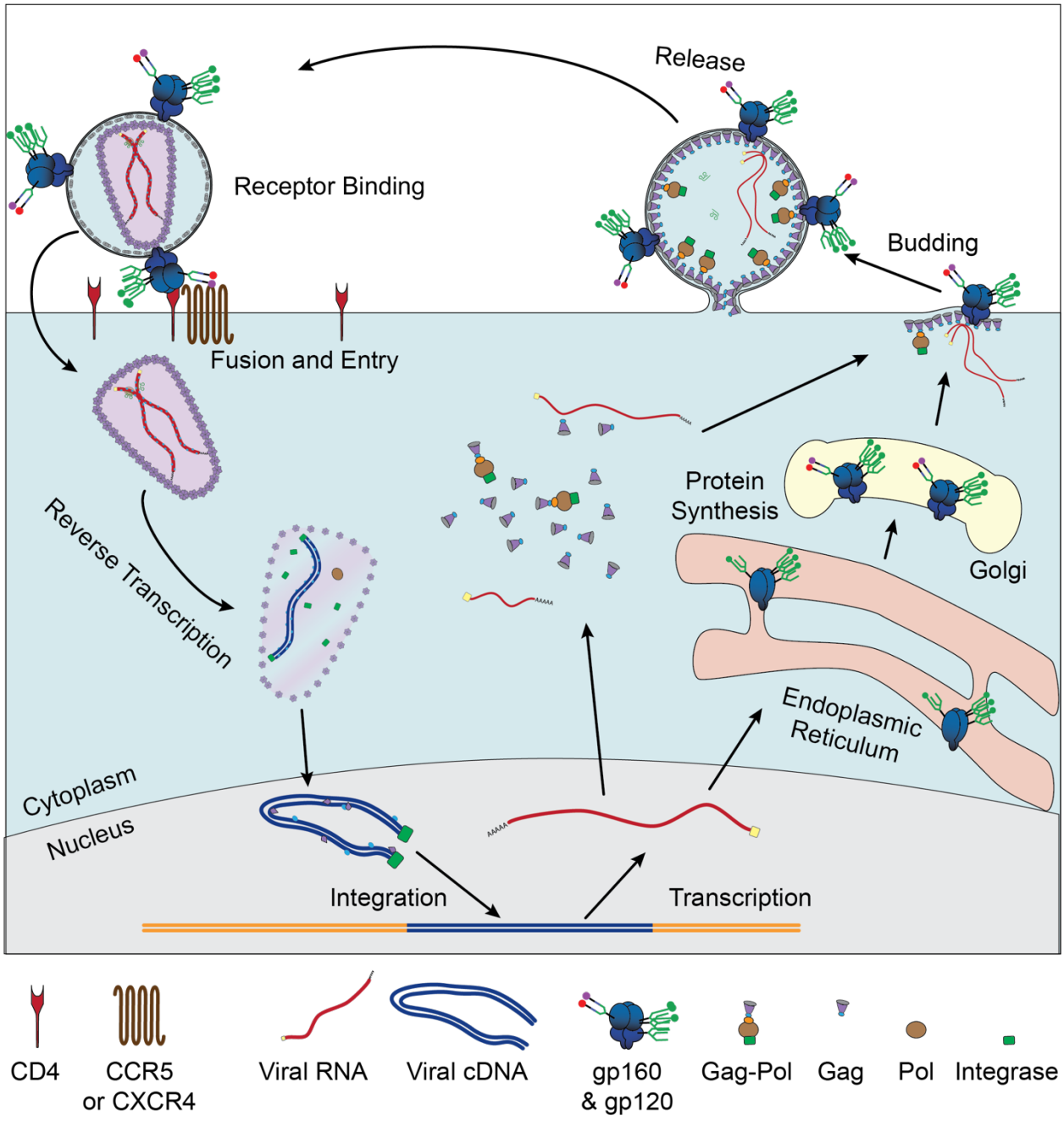


**Figure 1.2 Replication cycle of HIV**[2] Graphical depiction of the major events of the HIV replication cycle. Viral entry is mediated by binding of Env to CD4 and a co-receptor (CXCR4 or CCR5). After the capsid has entered the cytoplasm, the viral RNA genome is reverse transcribed by Pol into a double stranded cDNA genome. This viral genome is transported into the nucleus where it integrates into the host genome. The host RNA polymerase transcribes RNA copies of the genome, which are exported in an unspliced form to produce Gag and Gag-Pol or various spliced forms to produce the other viral

[2] This figure was adapted by Jay Lubow from earlier versions by Mark Painter and Thomas Zaikos.

proteins. Unspliced RNA genomes are also packaged into newly formed virions. Most viral protein translation occurs in the cytoplasm, but Env gp160 is translated into the lumen of the rough ER and transported through the secretory pathway where it is glycosylated and cleaved by furin into gp120 and gp41.

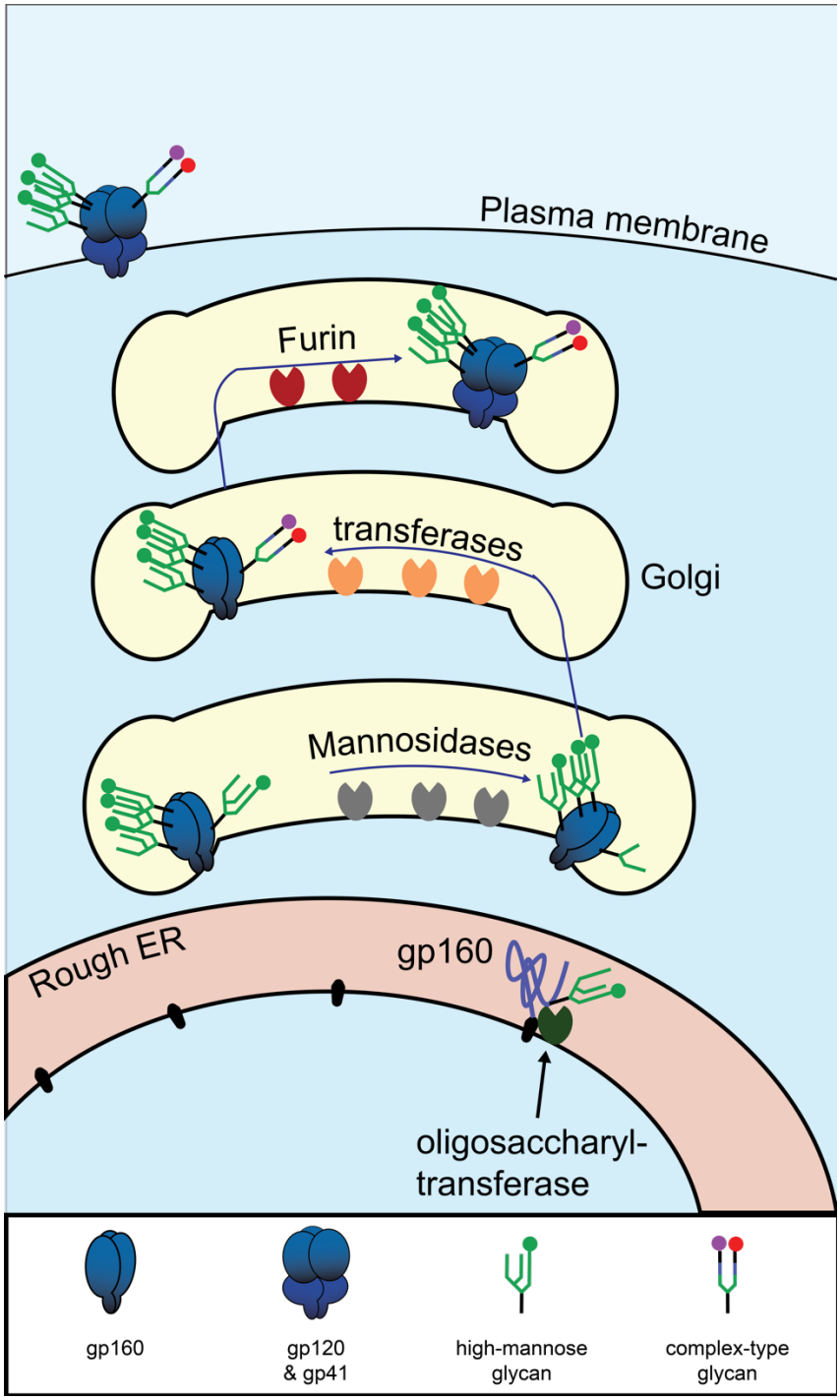


**Figure 1.3 Biosynthesis HIV Env in the secretory pathway**[3]**.** Graphical depiction of translation and glycosylation of Env in the endoplasmic reticulum followed by post-translational modifications in the Golgi. Mannose oligomers are depicted in green. The mannose patch is depicted as three densely packed glycans. For a detailed depiction of the monomers composing the glycans see Figure 1.4.

[3] This figure was generated by Jay Lubow.

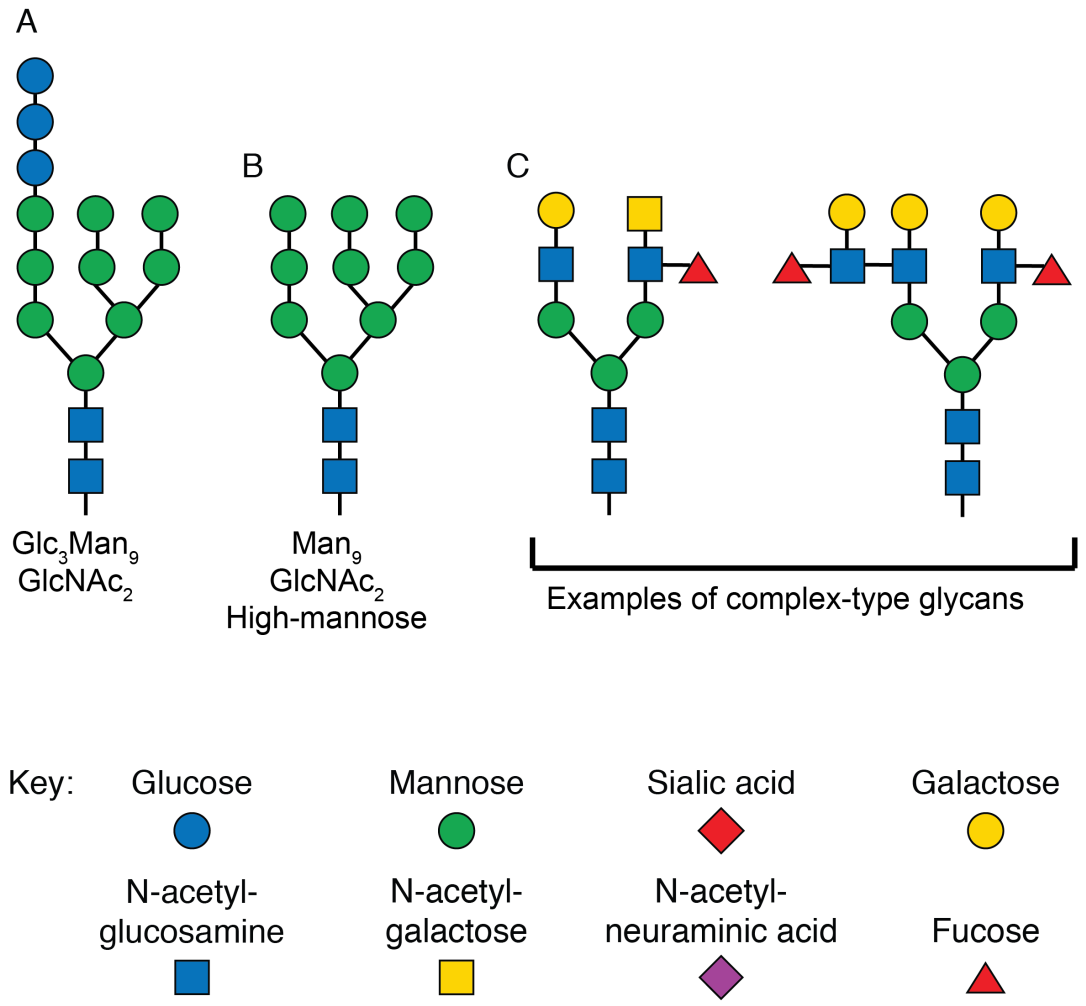


**Figure 1.4 Structures of glycans at various stages of N-linked glycosylation**[4]
**(A)** Graphical depiction of the glycan structure transferred to all N-linked glycosylation sites by the enzyme complex oligosaccharyltransferase in the rough ER. The three terminal glucose monomers are removed before exiting the ER. **(B)** Graphical representation of the high-mannose glycan at N-linked glycosylation sites as newly synthesized proteins enter the Golgi. For certain sites on HIV Env this is the final form of the glycan. **(C)** Graphical representation of two complex type glycans that are present on mature eukaryotic proteins. These are just two of the dozens of forms the final mature glycan structure can take. The sugar monomers are drawn according to the updated recommendations for symbol nomenclature for glycans [SNFG (Neelamegham et al., 2019)]

[4] This figure was generated by Jay Lubow

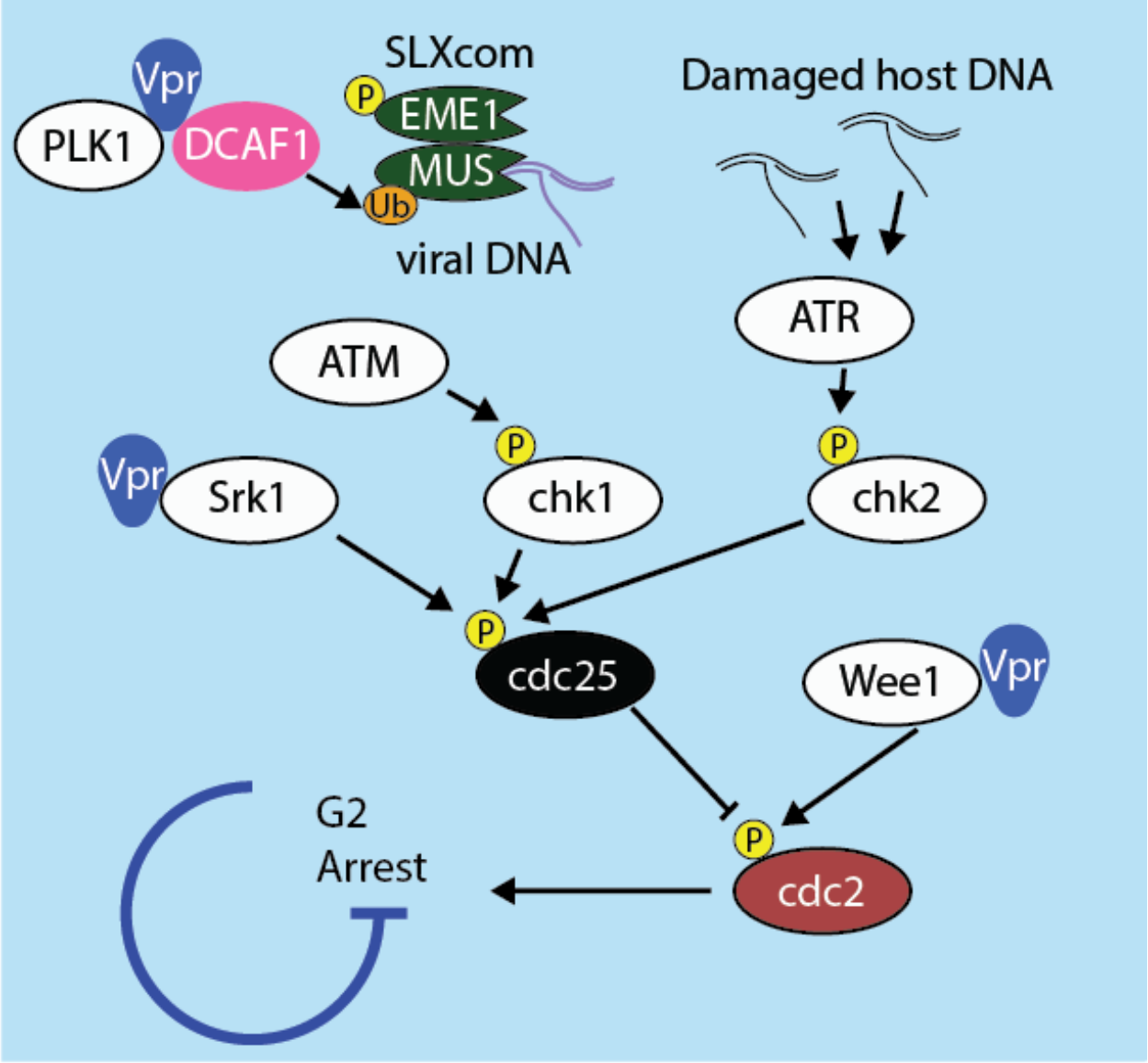


**Figure 1.5 Model of G2/M cell cycle arrest and mechanisms by which it is induced by Vpr[5].** Graphical depiction of the cdc2-mediated cell cycle arrest pathway, including points at which Vpr has been demonstrated to alter signaling in order to promote arrest. Kinases are depicted in white. The only phosphatase, cdc25, is depicted in black. EME1 and MUS81, depicted in green, are endonucleases that resolve Holliday junctions. Vpr activates MUS81 prematurely, which causes the SLX complex to cleave viral DNA and prevents induction of interferon. Vpr also degrades MUS81 which leads to accumulation of unresolved replication intermediates and ultimately causes cell cycle arrest via ATR (Laguette et al., 2014).

[5] This figure was generated by Jay Lubow

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## Chapter 2

## Vpr Promotes Macrophage-Dependent HIV Infection of CD4+ T Lymphocytes[1]


### Summary

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


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## Introduction

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

In this study, we describe cell culture conditions in which HIV-1 infection of primary T lymphocytes depended entirely on contact-dependent spread from macrophages; a mode of spread that evaded neutralization by some antibodies. Under these conditions, Vpr enhanced the formation of virological synapses (VS) between infected macrophages and primary T lymphocytes. Mechanistic studies revealed that Vpr functioned to prevent

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

## Results

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

To evaluate a role for Vpr in T lymphocyte infection that explained in vivo observations, we developed an assay to measure HIV-1 spread from primary macrophages to autologous CD4$^+$ T lymphocytes. As outlined in **Figure 2.1A**, we inoculated primary monocyte-derived macrophages (MDM) with HIV-1 and allowed infection to establish for two days before co-cultivation with activated autologous CD4$^+$ T lymphocytes for an additional two days to enable viral spread. MDM-T lymphocyte co-cultures produced an average of nine-fold more HIV-1 than infected MDM alone, suggesting that co-cultivation resulted in efficient spread between macrophages and T lymphocytes (**Figure 2.1B**).

To measure the frequency of infection in each cell type, we used flow cytometry to distinguish macrophages from T lymphocytes by expression of surface markers and measured infection by intracellular Gag staining (**Figure 2.2A**). Detection of Gag$^+$ cells

was dependent on reverse transcriptase activity demonstrating that our assay measures productive HIV-1 replication (**Figure 2.2B**). Although HIV-1 infects and depletes $CD4^+$ T lymphocytes to cause acquired immunodeficiency syndrome in vivo, infection of primary $CD4^+$ T lymphocytes by cell-free virus was inefficient in vitro after two days of continuous culture (**Figures 3.1A**, **3.2A**) using an inoculum comparable to the amount of virus present in MDM-T lymphocyte co-cultures (data not shown). In comparison, co-cultivation of activated T lymphocytes with infected MDM increased T lymphocyte infection by thirty-fold (**Figure 2.1C**).

The capacity for MDM to efficiently infect autologous primary $CD4^+$ T lymphocytes depended on direct cell-to-cell contact because infection was not detected when the cells were separated by a virus-permeable transwell insert (**Figures 3.1D,E**). Direct cell-to-cell transmission of HIV-1 across virological synapses between infected and target cells has been previously described and is known to be highly resistant to antibody neutralization (Durham et al., 2012; Schiffner et al., 2013). Consistent with this mode of spread, we observed that MDM-dependent spread to autologous primary $CD4^+$ T lymphocytes was highly resistant to neutralizing antibodies against Env gp120 (b12), Env gp41 (Z13E1) and CD4 (SIM2) that were capable of neutralizing greater than 95% of free virus infection of MDM (**Figure 2.1F**). When added at the time of co-cultivation, only 2G12, which has been previously demonstrated to block cell-to-cell spread (Duncan et al., 2014), was able to efficiently neutralize MDM-dependent T lymphocyte infection (**Figure 2.1F**).

Previous studies have demonstrated that uninfected dendritic cells and MDM can infect T lymphocytes through a "*trans*" mechanism in which virions bound to lectin receptors are transferred to T lymphocytes (**Figure 2.1G**) (McDonald, 2010; Peressin et

al., 2014). This contrasts with *"cis"* infection that requires HIV-1 replication in MDM. To determine the mode of infection that was active in our system, we used the protocol described in **Figure 2.1A** but substituted an HIV-1 molecular clone that can infect T lymphocytes but not macrophages (NL4-3). Similar to HIV-1 89.6, NL4-3 did not infect primary T lymphocytes when cultured with free virus. Consistent with previous reports (O'Doherty et al., 2000), however, this virus infected a high percentage of T lymphocytes upon spinoculation (**Figure 2.1H**). As expected, NL4-3 did not infect MDM (**Figure 2.1H**) and MDM treated with NL4-3 as outlined in **Figure 2.1A** did not spread infection to primary $CD4^+$ T lymphocytes (**Figure 2.1H**). Thus, spread of infection from MDM to primary $CD4^+$ T lymphocytes required productive HIV-1 replication in MDM under the conditions of our assay. In summary, efficient infection of primary $CD4^+$ T lymphocytes required contact-dependent, neutralizing antibody-resistant, *cis*-mediated virus transfer from HIV-1 infected macrophages.

*Vpr enables macrophage-dependent T lymphocyte infection.*

The HIV-1 Vpr protein is necessary for optimal infection and spread in MDM cultures but can actually be detrimental to spread of infection in actively replicating cells due to its inhibitory effects on cell cycle progression (Goh et al., 1998; Rogel et al., 1995; Stivahtis et al., 1997). Because $CD4^+$ T lymphocytes are the main target of HIV-1 in vivo, Vpr's role in HIV-1 infection and its evolutionary conservation across lentiviral species targeting a wide range of primates has remained enigmatic (Mashiba and Collins, 2013). We hypothesized that the mode of spread we describe here in which T lymphocyte infection is entirely dependent on infected MDM might reveal a crucial role for Vpr in

enabling efficient T lymphocyte infection. To address this, we co-cultivated activated primary $CD4^+$ T lymphocytes with autologous MDM infected by HIV-1 89.6 containing or lacking Vpr (**Figure 2.3A**). Indeed, we observed a striking enhancement of infection by Vpr in our co-culture assay as measured by virion production (nine-fold, **Figure 2.3B**) and frequency of T lymphocyte infection (three-fold, **Figure 2.3C**).

Because Vpr stimulates HIV-1 spread among macrophages (**Figure 2.3C**) (Connor et al., 1995; Mashiba et al., 2014), it was possible that the stimulation of T lymphocyte infection we observed may result from an increase in the number of infected MDM that could amplify virus production. To address this, we measured spread of HIV-1 from infected MDM to T lymphocytes under conditions in which HIV-1 could only infect MDM for a single round and subsequent spreading infection could only occur in T lymphocytes. This was accomplished by using T-lymphotropic HIV-1 NL4-3 pseudotyped with macrophage-tropic YU2 Env (**Figure 2.3D**). This virus utilizes YU-2 Env protein to efficiently infect MDM for one round of viral replication. However, *de novo* virions produced by the infected macrophages express only NL4-3 Env and thus can only infect T lymphocytes. As previously reported (Mashiba et al., 2014), this virus initially infected macrophages equally in the presence or absence of Vpr expression (**Figure 2.3E**). Remarkably, however, Vpr significantly enhanced spread of HIV-1 from infected MDM to T lymphocytes (four-fold, **Figure 2.3E**). In contrast, Vpr did not stimulate direct infection of primary T lymphocytes via spinoculation (**Figure 2.3E**), consistent with previous studies (Balliet et al., 1994). These data indicate that Vpr promotes the directional spread of HIV-1 from macrophages to T lymphocytes and that this activity of Vpr is conserved in diverse HIV-1 isolates.

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

Vpr interacts with the cellular protein DDB1-and-CUL4-associated factor 1 (DCAF1, also known as VprBP) to modulate ubiquitylation and proteasomal degradation pathways (Ahn et al., 2010; Belzile et al., 2010; Casey Klockow et al., 2013; Collins and Collins, 2014). Recent work has demonstrated that DCAF1 is an essential co-factor for Vpr to evade the induction of a type I interferon response, and thereby counteract macrophage restriction of Env and virion production (Laguette et al., 2014; Mashiba et al., 2014). To determine whether this pathway was required for spread of HIV-1 from infected MDM to primary T lymphocytes, we employed the Vpr Q65R mutant of 89.6 that is deficient at interacting with DCAF1 and relatively defective at inducing DCAF1-dependent cell cycle arrest (DeHart et al., 2007; Mashiba et al., 2014). We found that Vpr Q65R was proportionally defective at enhancing HIV-1 spread from MDM to $CD4^+$ T lymphocytes (**Figure 2.4A**). To more directly address the requirement of DCAF1 for Vpr-dependent spread, we silenced DCAF1 in infected MDM and co-cultured these cells with autologous T lymphocytes (**Figure 2.4B**). Remarkably, we found that DCAF1 silencing abrogated the ability of Vpr to stimulate transmission of HIV-1 from MDM to $CD4^+$ T lymphocytes (**Figure 2.4C**). These data demonstrate that Vpr requires DCAF1 to promote MDM-to-T lymphocyte spread of HIV-1.

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

MDM infected by HIV-1 lacking Vpr mount an innate immune response that restricts Env expression by accelerating lysosomal degradation of Env, and Vpr prevents

the induction of this pathway via a DCAF1-dependent mechanism (Mashiba et al., 2014). Because DCAF1 was also required for Vpr-dependent MDM-T lymphocyte spread of HIV-1 (**Figures 3.4A-C**), we assessed whether this pathway was active in MDM-T lymphocyte cocultures. We analyzed co-culture whole-cell lysates for steady-state Env expression by quantitative immunoblot in the presence or absence of Vpr (**Figure 2.4D**). Indeed, we observed a loss of Env gp160, gp120 and gp41 relative to the HIV Gag precursor pr55 in the absence of Vpr in cocultures (**Figure 2.4E**), similar to what was previously reported in HIV-1 infected MDM (Mashiba et al., 2014).

Because virions incorporate HIV-1 Env and because Env is required for Vpr-dependent changes in virion production (Mashiba et al., 2014), we hypothesized that in the absence of Vpr, Env-containing virions are targeted for lysosomal degradation in macrophages. To test this, we examined the localization of mature virions (Gag MAp17$^{+}$) with LAMP1, a marker of lysosomes. Because HIV-infected cells form syncytia, infected MDM are frequently multinucleated, which we also observed (**Figure 2.5A**). Remarkably, in the absence of Vpr, mature virions (magenta puncta in **Figure 2.5A**, lower two panels) frequently co-localized with LAMP1. In comparison, expression of Vpr reduced co-localization of mature virions with lysosomal markers (**Figures 3.5A,B**). In addition, we observed more virions present in LAMP1$^{+}$ compartments when lysosomal acidification was blocked by $NH_4Cl$ treatment, indicating that colocalization with LAMP1 represents bona fide lysosomal targeting that results in significant degradation (**Figure 2.5B**).

Vpr also prevented targeting of virions to lysosomes in MDM infected by YU-2 Env-pseudotyped HIV-1 NL4-3. Moreover, lysosomal targeting of virions was not observed without expression of Env from the integrated provirus (YU-2 Env-pseudotyped HIV-1

NL4-3*env*$^{-}$, **Figure 2.5C**). These studies reveal that in the absence of HIV-1 Vpr, MDM restrict HIV-1 by targeting Env-containing virions for lysosomal degradation. Furthermore, the capacity of Vpr to counteract this restriction is conserved among disparate HIV-1 clones.

Because restriction of Env expression and virion release by infected MDM is inducible by type I interferon (Mashiba et al., 2014), we treated MDM with exogenous IFNα to assess its effects on virion localization. Interestingly, IFNα stimulated lysosomal targeting of virions even in MDM expressing Vpr (**Figure 2.5B**), confirming the model that Vpr acts primarily by preventing the induction of an innate immune restriction pathway.

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

Infection of T lymphocytes in our culture system occurs by direct cell-to-cell spread, which requires formation of a transient VS between the infected cell and its target. Formation of VS requires interactions between HIV-1 Env on infected cells and CD4 on target cells (Jolly et al., 2004). Upon VS formation, high concentrations of mature virions localize to VS to mediate cell-to-cell spread (Dale et al., 2013). Because Vpr rescues Env and Env-containing virions from lysosomal degradation, we hypothesized that Vpr would also enable the formation of VS in the co-culture system. To determine whether Vpr affects VS formation between MDM and primary T lymphocytes, we used laser-scanning confocal microscopy to visualize areas of co-localization between surface CD4 on T lymphocytes and mature virions in MDM. We pre-stained T lymphocytes with an anti-CD4 antibody (DK4003) that does not disrupt the ability of CD4 to bind Env, and co-cultured

these cells with infected MDM briefly to allow formation of cellular contacts. We then washed away unbound cells and stained with an antibody against Gag MAp17 to visualize mature virions, as previously described (Duncan et al., 2014; Giese and Marsh, 2014; Groot et al., 2008). Virological synapses were identified as regions of co-localization between CD4 (green puncta in **Figure 2.6A**) on T lymphocytes and mature Gag on MDM (red puncta in **Figure 2.6A**). We identified similar numbers of MDM infected with wild type and mutant virus, and infected MDM were frequently multi-nucleated syncytia (**Figure 2.6A**). However, we consistently observed significantly more VS per infected MDM in the presence of Vpr (**Figures 3.6A,B**). These results explain why spread of HIV-1 from MDM to T lymphocytes is dramatically enhanced by Vpr.

As has been shown for other types of cell-to-cell spread (Jolly et al., 2004), we observed that VS between MDM and primary T lymphocytes did not form in the absence of de novo Env expression (YU2 Env-pseudotyped HIV-1 89.*6env*$^{-}$) (**Figure 2.6C**). Furthermore, consistent with a previous report (Massanella et al., 2009), VS formation was efficiently blocked by treating infected MDM with a high concentration (10 μg/ml) of the broadly-neutralizing anti-Env gp120 antibody b12 at the time of co-culture (**Figure 2.6C**). Thus, VS formation between HIV-1 infected MDM and primary T lymphocytes requires HIV-1 Env expression and is dramatically enhanced by expression of Vpr in MDM.

Vpr enhances Env expression by preventing the induction of a type I interferon-inducible restriction that degrades Env (Mashiba et al., 2014) and targets Env-containing virions for lysosomal degradation (**Figure 2.5**). Therefore we asked whether the addition of IFN-α to infected macrophages affected VS formation with T lymphocytes. Indeed, we

observed that IFN-α significantly reduced the number of VS detected per infected MDM even when MDM expressed Vpr (**Figure 2.6C**). In sum, these results are consistent with a model in which Vpr increases HIV-1 infection of T lymphocytes by preventing the induction of a type I interferon-mediated restriction of Env-dependent VS formation in MDM.

## Discussion

Vpr is a highly conserved HIV-1 protein that is required for full pathogenesis in vivo by a mechanism that is poorly understood. Here we show that under conditions in which efficient CD4+ T lymphocyte infection required contact-dependent VS formation with infected MDM, Vpr promoted VS-mediated transmission of HIV-1. Moreover, we provide evidence that Vpr promoted infection by counteracting an IFN-inducible restriction of HIV-1 Env expression in MDM.

Although CD4+ T lymphocytes are the most abundant HIV-1-infected cell type in vivo and are responsible for much of its pathogenesis, T lymphocytes are relatively refractory to infection by cell-free HIV-1 in vitro. In contrast, we observed significantly more HIV-1 infection of activated primary CD4+ T lymphocytes when T lymphocytes were co-cultured with autologous infected MDM, despite similar amounts of free virus in the co-culture supernatant. These results are consistent with research from other investigators showing cell-to-cell spread is much more efficient than infection of T lymphocytes by cell-free virus (Del Portillo et al., 2011; Groot et al., 2008). We also observed that once low-level initial infection of T lymphocytes by cell-free virus was established, subsequent spread within the culture became highly efficient and Vpr-independent. Thus, in the in

vitro co-culture system, Vpr and macrophages help the virus overcome a bottleneck to initial infection, accelerating infection of T lymphocytes. In this respect, the co-culture system recapitulated the in vivo requirement for Vpr for maximal T lymphocyte infection and provides a mechanism that helps explain its evolutionary conservation.

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

Our previous work indicates that Vpr increases MDM infection by preventing lysosomal degradation of Env and amplifying release of Env-containing virions (Mashiba et al., 2014). We report herein that in the absence of Vpr, virions containing Env were targeted to macrophage lysosomes and fewer virions were localized to Env-dependent VS between MDM and T lymphocytes. Indeed, our results illustrate that Vpr from multiple HIV-1 isolates promoted efficient macrophage-dependent T lymphocyte infection by this mechanism. This conserved function of Vpr provides a mechanistic explanation for its evolutionary conservation.

Finally, we provide confirmatory evidence that Vpr prevents the activation of an innate immune restriction of HIV-1 in MDM. Vpr activates the SLX4 endonuclease complex through its adaptor protein, DCAF1, allowing HIV-1 to evade the induction of a type I IFN response (Laguette et al., 2014). This pathway is active in MDM and may

explain how Vpr prevents macrophage-specific restriction of Env (Mashiba et al., 2014). Consistent with this, we demonstrated that treatment of infected MDM with exogenous IFN increased Env-dependent lysosomal targeting of virions and impaired Env-dependent VS formation with T lymphocytes. While the involvement of DCAF1 and IFN in Vpr-dependent HIV-1 spread from MDM to T lymphocytes supports a potential role for SLX4-mediated immune evasion, this has not yet been directly demonstrated.

IFN has several well-documented antiviral effects and likely acts through multiple mechanisms to inhibit HIV-1 infection and spread. While we cannot exclude the possibility that IFN affects VS formation through additional mechanisms, our results suggest that the Env-dependent restriction observed in MDM in the absence of Vpr is inducible by exogenous IFN treatment. Whether the restriction observed in Vpr-null-HIV-1-infected MDM requires secreted IFN is an interesting possibility that requires further study. Restriction of HIV-1 by IFN is of particular interest in light of recent evidence that IFN treatment may shrink the HIV-1 reservoir (Azzoni et al., 2013; Sun et al., 2014). Further elucidation of this pathway, including the mechanism by which HIV-1 is detected and the identity of the IFN-stimulated macrophage restriction factor are important areas for future investigation.

In sum, we report a novel role for Vpr in promoting VS-mediated HIV-1 infection of T lymphocytes by counteracting IFN-inducible restriction of Env in MDM. These results underscore the importance of macrophages in HIV-1 pathogenesis and antiviral immunity, and provide a compelling explanation for the in vivo function and evolutionary conservation of Vpr.

## Materials and Methods

*Antibodies*

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

*Viral constructs*

p89.6 and pNL4-3 were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalogs 3552 and 114 from Dr. Ronald G. Collman and Dr. Malcolm Martin, respectively (Adachi et al., 1986; Collman et al., 1992; Li et al., 1991). p89.6*vpr*,

p89.6*env*⁻, p89.6*vpr*Q65R, pNL4-3*env*⁻, pNL4-3*vpr*⁻, and pNL4-3*vpr*⁻*env*⁻ were constructed as previously described (Mashiba et al., 2014). pSIV3⁺, psPAX2, pAPM-1221 (shNC) and pDCAF-APM.1-3 (shDCAF1) were obtained from Dr. Jeremy Luban (Pertel et al., 2011). pYU-2*env* was obtained from Joseph Sodroski (Sullivan et al., 1995).

*Virus preparation*

Virus stocks were obtained by transfection of 293T cells with virus expression plasmids using polyethylenimine, as described (Mashiba et al., 2014; McNamara et al., 2012). Pseudotyped virus was produced by co-transfecting 293T cells with provirus and Env expression plasmid, as described (Mashiba et al., 2014). Viral supernatants were collected at 48h and centrifuged at 1500 rpm to remove cell debris. Virus was stored at -80°C and quantified by CAp24 ELISA, as described (Mashiba et al., 2014).

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

Leukocytes isolated from anonymous donors by apheresis were obtained from New York Blood Center Component Laboratory. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll density gradient separation, as described (Norman et al., 2011). CD14⁺ monocytes and CD4⁺ T lymphocytes were isolated as previously described (Mashiba et al., 2014). Briefly, monocytes were isolated by positive selection with an EasySep magnetic sorting kit (StemCell Technologies). Monocyte-derived macrophages (MDM) were obtained by culturing monocytes in R10 [RPMI-1640 with 10% Certified endotoxin-low fetal bovine serum (Gibco, Invitrogen)], penicillin (10 Units/ml), streptomycin (10 µg/ml), L-glutamine (292 µg/ml), carrier-free M-CSF (50 ng/ml, R&D

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

*Flow cytometry*

Surface staining for CD3 and CD14 was performed before fixation and intracellular staining for Gag CAp24 as described previously (Carter et al., 2010; Mashiba et al., 2014). Flow cytometric data was acquired using a FACSCanto instrument with FACSDiva collection software (BD) or a FACScan (BD, Cytek) with FlowJo software (TreeStar) and analyzed using FlowJo. Cell cycle analysis of 293T cells was performed previously (Mashiba et al., 2014).

*Immunoblot*

MDM or MDM-T lymphocyte cocultures were lysed in Blue Loading Buffer (Cell Signaling), sonicated with a Misonix sonicator (Qsonica, LLC.) and clarified by

centrifugation at 13000 RPM. Lysates were analyzed by SDS-PAGE immunoblot and protein levels were quantified using Adobe Photoshop as described (Mashiba et al., 2014; Norman et al., 2011).

*CAp24 ELISA*

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

*RNAi*

Short hairpin RNA-mediated knockdown of DCAF1 was performed as previously described (Mashiba et al., 2014; Pertel et al., 2011). Briefly, we spinoculated primary monocytes with VSV-G-pseudotyped SIV3$^+$ for 2 hours with 10 µg/ml polybrene to allow Vpx-dependent downmodulation of SAMHD1. Cells were then incubated overnight in R10 with M-CSF (50 ng/ml) and GM-CSF (50 ng/ml) plus 20 µg VSV-G-pseudotyped lentivirus containing a shRNA cassette targeting luciferase (Control) or DCAF1. Following an overnight incubation, the cells were cultured for 3 days in fresh medium before addition of 10 µg/ml puromycin for 3 additional days prior to HIV-1 infection.

*Laser-scanning confocal microscopy (LSCM)*

LSCM of MDM or MDM-T lymphocyte VS was performed as described previously (Duncan et al., 2014; Groot et al., 2008), with modifications. Briefly, MDM were differentiated on Nunc Lab-Tek 4-well chambered borosilicate cover glass (Thermo Fisher). For VS visualization, autologous, PHA/IL-2-activated CD4$^+$ T lymphocytes were

pre-stained for surface CD4 for one hour with primary antibody plus 30 minutes with secondary antibody and cocultured for four hours at room temperature with MDM before gentle washing with warm RPMI. For experiments using exogenous IFN, infected MDM were treated with 500U/mL recombinant IFNα (Calbiochem) two days before harvest. For LAMP1 staining, infected MDM were treated with 20 μM ammonium chloride for the final eight hours to prevent lysosomal acidification. Cells were fixed in 4% paraformaldehyde for one hour at room temperature and permeabilized with 0.1% saponin (Sigma) in 10% pooled human AB and goat sera for $F_C$-receptor blocking for one hour at room temperature, and endogenous biotin was blocked using endogenous biotin-blocking kit (Life Technologies) before staining for Gag p18 and/or LAMP1 for one hour primary and 30 minutes secondary using the antibodies listed above. Actin cytoskeleton was visualized by Phalloidin-TRITC (Sigma) and nuclei were stained using DAPI (Fisher Scientific). Cells were preserved in ProLong Gold anti-fade (Life Technologies) and visualized on a Leica SPX5 inverted confocal microscope at the University of Michigan Microscopy and Image-Analysis Laboratory. Images of optical sections of approximately 1 μm depth were captured at 20X dry or 100X oil-immersion objective magnification. Images were processed using ImageJ (NIH) and co-localization was quantitated by automated spots analysis using Imaris (BitPlane). Each Gag MAp17$^+$ puncta with signal 2-fold or greater above background based on a raltegravir-inhibited infected MDM control was identified in an automated manner, and fluorescence intensity in each channel was quantitated for each Gag$^+$ spot. Co-localization was defined as the number of Gag$^+$ spots that were also positive for LAMP1 or CD4 (VS) two-fold or greater above isotype staining controls, per Gag$^+$ cell imaged.

**Acknowledgements**

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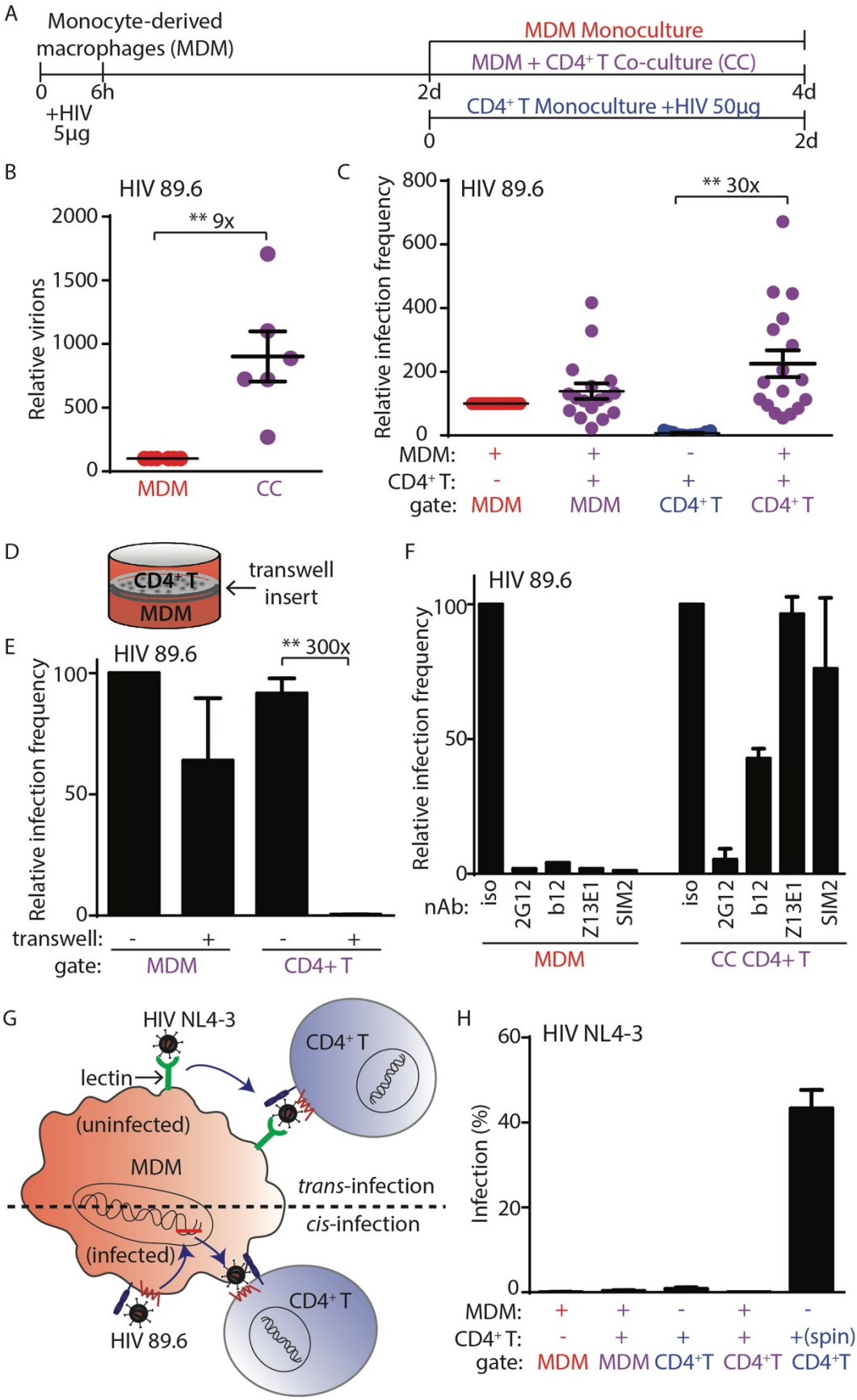
A
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MDM Monoculture
MDM + CD4+ T Co-culture (CC)
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2d
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Relative virions
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MDM
CC
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**Figure 2.1: Efficient HIV infection of T lymphocytes requires contact with infected macrophages.** (**A**) Graphical outline of experimental setup depicting HIV-1 infection of macrophages and co-cultivation with autologous, PHA-activated $CD4^+$ T lymphocytes as detailed in Methods. (**B**) Summary graph of relative quantity of virions released into culture supernatant as measured by Gag CAp24 ELISA (n=6 donors). (**C**) Summary graph of relative infected cell frequency in the indicated cultures as measured by flow cytometry (n=11 donors for $CD4^+$ T or 17 donors for MDM and CC). (**D**) Diagrammatic representation of virus-permeant transwell. (**E**) Summary graph of relative infected cell frequency in cocultures prepared as shown in A in the presence or absence of transwell inserts (n=4 donors). (**F**) Summary graph of relative infected cell frequency in the indicated cultures prepared as shown in A. Neutralizing antibodies to HIV Env gp120 (2G12, b12), gp41 (Z13E1) or CD4 (SIM.2) were added at the time of initial infection (MDM) or at the time of $CD4^+$ T addition and cocultivation (CC). (**G**) Diagram illustrating *trans*- and *cis*-infection of T lymphocytes. (**H**) Summary graph of infected cell frequency in the indicated cell type after addition of HIV-1 NL4-3 as described in A. For "spin" condition, PHA activated $CD4^+$ primary T lymphocytes were centrifuged at 2500 RPM with 50µg HIV-1 NL4-3 in polybrene (n=4 donors). Error bars represent SEM. $^{**}p<0.01$, student's paired t-test.[1]

[1] The data in this figure was created by David Collins.

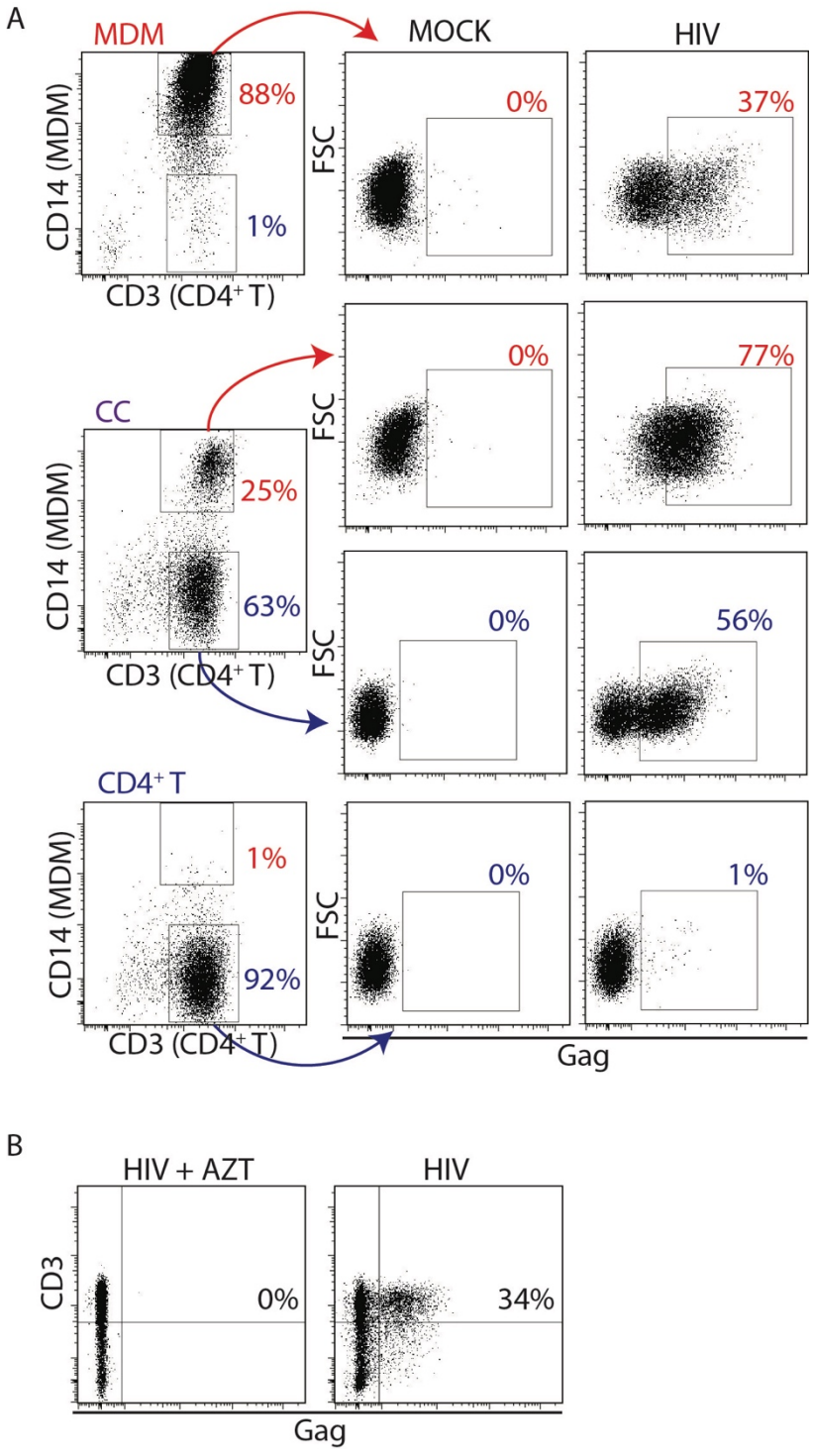


**Figure 2.2: Flow cytometric analysis of HIV infection in macrophage-T lymphocyte cocultures.** (**A**) Representative flow cytometric dot plots illustrating segregation of $CD14^+$ MDM from $CD3^+$ T lymphocytes in cocultures and subsequent assessment of HIV-1 infection by intracellular Gag CAp24 stain after treatment of the indicated cultures treated as shown in Figure 2.1A. (**B**) Representative flow cytometric dot plots of intracellular Gag CAp24 staining (x-axes)

vs. surface CD3 expression (y-axes) of MDM-CD4$^+$ T lymphocyte cocultures infected as shown in Figure 2.1A in the presence or absence of the reverse transcriptase inhibitor zidovudine (AZT).[2]

[2] The data in this figure was created by David Collins.

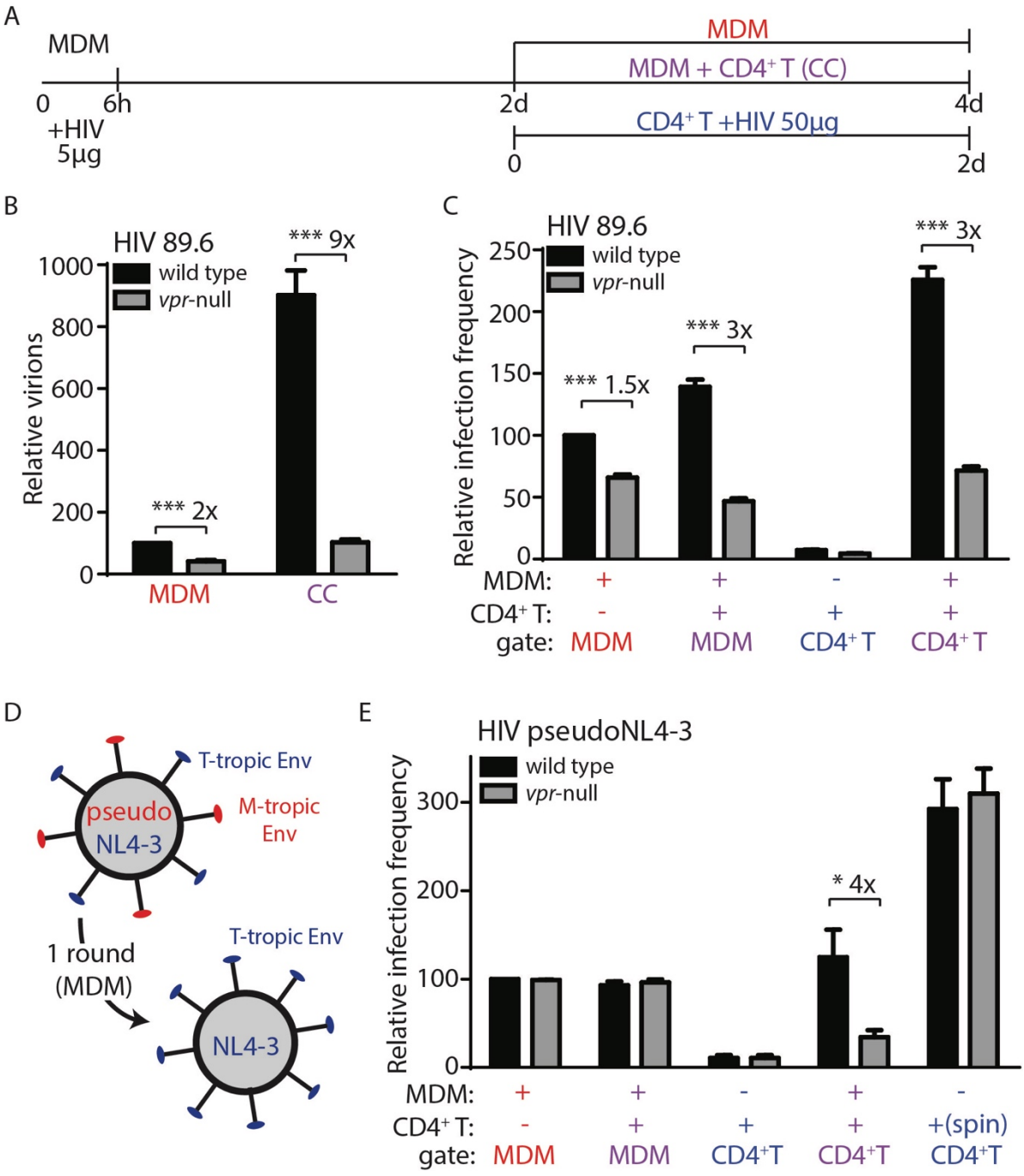


**Figure 2.3: Vpr enhances macrophage-dependent infection of CD4$^+$ T lymphocytes.** (**A**) Graphical outline of experimental setup as in Figure 2.1A. (**B**) Summary graph of relative quantity of virions released into the supernatant of the indicated cultures after inoculation with type (black bars) or *vpr*-null (gray bars) HIV-1 89.6 (n=6 donors). (**C**) Summary graph of relative infected cell frequency in the indicated cultures (n=11 donors for CD4$^+$ T or 17 donors for MDM and CC). (**D**) Diagram illustrating HIV-1 NL4-3 pseudotyped with YU-2 Env (Buchacher et al.) to infect macrophages for a single round and subsequently spread to T lymphocytes using NL4-3 Env (blue). (**E**) Summary graph of relative infected cell frequency in the indicated cell type after addition of HIV-1 YU-2 pseudo-NL4-3 as described in A. For "spin" condition, PHA activated CD4$^+$

primary T lymphocytes were centrifuged for 2500 RPM with 50µg HIV-1 NL4-3 in polybrene (n=3 donors). Data normalized relative to wild-type MDM. Error bars represent SEM. *p<0.05, ***p<0.001, student's paired t-test.[3]

[3] The data in this figure was created by David Collins and Jay Lubow.

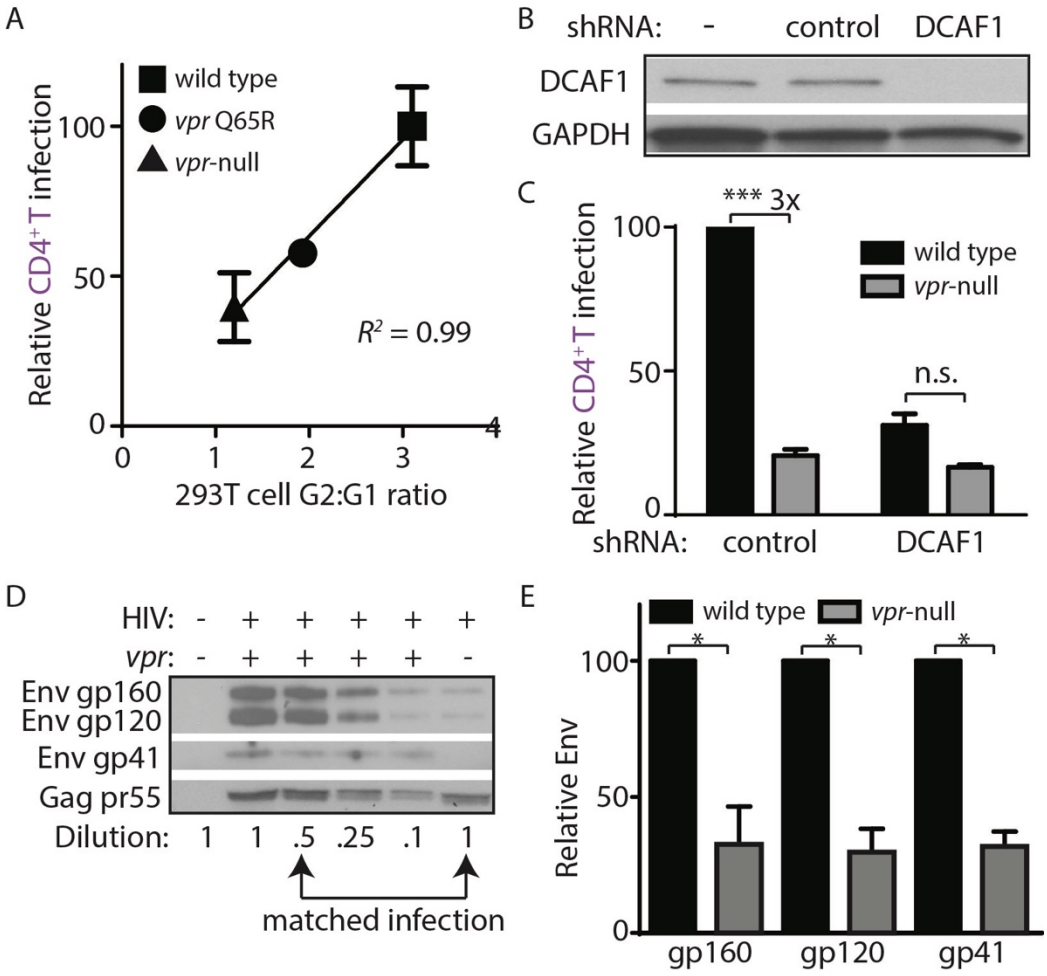


**Figure 2.4: DCAF1 is required for Vpr-dependent HIV-1 spread from macrophages to CD4$^+$ T lymphocytes.** (**A**) Scatter-plot of Vpr-dependent cell cycle arrest of 293T cells (x-axis) versus Vpr-dependent increase in cocultured T lymphocyte infection (y-axis). Best-fit curve from linear regression analysis, $R^2$=0.99 (n=4 donors). (**B**) Immunoblot of DCAF1 and GAPDH in MDM seven days after transduction with lentivirus encoding shRNA targeting luciferase ("control") or DCAF1. (**C**) Summary graph showing infection frequency of T lymphocytes in co-culture (Figures 3.1A and 3.3A) by MDM treated with the indicated shRNA (n=3 donors). (**D**) Immunoblot of HIV-1 89.6 Env and Gag in MDM-T lymphocyte coculture whole-cell lysates diluted as indicated. Arrows denote lysates with comparable levels of Gag pr55 in the presence and absence of Vpr. (**E**) Summary graph of Env levels quantified by densitometry and normalized to Gag pr55 levels (n=4 donors). Error bars represent SEM. *p<0.05, ***p<0.001, "n.s."p>0.05, student's paired t-test.[4]

[4] The data in this figure was created by David Collins and Jay Lubow.

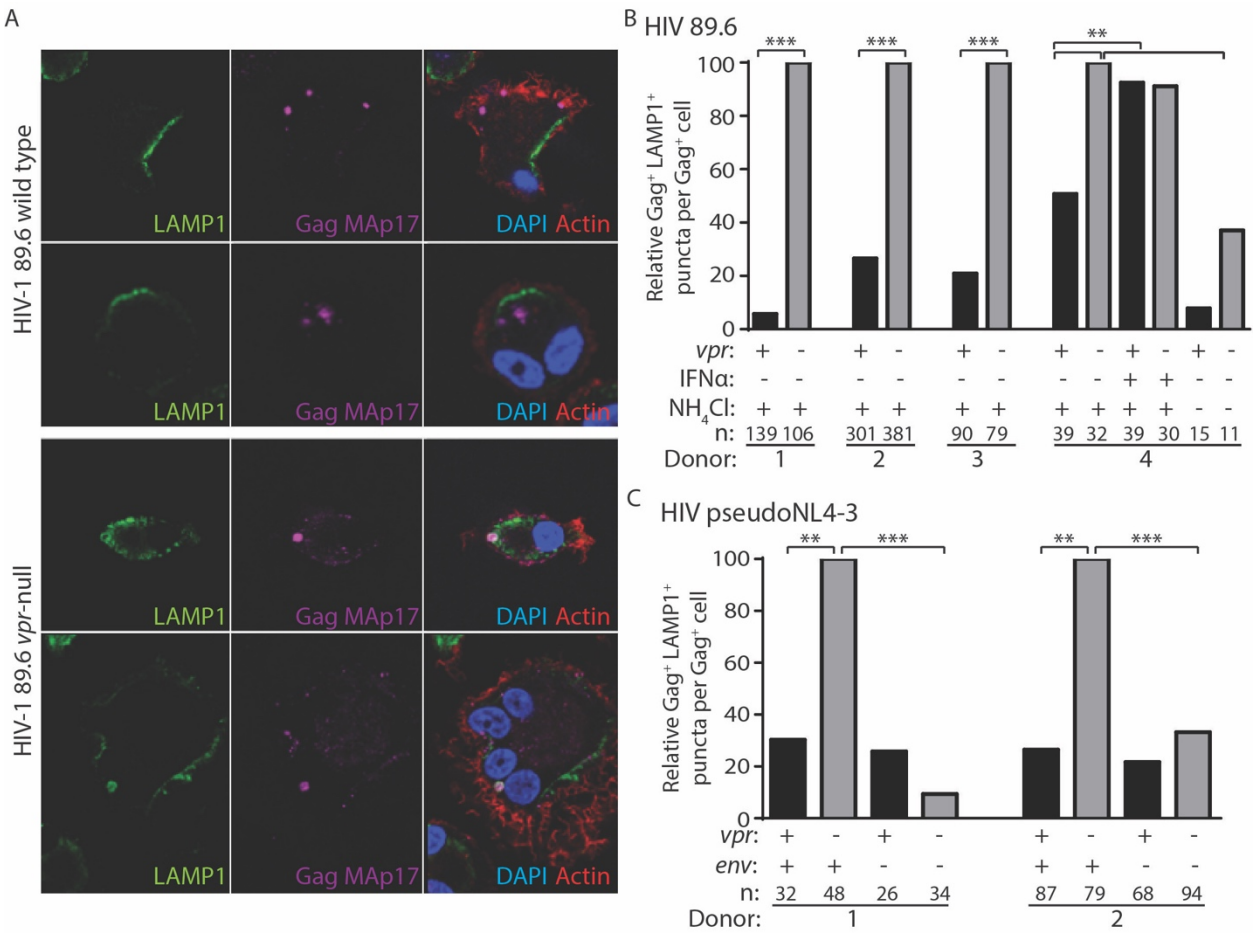


**Figure 2.5: Vpr prevents Env-dependent targeting of HIV virions to lysosomes in macrophages.** (**A**) Representative confocal micrographs depicting subcellular localization of lysosome marker LAMP1 (green) and HIV-1 Gag MAp17 (magenta) in MDM infected by wild type or Vpr-null HIV-1 89.6 for ten days. Merged images (right panels) include phalloidin staining of actin cytoskeleton (Buchacher et al.) and DAPI staining of nuclei (blue). (**B**) Summary graph of LAMP1$^+$ Gag MAp17$^+$ co-localized puncta per Gag$^+$ cell across 'n' number of MDM from four donors infected as shown in Figure 2.1A expressed as percent colocalization relative to *vpr*-null. MDM from donor 4 were treated with IFNα for the final two days of infection. Lysosomal acidification was blocked with $NH_4Cl$ for the final 8 hours where indicated. (**C**) Summary graph as in B of MDM from two donors infected with wild-type, *vpr*-null, *env*-null, or *vpr*- and *env*-null NL4-3 pseudotyped with YU-2 Env and treated with $NH_4Cl$ for the final 8 hours. **p<0.01, ***p<0.001, Fisher's exact test.[5]

[5] The data in this figure was created by David Collins.

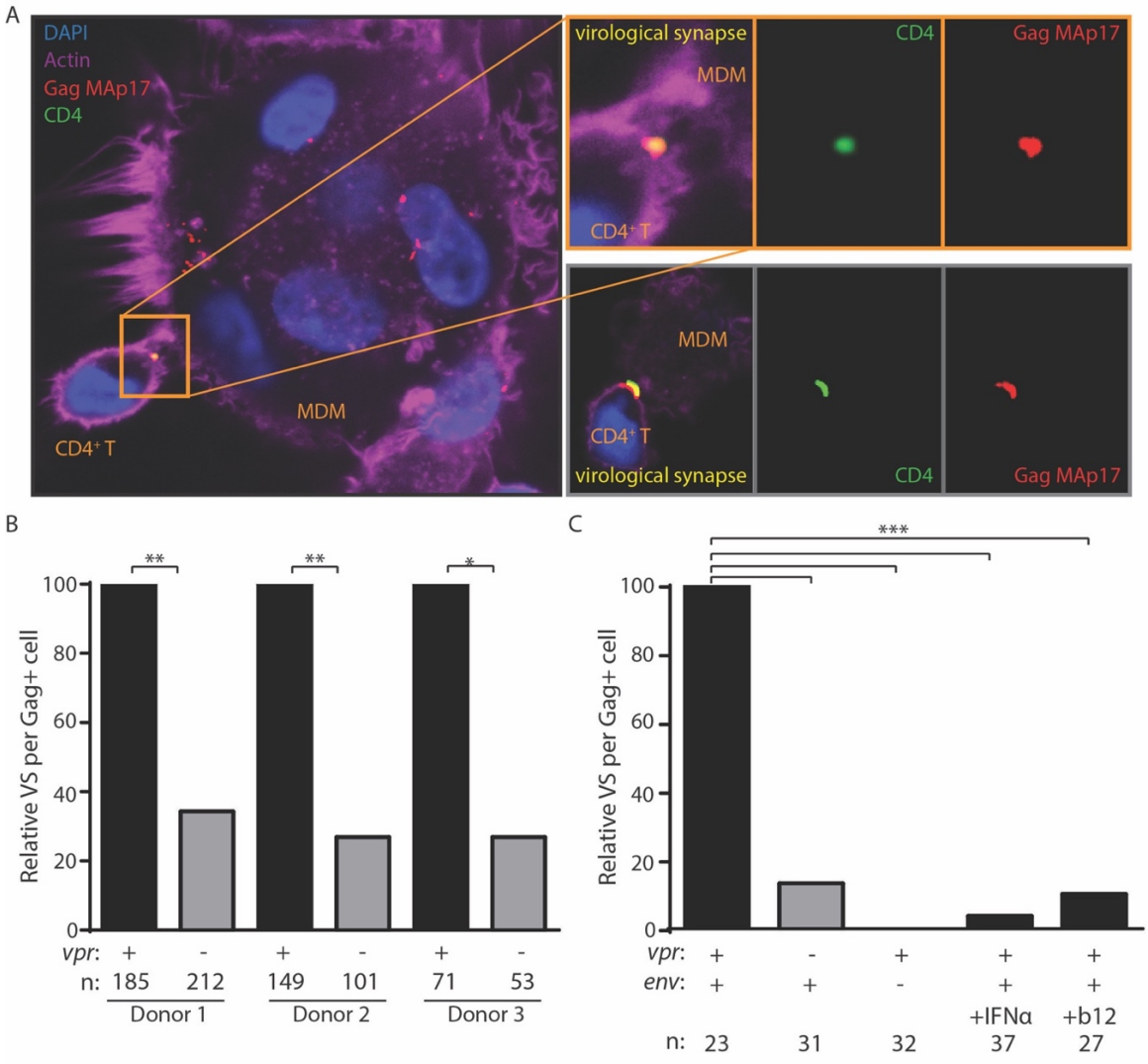


**Figure 2.6: Vpr promotes Env-dependent virological synapse formation between macrophages and CD4⁺ T lymphocytes.** (**A**) Representative confocal micrographs of MDM infected as shown in Figures 3.1A and 3.3A and briefly cocultured with CD4⁺ T lymphocytes pre-stained for surface CD4. Co-localization between HIV-1 Gag MAp17 (Buchacher et al.) in MDM and surface CD4 (green) on T lymphocytes at 100X magnification is indicated as virological synapses (VS). Merged images include phalloidin staining of actin (magenta) and DAPI staining of nuclei (blue). Inset depicts magnified VS from same image (top) or from a different representative image (bottom). (**B**) Summary graph of VS observed at 20X magnification per 'n' number of Gag⁺ MDM from three donors infected by wild type or *vpr*-null HIV-1 89.6. (**C**) Summary graph of VS as in B of MDM infected with YU-2 Env-pseudotyped *env*-null 89.6 (third column), wild type 89.6-infected MDM treated with anti-Env gp120 neutralizing antibody b12 during co-cultivation with CD4⁺ T lymphocytes (fourth column) or treated for two days prior to coculture with interferon-α (IFNα, final column). *p<0.05 **p<0.01 ***p<0.001, Fisher's exact test.[2]

[2] The data in this figure was created by David Collins.

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## Chapter 3

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


### Summary

Earlier publications and Chapter 2 of this dissertation provided significant evidence of the existence of a macrophage-specific restriction factor 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 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 response system to facilitate infection while evading MR's normal role, which is to trap and destroy mannose-expressing pathogens.


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## 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 $\alpha$-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 SDXXL$\Phi$ 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 3.1A 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 3.1C 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 3.2**). Relative expression of known restriction factors GBP5 and IFITM3 varied in infected MDM from multiple donors (**Figure 3.2**), 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 3.1E 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, $p<0.001$).

*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 defective HIV with a GFP marker (NL4-3 ΔGPE-GFP, **Figure 3.3A**) 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 3.3B**). For these experiments, primary MDM were harvested earlier than the experiments described in **Figure 1** (five days versus ten days) because the viruses could not replicate and the GFP marker allowed identification of transduced cells (**Figure 3.3**). 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 3.3C-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 3.3E**). In addition, complete elimination of downmodulation required mutation of both Vpr and Nef (**Figure 3.3C-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.

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 3.3A**), confirmed expression in transfected 293T cells (**Figure 3.3F**) 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 3.3E**). 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 transduced with the mutant viral constructs had roughly similar transduction rates as the parental construct (**Figure 3.3G**) but demonstrated less MR downmodulation (**Figure 3.3E**).

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 3.3H**) similar to what was observed using the HIV reporter construct (**Figure 3.3E**). 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 3.4**). 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.

*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 3.5A**) using cells isolated based on GFP expression (**Figure 3.5B**). 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 3.5C 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 3.5D**, p<0.01). Similar results were obtained when each gene was normalized to *ACTB* instead of *GAPDH* (**Figure 3.6A-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 3.6C-F**).

*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 3.7A**) or release of virions as assessed by measuring Gag p24 into the supernatant by ELISA (**Figure 3.7B**). 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 3.7C 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 3.7D**, 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 3.7C**) and Gag pr55 western blot (**Figure 3.7E**) 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 3.7E**, 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 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 3.8A**). 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 3.8B**).

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 3.8C**, a fragment of the YU-2 genome containing most of *env* but none of *vpr* (**Figure 3.8C**, 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 3.8D 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- 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 3.8F**)]. 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.

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 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 3.8G**). Moreover, MDM infected with the chimeric HIV had a reduced requirement for Vpr for maximal virion release (**Figure 3.8H and Figure 3.9**). 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 3.10A**). 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 $\alpha$-mannosidases (Pritchard et al., 2015). These substitutions (N230D and N339E) in 89.6

did not alter virion production (**Figure 3.10B**) or Env protein expression (**Figure 3.10C**) 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 3.10D**, 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 3.10E**). 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 3.10E**, 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 3.10F**, left panel). However, infection of MDM by wild-type HIV-1 was inhibited up to 16-fold by mannan (**Figure 3.10F**, 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. 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 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 3.10G**, $p<0.001$). In addition, in single-round infections of MDM, the mannose-deficient Env had a reduced requirement for both Nef and Vpr (**Figure 3.10H** and **Figure 3.11**, $p<0.001$). 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 3.10I**, right

side, black bars) and Nef (**Figure 3.10I**, 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.

*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 3.12A**). 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 3.13A**. Co-cultured cells were stained for CD3 to distinguish T cells and CD14 to distinguish MDM as shown in **Figure 3.13B**, accounting for differences in autofluorescent background in the two cell types by using isotype controls (**Figure 3.13C**) We confirmed our prior finding that Vpr enhances HIV-1 89.6 spread from MDM to T cells (**Figure 3.13D**) and extended this finding to the transmitted/founder (T/F) clone REJO (**Figure 3.13E**). 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 3.12B**. Using the gating strategy shown in **Figure 3.13B**, infected MDM and infected T cells were identified by intracellular Gag stain (**Figure 3.12C**). 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 3.12D**). 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.

## 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.

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 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 TCTCGAGTTTAAA CTTATAGCAAAGCCCTTTCCA). 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 TCGAGAAGGTATATTGCTGTTGACAGTG AGCGAGTAACTTGACTGATAATCAATTAGTGAAGCCACAGATGTAATTGATTATCAG TCAAGTTACTTGCCTACTGCCTCGG (forward) and AATTCCGAGGCAGTAGGCAA GTAACTTGACTGATAATCAATTACATCTGTGGCTTCACTAATTGATTATCAGTCAAG TTACTCGCTCACTGTCAACAGCAATATACCTTC (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.

**Acknowledgments**

We are grateful to the University of Michigan Vector Core and the NIH AIDS Reagent Program for reagents and to the University of Michigan Flow Cytometry Core for assistance with experiments. We thank Dr. Estelle Chiari for providing pMSCV 3xFLAG UNG2 IRES-GFP

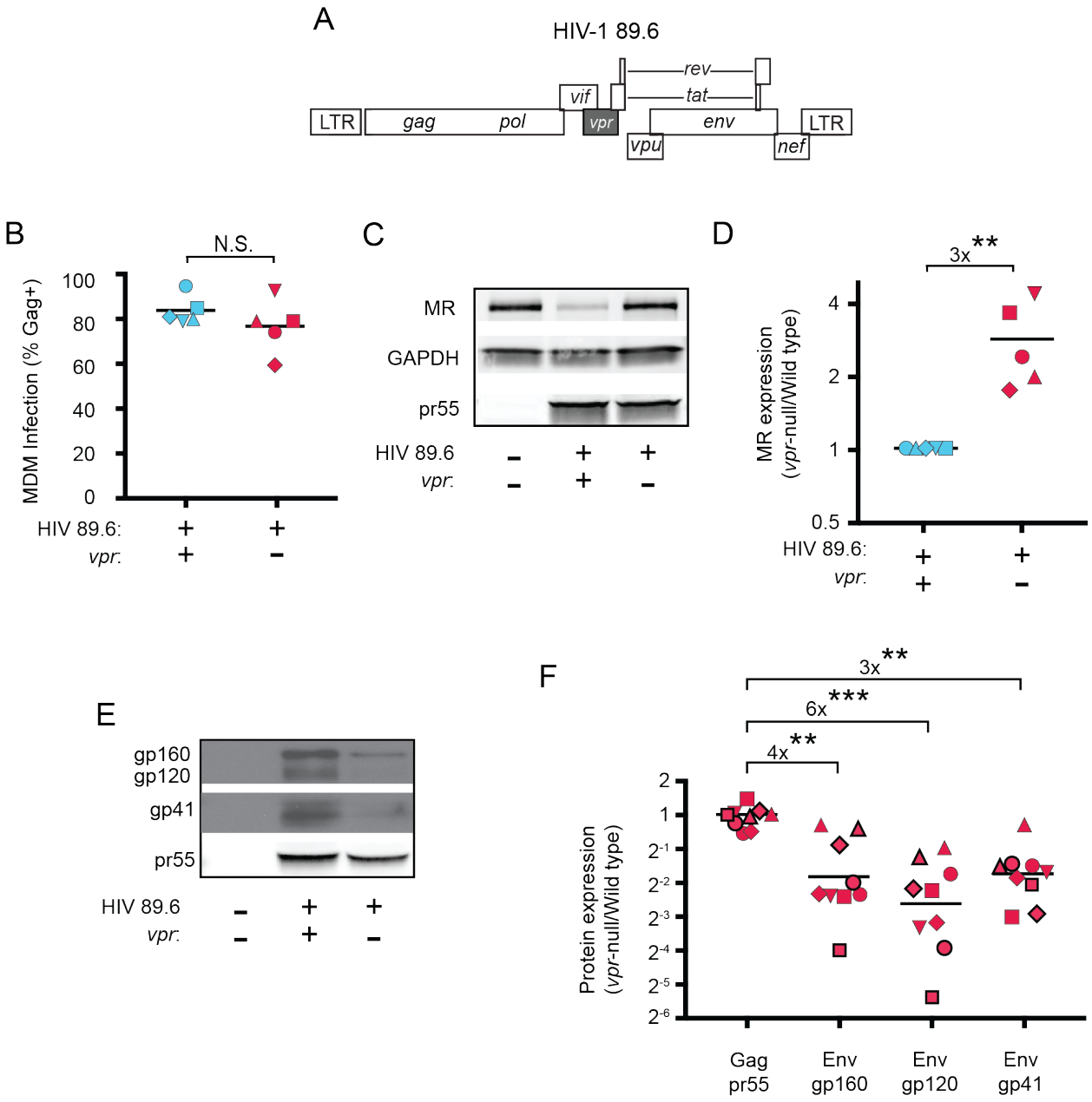


**Figure 3.1: HIV Vpr reduces steady state levels of host mannose receptor in MDM and increases steady state levels of viral Env protein[2]. (A)** Diagram of the HIV 89.6 proviral genome. The shaded box shows the location of *vpr,* which was disrupted by a frame shift mutation to create the Vpr-null 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 MDM prepared as in B. **(D)** Summary graph displaying relative expression of MR in wild-type and

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

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.

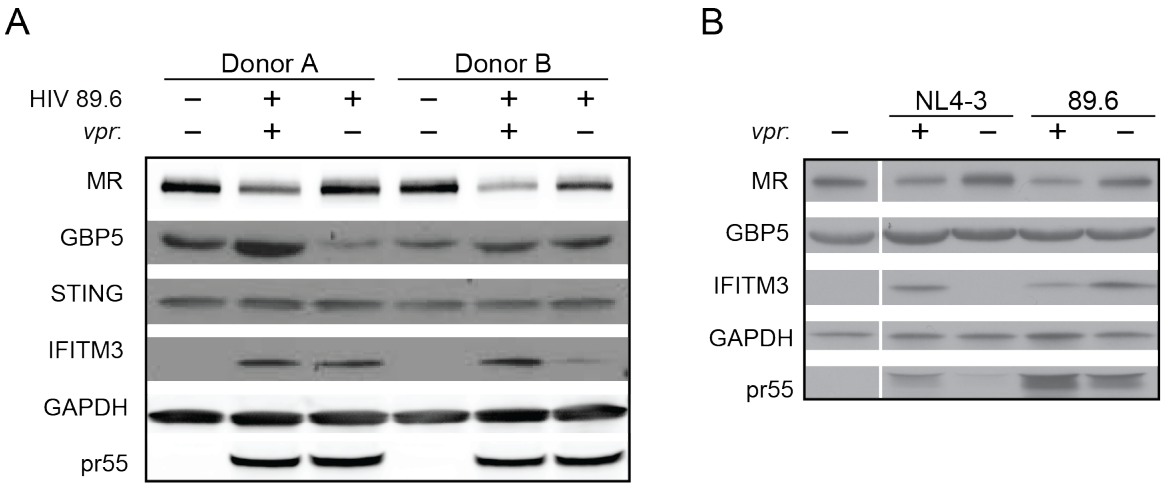


**Figure 3.2: HIV Vpr reduces steady state levels of MR but not GBP5, STING or IFITM3[3]. (A)** Western blot analysis of whole cell lysates from MDM infected with wild-type or *vpr*-null HIV-1 89.6 for 10 days. **(B)** Western blot analysis of whole cell lysates from MDM infected with wild-type or *vpr*-null HIV-1 89.6 and YU2-pseudotyped NL4-3 for 10 days. *n*=3 independent donors.

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

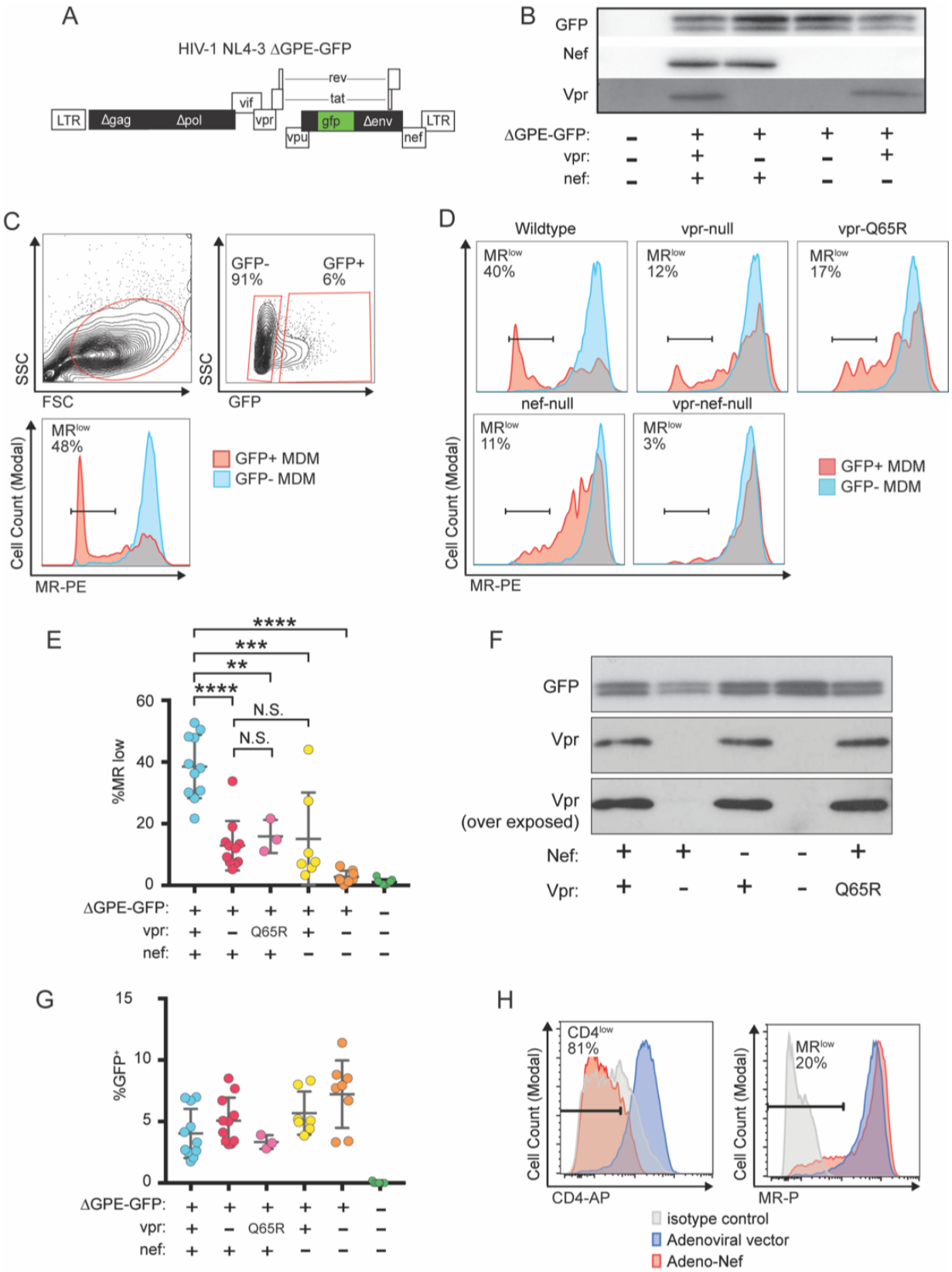
A
HIV-1 NL4-3 ΔGPE-GFP
LTR
Δgag
Δpol
vif
vpr
rev
tat
vpu
gfp
Δenv
nef
LTR
B
GFP
Nef
Vpr
ΔGPE-GFP:
vpr:
nef:
C
SSC
FSC
GFP-
91%
GFP+
6%
GFP
MR^low
48%
Cell Count (Modal)
MR-PE
GFP+ MDM
GFP- MDM
D
Wildtype
vpr-null
vpr-Q65R
MR^low
40%
MR^low
12%
MR^low
17%
nef-null
vpr-nef-null
MR^low
11%
MR^low
3%
Cell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

**Figure 3.3: 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, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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

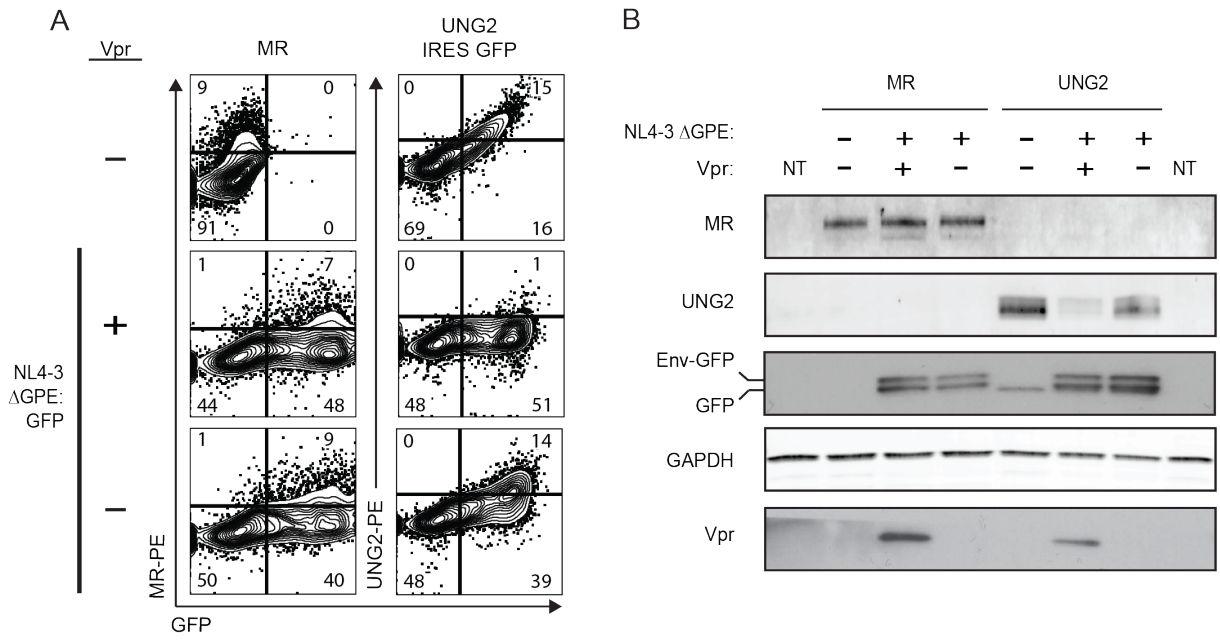


**Figure 3.4: HIV Vpr reduces steady state levels of UNG2 but not MR in co-transfected 293T cells[5]. (A)** Flow cytometric plots of 293T cells co-transfected with NL4-3 ΔGPE-GFP, pCDNA3.1-hMR, and pMSCV 3x FLAG UNG2 IRES-GFP as indicated. **(B)** Western blot analysis of 293T cells co-transfected exactly 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.

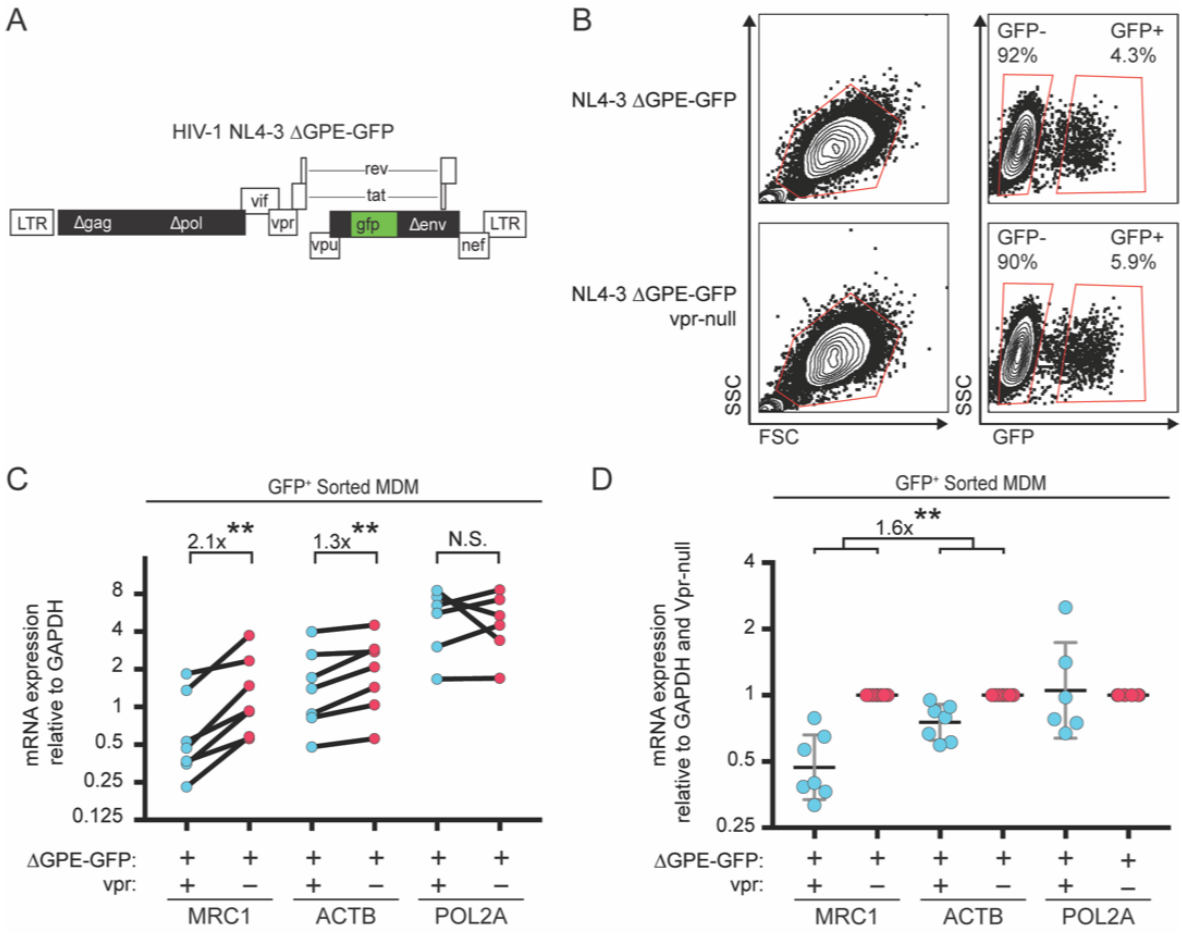


**Figure 3.5: Vpr reduces transcription of *MRC1*[6]. (A)** Diagram of HIV NL4-3 ΔGPE-GFP. **(B)** Flow cytometry plots indicating the gating strategy used to sort live GFP$^+$ vs GFP$^-$ cells for subsequent qPCR analysis. **(C)** Summary graph of mannose receptor (*MRC1*), β-actin (*ACTB*) and RNA 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, Francisco Gomez-Rivera, and Gretchen Zimmerman.

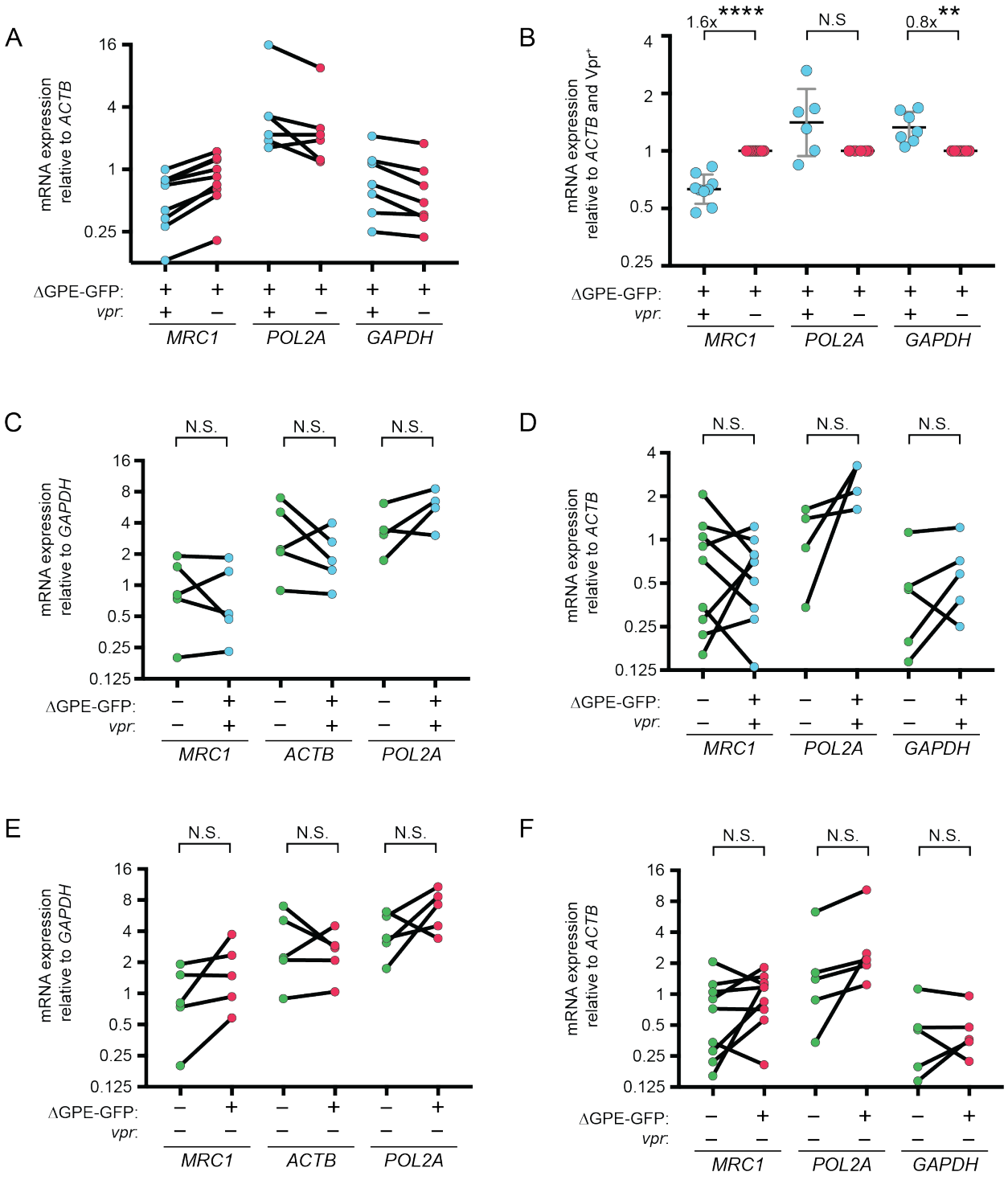


**Figure 3.6: Vpr does not reduces transcription of housekeeping genes[7]. (A)** Summary graph of mannose receptor (*MRC1*), RNA Polymerase 2A (*POL2A*) and *GAPDH* mRNA expression in MDM transduced with Vpr-competent or Vpr-null HIV NL4-3 ΔGPE-GFP and sorted for GFP expression by FACS. All data are 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).

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

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, ** $p<0.01$, **** $p<0.0001$.

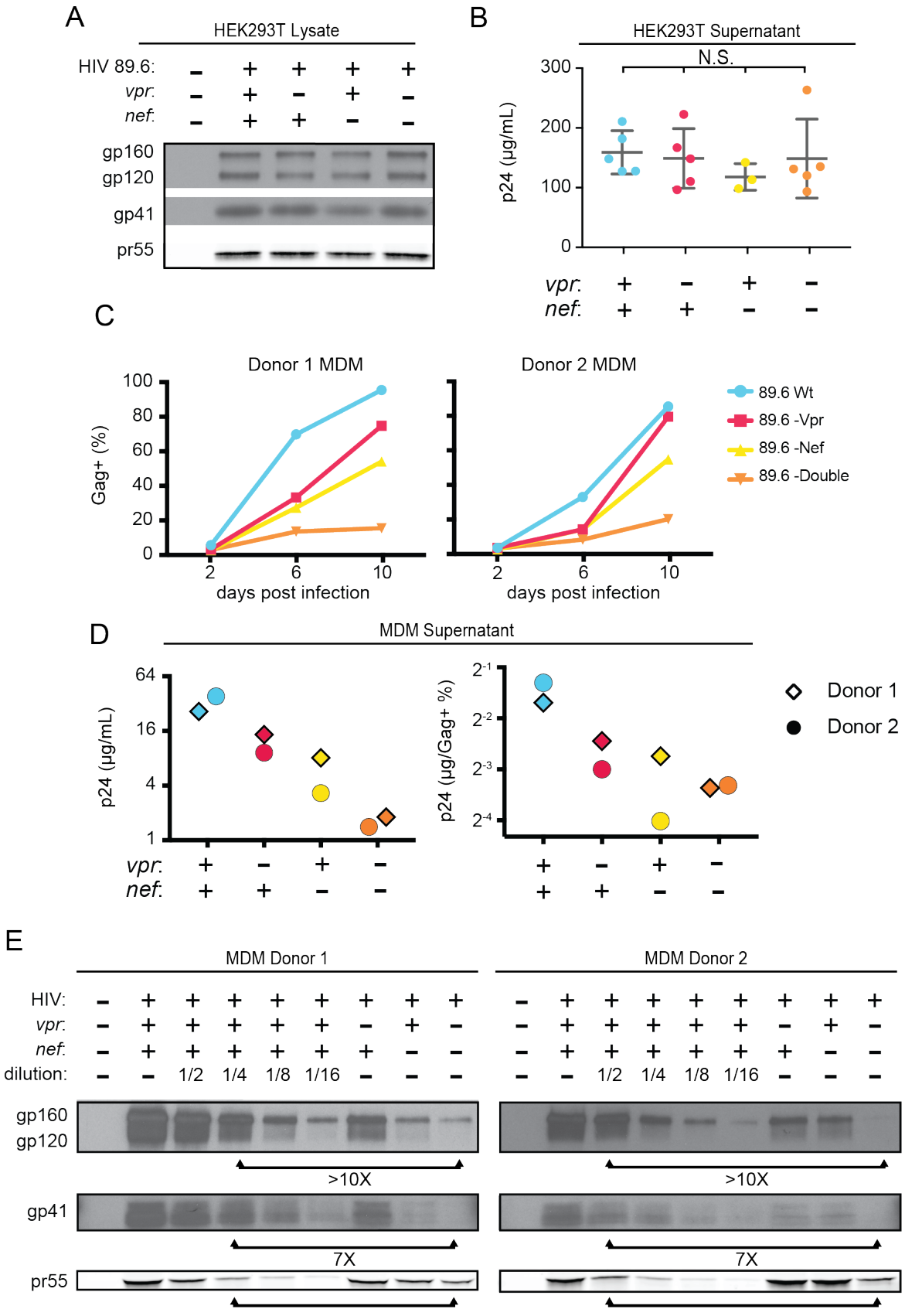
A
HEK293T Lysate
HIV 89.6:
vpr:
nef:
gp160
gp120
gp41
pr55
B
HEK293T Supernatant
N.S.
p24 (µg/mL)
300
200
100
0
vpr:
nef:
C
Donor 1 MDM
Donor 2 MDM
Gag+ (%)
100
80
60
40
20
0
2
6
10
days post infection
89.6 Wt
89.6 -Vpr
89.6 -Nef
89.6 -Double
D
MDM Supernatant
p24 (µg/mL)
64
16
4
1
p24 (µg/Gag+ %)
2-1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

**Figure 3.7: 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.

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

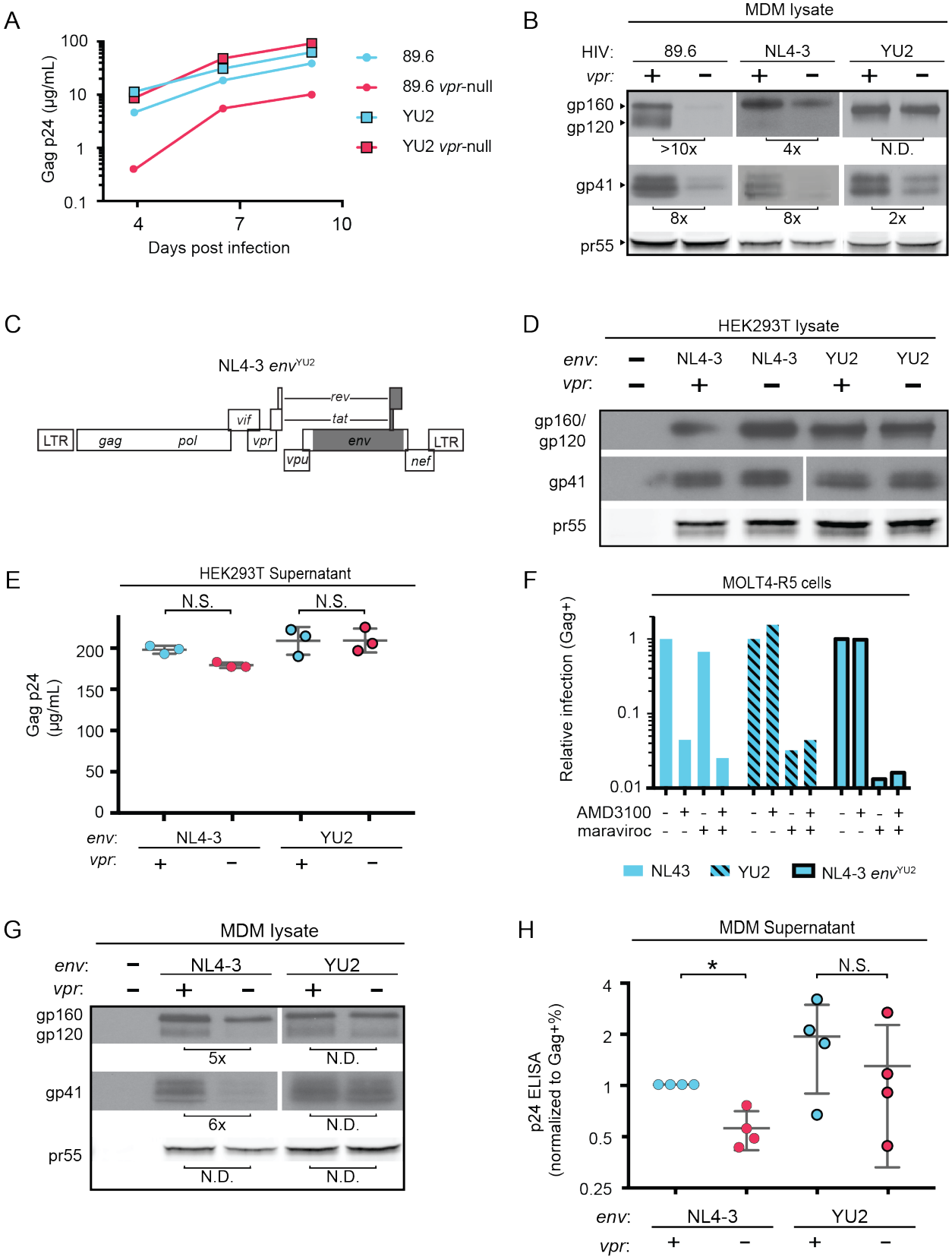
A
Gag p24 (µg/mL)
100
10
1
0.1
4
7
10
Days post infection
89.6
89.6 vpr-null
YU2
YU2 vpr-null
B
MDM lysate
HIV:
89.6
NL4-3
YU2
vpr:
+ − + − + −
gp160
gp120
>10x
4x
N.D.
gp41
8x
8x
2x
pr55
C
NL4-3 env^YU2
rev
tat
vif
LTR
gag
pol
vpr
env
LTR
vpu
nef
D
HEK293T lysate
env:
− NL4-3 NL4-3 YU2 YU2
vpr:
− + − + −
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 3.8: 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, * $p<0.05$.

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

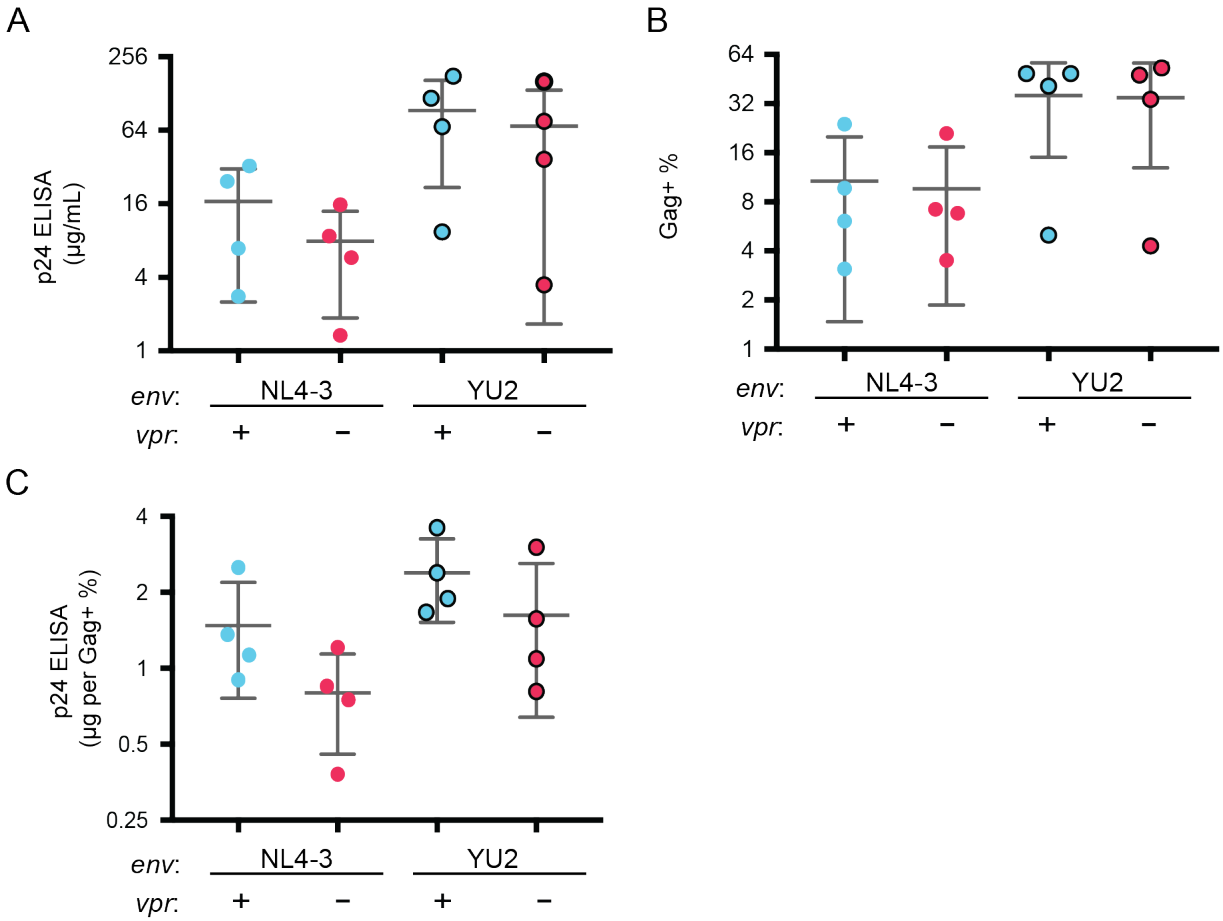


**Figure 3.9: Raw p24 ELISA and intracellular gag data 10 days post infection by NL43*env*$^{YU2}$.**[10] **(A)** Summary graph showing Gag p24 concentration of supernatant from MDM cultures 10 days post infection with the indicated virus. **(B)** Summary graph showing the fraction of MDM that are Gag+ 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.

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

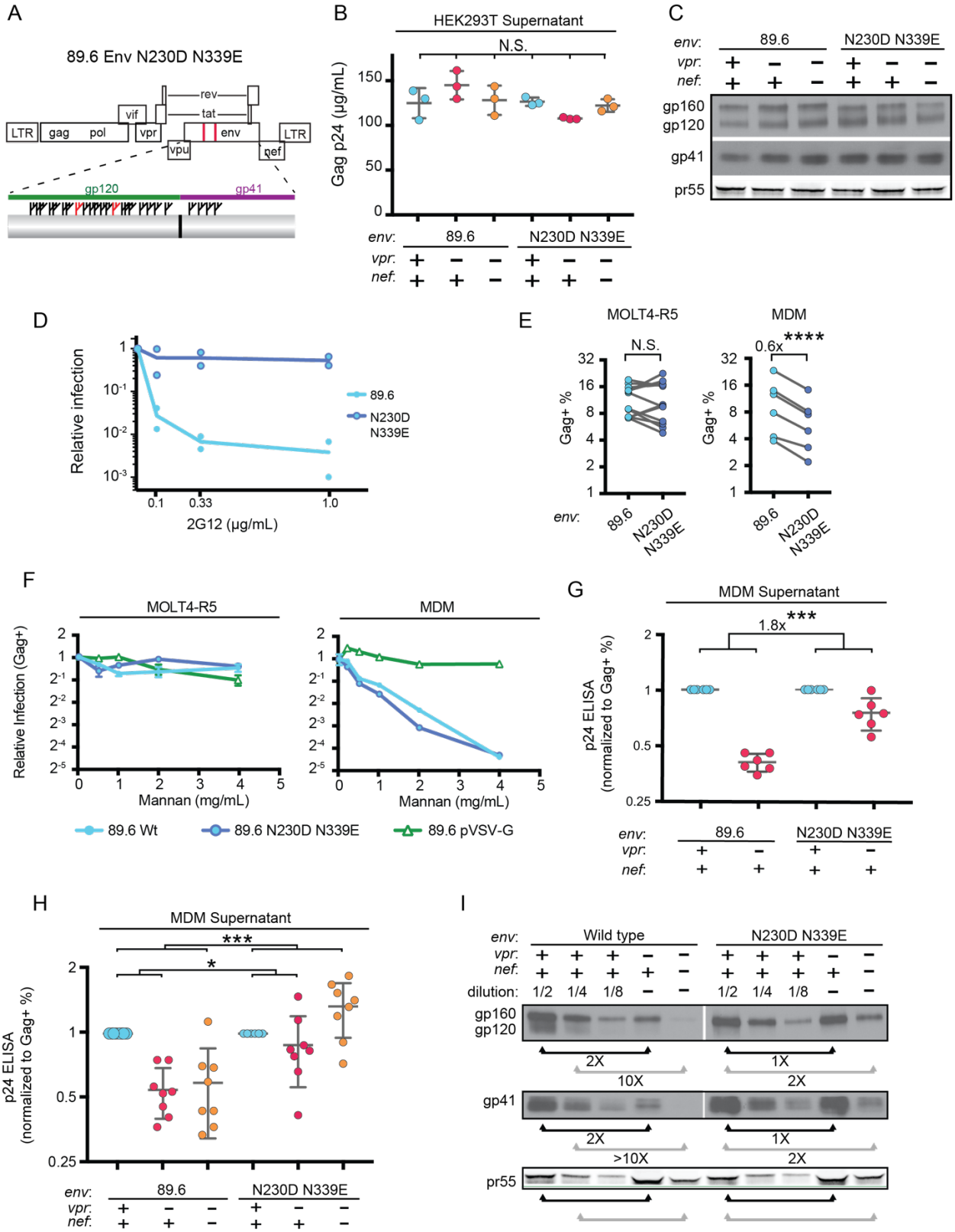
A
89.6 Env N230D N339E
rev
tat
vif
LTR
gag
pol
vpr
env
LTR
vpu
nef
gp120
gp41
B
HEK293T Supernatant
N.S.
Gag p24 (µg/mL)
150
100
50
0
env:
89.6
N230D N339E
vpr:
nef:
C
env:
89.6
N230D N339E
vpr:
nef:
gp160
gp120
gp41
pr55
D
Relative infection
1
10-1
10-2
10-3
0.1
0.33
1.0
2G12 (µg/mL)
89.6
N230D N339E
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
2X
10X
1X
2X
gp41
2X
>10X
1X
2X
pr55

**Figure 3.10: 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.

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

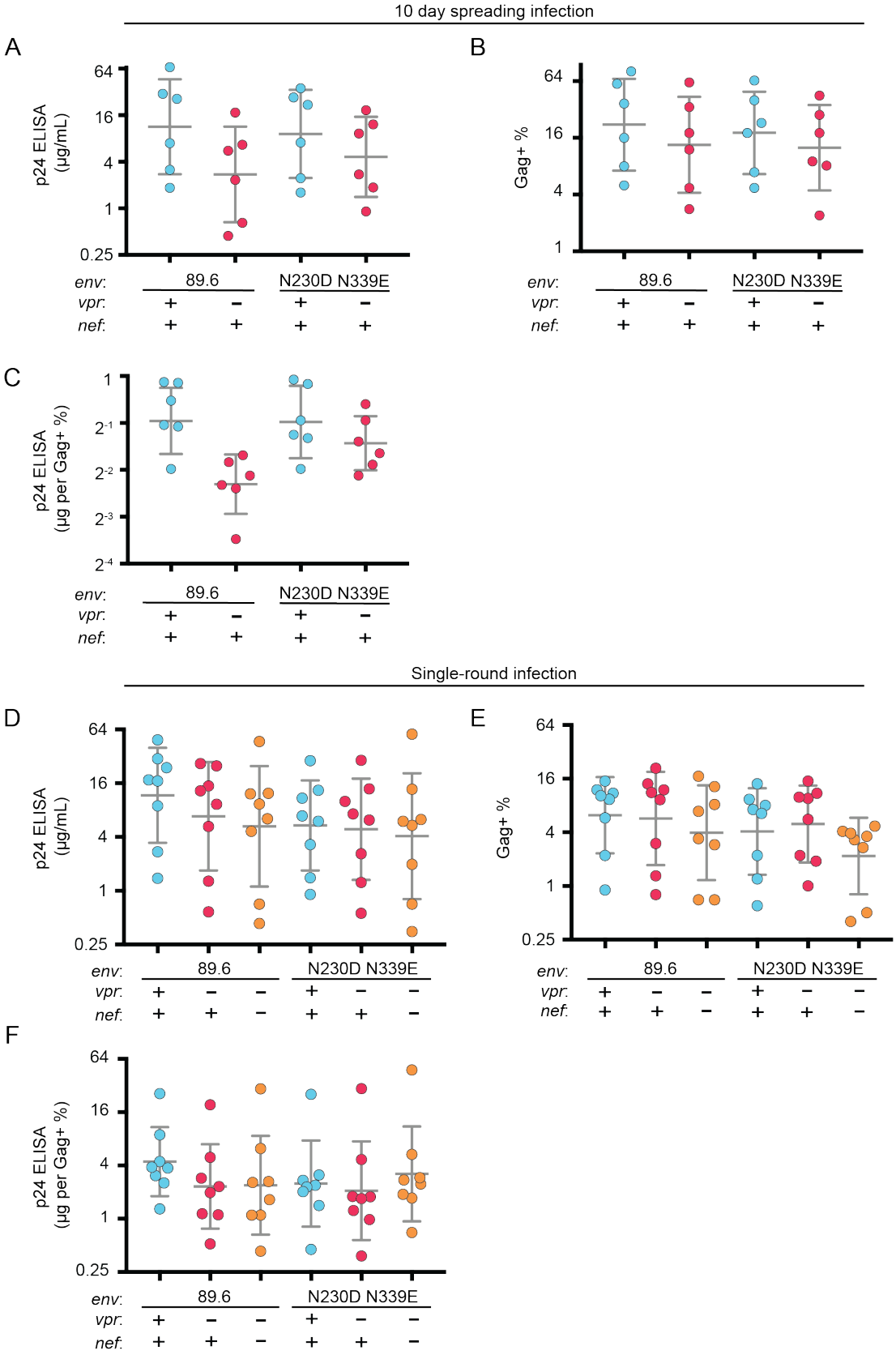
10 day spreading infection
A
p24 ELISA (µg/mL)
64
16
4
1
0.25
B
Gag+ %
64
16
4
1
env:
89.6
N230D N339E
vpr:
+
−
+
−
nef:
+
+
+
+
C
p24 ELISA (µg per Gag+ %)
1
2-1
2-2
2-3
2-4
Single-round infection
D
p24 ELISA (µg/mL)
64
16
4
1
0.25
E
Gag+ %
64
16
4
1
0.25
env:
89.6
N230D N339E
vpr:
+
−
−
+
−
−
nef:
+
+
−
+
+
−
F
p24 ELISA (µg per Gag+ %)
64
16
4
1
0.25

**Figure 3.11: 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 3.10G. **(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 3.10H. **(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.

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

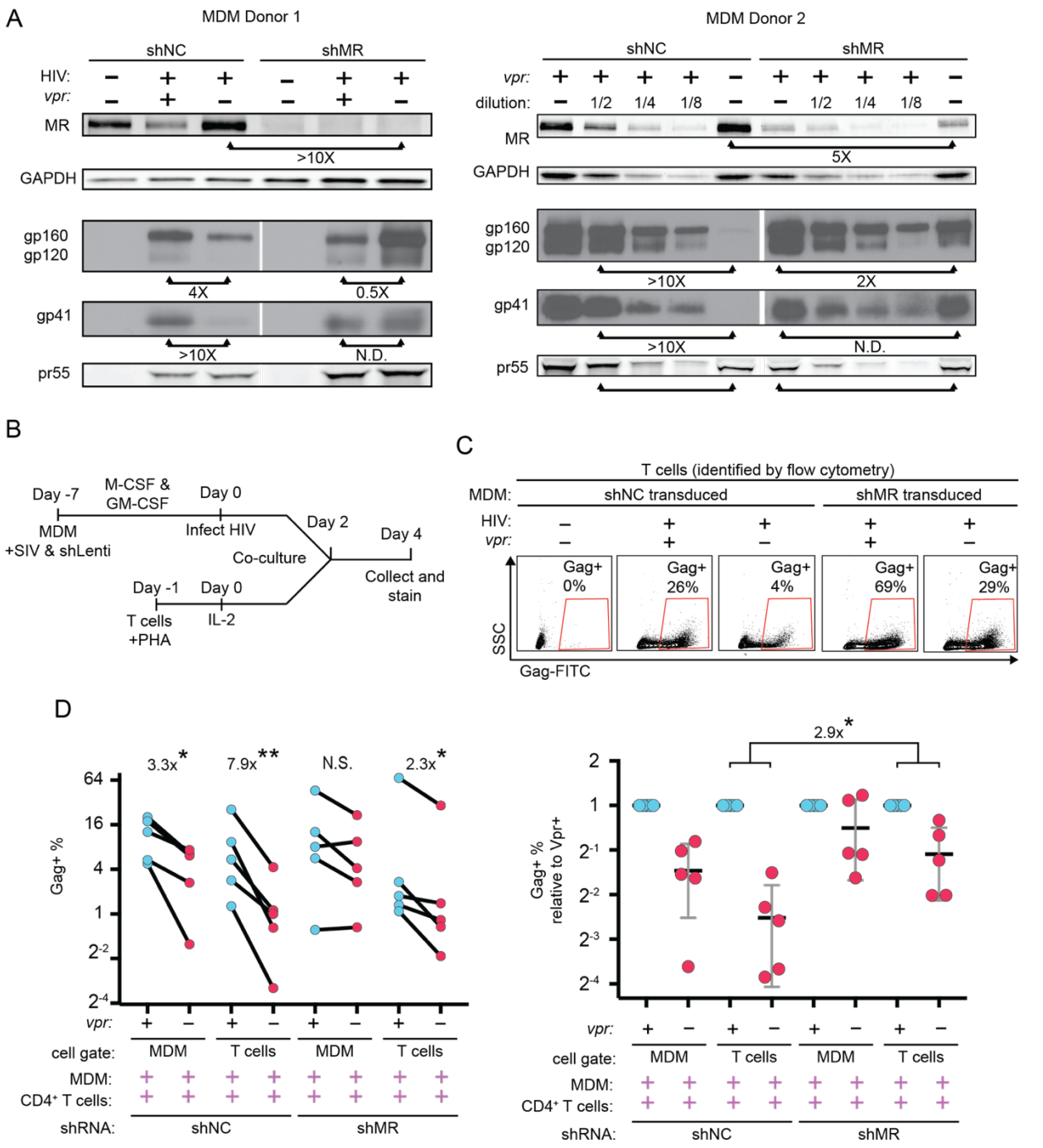


**Figure 3.12: Knockdown of MR enhances Env expression and spread to T cells in *vpr*-null infection of MDM[13]. (A)** Western blot analysis of MDM from two independent donors treated with the indicated silencing vector and infected with the indicated HIV for 10 days. The shRNA sequences encoded by the 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 ($Gag^+$) 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 3.13B. **(D)**

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

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.

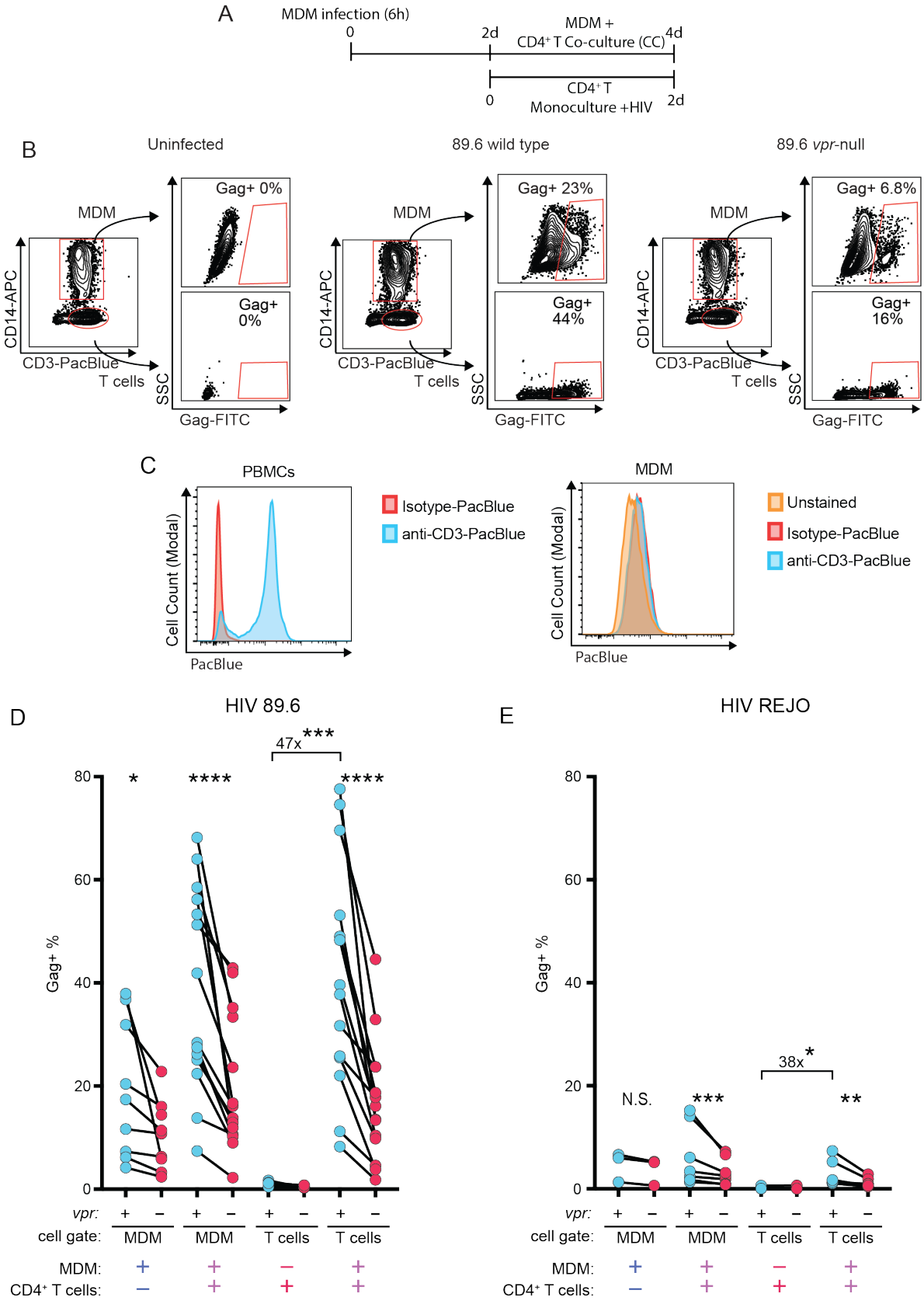
A
MDM infection (6h)
0
2d
MDM +
CD4+ T Co-culture (CC)
4d
CD4+ T
Monoculture +HIV
0
2d
B
Uninfected
89.6 wild type
89.6 vpr-null
MDM
CD14-APC
CD3-PacBlue
T cells
SSC
Gag-FITC
Gag+ 0%
Gag+ 0%
Gag+ 23%
Gag+ 44%
Gag+ 6.8%
Gag+ 16%
C
PBMCs
MDM
Cell Count (Modal)
PacBlue
Isotype-PacBlue
anti-CD3-PacBlue
Unstained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

**Figure 3.13: 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.

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

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# Chapter 4

# Discussion

## Summary of results

The results presented here are the latest addition to a long list of evidence for the importance of macrophages in HIV infection. This has included detection of HIV infected macrophages at numerous anatomical sites *in vivo* (Embretson et al., 1993; Ganor et al., 2019; Kandathil et al., 2018; Koenig et al., 1986) and evidence from cultures of monocyte derived macrophages *in vitro* (Crowe et al., 1994; Gendelman et al., 1988; Orenstein et al., 1988). Perhaps the most convincing line of evidence for the importance of macrophages in HIV infection comes from the conservation of HIV accessory proteins that enhance macrophage infection (Balliet et al., 1994). Vpr enhances infection of macrophages dramatically but has only a minor effect on the direct infection of T cells (Mashiba et al., 2014).

The results described in Chapter 2 highlight the importance of Vpr in macrophages by demonstrating that Vpr drives viral transmission to neighboring $CD4^+$ T cells. Earlier work had observed that Vpr enhances infection of $CD4^+$ T cells in human lymphoid tissue (Eckstein et al., 2001), but the exact mechanism by which this occurs was not known.

Separately other groups demonstrated that HIV efficiently transmits across virological synapses, connections that form when Env on an infected cell binds CD4 on a neighboring uninfected cell (Groot et al., 2008; Jolly et al., 2004; Schiffner et al., 2013). Our findings demonstrated that these two processes are intimately related. We observed that virological synapses formed between infected macrophages and uninfected CD4+ T cells, which led to significantly higher infection rates than incubation of cell-free HIV with $CD4^+$ T cells alone. HIV spread from macrophages to T cells was boosted by expression of Vpr. To ensure that the result observed in T cells was not simply due to Vpr's well established ability to enhance infection within macrophage cultures, we used pseudotyped virions to establish a one-way infection of macrophages in which HIV can spread from macrophages to T cells but not to uninfected macrophages. This allowed us to achieve an equal infection of wild-type and Vpr-null MDM, and under these conditions we still observed significantly more spread to T cells by the wild-type virus. To our knowledge this is the first demonstration that Vpr enhances T cell infection in short term assays. This finding explains earlier observations that Vpr was required to drive high infection and depletion of $CD4^+$ T cells in *ex vivo* human lymphoid tissue (Rücker et al., 2004).

The results presented in Chapter 2 detail the mechanism by which Vpr enhances cell-to-cell spread. We used confocal microscopy to identify virological synapses, points at which HIV Gag in an infected macrophage co-localizes with CD4 on an infected T cell at junctions between these two cells. Co-localization of Gag and CD4 was entirely dependent on Env, indicating that these were virological synapses and not immunological synapses. Vpr significantly enhanced expression of Env and this correlated with

enhanced formation of virological synapses. The effect of Vpr on synapse formation could be abrogated by addition of exogenous IFNα, suggesting that Vpr acts by preventing an interferon-mediated response. This is consistent with previous reports that Vpr prevents induction of type I interferons (IFNα and IFNβ) and MxA, an interferon stimulated gene (Laguette et al., 2014; Mashiba et al., 2014). Additional microscopy experiments demonstrated that in macrophages not expressing Vpr, Env-containing virions were targeted to lysosomes. Crucially, when Env was not present, virions were not observed in lysosomes, adding to the evidence presented by Mashiba et al. that Env is the proximal target of a macrophage-specific restriction factor.

Chapter 3 details multiple lines of evidence that indicate mannose receptor (MR) is the restriction factor that targets HIV Env and is counteracted by Vpr. In MDM cultures, MR expression was decreased by HIV infection and this effect was dependent on Vpr. Our findings demonstrated that unlike other host targets, e.g. UNG2, Vpr did not degrade MR via direct ubiquitylation using a host ligase complex, but rather decreased *MRC1* mRNA levels. Unexpectedly, when we investigated MR expression in individual cells by flow cytometry, we observed that Vpr-null virus decreased MR expression, although this effect was smaller than by wildtype virus. This partial phenotype indicated that MR was targeted by one or more additional HIV proteins. Because an earlier study indicated that Nef dysregulates MR trafficking (Vigerust et al., 2005), we tested the ability of a Nef-null mutant to decrease MR expression. We found that the Nef-null mutant produced partial downmodulation of MR similar to that displayed by the Vpr-null mutant. We then generated a Vpr-null, Nef-null double mutant, and found this virus had no effect at all on MR. This indicated that Vpr and Nef worked separately, but additively to, reduce MR

expression. A previous study had implicated HIV Tat in MR downmodulation (Caldwell et al., 2000), however, in our assay we did not observe any evidence of an effect by Tat in the absence of Vpr and Nef.

If MR is responsible for the restrictions observed in the absence of Vpr (Mashiba et al., 2014), and if Nef also downmodulates MR, we reasoned that Nef-null mutants should display defects in Env expression and virion release similar to those displayed by Vpr-null mutants. Indeed, we observed that in infection by Nef-null HIV Env expression and virion release was reduced compared to wildtype HIV. These effects were even more striking in a Vpr-null, Nef-null double mutant, which spread very poorly and produced much less Env than wild type virus.

These results led us to a model of restriction in which mannose receptor directly binds to Env, most likely via a structure of dense mannose-containing glycans known as the mannose patch (Coss et al., 2016; Sanders et al., 2002). We investigated two viral Envs that lack the mannose patch, YU-2 and a mutated form of 89.6 engineered to mimic YU-2. We observed that both Envs were less dependent on Vpr than wildtype 89.6 or NL4-3. These experiments also demonstrated that the mannose deficient form of 89.6 was significantly less infectious in MDM than wildtype 89.6. Infection of MDM by 89.6 Env could be competitively inhibition by D-mannan, a natural ligand of MR (Shibata et al., 1997), indicating that interactions between MR and Env facilitate HIV entry of MDM. This is consistent with a previous study that found that MR on the plasma membrane of macrophages binds HIV and passes the virus to T cells (Nguyen and Hildreth, 2003), but this study did not investigate cis-infection of macrophages themselves. Endocytosis and degradation of pathogens is a primary function of MR, so it is surprising that HIV can use

it to enhance infection. However MR has also been implicated in enhancing Dengue virus infection (Miller et al., 2008), indicating that at least one other virus can bind MR and evade degradation. Further work is needed to determine if MR's interaction with HIV Env affects HIV tropism and transmission.

To confirm that the factor responsible for restricting Env was MR, rather than another mannose binding lectin, we performed a series of experiments using MDM in which MR had been silenced. In these assays we observed that Env expression and spread from MDM to autologous $CD4^+$ T cells were no longer Vpr dependent. This provided conclusive evidence that MR is the restriction factor counteracted by Vpr. Because Nef counteracts the restriction factors SERINC3 and SERINC5 (Usami et al., 2015) and downmodulates HIV receptors that impede viral egress, we do not expect that knockdown of MR would rescue defects observed in Nef-null HIV infection. Nonetheless, our combined results indicate that Vpr and Nef work together to dramatically lower MR expression and facilitate viral spread within macrophages and from macrophages to T cells.

Because macrophages are present at mucosal surface of the genitals (Anderson et al., 2011; Ganor et al., 2013; Shen et al., 2009) and rectum (Smith et al., 2003), they may play an important role in sexual transmission of HIV. The ability of HIV isolated in early stages of infection to infect macrophages has a subject of considerable debate (Joseph and Swanstrom, 2018), although it has been clear for decades that HIV can be detected in macrophages at the earliest stages of infection (Popovic and Gartner, 1987). In recent years attempts to computationally reconstruct the genome sequences of transmitted HIV have produced a set HIV clones known as transmitted/founder (T/F)

viruses. One study investigated infection by T/F viruses and found that they replicated to higher titers in T cells than in macrophages (Ochsenbauer et al., 2012). However, in our assays comparing infection by the T/F clone REJO in mono-cultures of T cells, mono-cultures of macrophages, or co-cultures of both, we observed maximal infection in co-cultures followed closely by macrophages. T cells alone displayed the least infection, in contrast to the findings reported by Ochsenbauer et al.

A number of features of our co-culture assay may explain the difference between our findings and those described by Ochsenbauer et al. First, we investigated infection by measuring the fraction of cells expressing Gag 48 hours after inoculation rather than by supernatant ELISA over 14 days. This allows us to investigate very early events, which may be critical in establishing infection before immune effectors are activated. Second, our assays included a co-culture condition, which likely model physiological conditions more accurately. Finally, our protocol for isolation of monocytes and stimulation of macrophages differs significantly from Ochsenbauer et al. The stimulation conditions of MDM dramatically affect expression of surface proteins, including MR, CD4, and CCR5 (Lee et al., 1999). Given our finding that interactions between MR and Env enhance viral entry, further work is needed to determine if MR expression increases the infectability of macrophages. If so, earlier findings about the ability of HIV clones to infect macrophages may need to be re-tested in $MR^{hi}$ and $MR^{low}$ macrophages.

**Working Model**

Findings from other laboratories and from our own (Mashiba et al., 2014) presented significant evidence that Vpr enhances pathogenesis of HIV *in vivo* and

infection of primary macrophages cultures *in vitro*. Based on their observations we constructed a model in which Vpr evades the activation of an interferon response, which prevents activation of an unidentified host restriction factor. In the absence of Vpr, or when exogenous interferon is added, this restriction factor degrades Env and Env-associated virions, reduces virion release, and inhibits spread. Because the reduction in virion release is abrogated in ΔEnv infections, we proposed that Env is the direct target of the restriction factor and through Env the factor degrades the whole virion. Vpr-mediated evasion of the restriction factor is dependent on Vpr's association with DCAF1. Most likely Vpr uses DCAF1 to redirect the DCAF1-DDB1-Cullin4 ubiquitin ligase to target novel substrates.

The findings presented in this dissertation have allowed us to expand and refine this model considerably, most notably by increasing its scope to include viral spread from macrophages to T cells and identifying the factor that restricts Env (and therefore the entire HIV replication cycle). In the updated model (**Figure 4.1)**, MR binds Env via high-mannose glycans, especially the mannose patch, a structure formed by a high density of N-linked glycosylation sites in close proximity in the 3 dimensional structure of Env trimers (Checkley et al., 2011; Coss et al., 2016; Stewart-Jones et al., 2016). On the plasma membrane, the interaction between MR and Env enhances viral entry and infection, but when progeny virions are produced the interaction leads to degradation of Env, reduced formation of virological synapses, and reduced virion release. Combined these effects significantly hinder spread of HIV to new macrophages and $CD4^+$ T cells. To counter this restriction, HIV utilizes two accessory proteins. Vpr reduces transcription of *MRC1*, the gene that produces MR. We confirmed that Vpr-mediated reduction of MR expression

requires Vpr's interaction with DCAF1, and therefore likely involves ubiquitylation of a host factor, but that ubiquitylated factor is not MR itself. An earlier study by Vigerust et al. demonstrated that Nef dysregulates trafficking of MR, and our experiments demonstrated that the combined effect of Vpr and Nef dramatically lowers MR expression. When MR expression is low, Env expression is high, which allows formation of virological synapses and spread via direct cell-to-cell contact (**Figure 4.2**). Additionally low MR expression allows virions to be efficiently released into the supernatant. Combined these effects accelerate infection in cell culture and enhance pathogenesis *in vivo.*

**Future Directions**

The proposed model can continue to be refined and the relevance of the work increased by investigating broader implications of macrophage infection. The most important questions remaining about the model pertain to the mechanism by which Vpr reduces MR expression. We found that Vpr reduces steady state levels of *MRC1* mRNA, but there are several mechanisms by which Vpr might do this. It has been demonstrated that transcription of murine *MRC1* is controlled, at least in part, by the transcription factors PU.1, which is myeloid specific, and SP1, which is ubiquitous (Eichbaum et al., 1997). A study of the promoter of rat *MRC1* demonstrated that PU.1 and USF were required for transcription, but SP1 was not (Egan et al., 1999). The 400bp preceding the transcription start site of human MRC1 contains 3 PU.1 binding sites (TTCCT), one of which is conserved in both mouse and rat, suggesting it may be particularly important in gene regulation. Experiments to determine if the human promoter is bound and activated by PU.1, SP1, and USF would be highly informative. Western blots of lysates of infected

cells could detect Vpr-mediated changes in steady state levels of these transcription factors, but the TFs may not be abundant enough to detect. Assessing transcription of other genes known to be regulated by these factors may be a more sensitive way to detect Vpr-mediated changes in these transcription factors. A worthwhile target is macrophage colony stimulating factor (M-CSF), of which transcription is induced by PU.1 (Zhang et al., 1994). When transcription regulators have been identified, Vpr's ability to degrade them can be tested using straightforward transfection experiments similar to those in Chapter 3 that demonstrated UNG2 is directly degraded by Vpr, but MR is not.

Although a simple model in which Vpr directly degrades factors that control transcription of *MRC1* is appealing, it is possible that Vpr-mediated reduction is the result of the numerous changes in proteins that regulate cell cycle checkpoints and interferon signaling induced by Vpr. The mechanism by which Vpr induces cell cycle arrest has been studied extensively but is not completely understood. There is strong evidence that Vpr activates the ATR-mediated DNA damage response, which begins a signal cascade that arrests the cell cycle until the damaged DNA has been repaired. Several competing models for Vpr-mediated ATR activation have been proposed, but none demonstrated definitively. One provocative study found that Vpr uses DCAF1 and PLK1 to activate the SLX complex, specifically by interactions with the subunits SLX4 and MUS81 (Laguette et al., 2014). According to their model, in the absence of Vpr multiple copies of partially reverse transcribed viral cDNA genomes accumulate in the cell, which activates the ATR-mediated DNA damage response. However, when Vpr is present these partial genomes are degraded so ATR is not activated, which in turn prevents induction of type I interferons and induces cell cycle arrest. Other groups have presented data indicating that SLX4 and

MUS81 are not required for Vpr-mediated cell cycle arrest (Fregoso and Emerman, 2016; Zhou et al., 2016), but they did not investigate whether these host factors are required for Vpr-mediated evasion of interferon, which is arguably the more relevant phenotype. These studies, while informative, were performed in cell lines; given that many Vpr phenotypes we have described are macrophage-specific, confirmatory experiments in primary MDM would be highly informative. Previous work in our lab has confirmed that Vpr reduces steady state levels of PLK1 in MDM (Mashiba et al., 2014). We have also demonstrated in MDM that DCAF1 is required for Vpr to reduce MR expression. Using MR expression as a read out, future experiments can quickly determine if the other host factors identified by Laguette et al. (PLK1, SLX4 and MUS81) are part of this pathway. As the determinants for Vpr's interactions with PLK1, SLX4 and MUS81 are not currently know, gene knockouts similar to those we employed for MR will be required.

In addition to providing clues about Vpr, these studies would illuminate our knowledge of MR expression more broadly. As MR is required for collagen recycling, innate immunity, antigen presentation (especially cross presentation) and infection by some intracellular pathogens, the regulation of its expression is of interest to many aspects of human health. Methods to increase MR activity could be beneficial for treating certain infections or establishing immunity by vaccination. Methods to reduce MR expression could be used to treat infections of pathogens that enter cells using MR, such as Dengue virus. Using monocyte derived macrophages, one group has already demonstrated that vitamin D3 reduces MR expression on macrophages, which led to restricted infection by Dengue virus and reduced production of TNF-$\alpha$, IL-1$\beta$, and IL-10 (Alzate et al., 2017). Pro-inflammatory TNF-$\alpha$ has been implicated as a primary mediator

of Dengue-induced hemorrhage (Chen et al., 2007; Dewi et al., 2004), indicating that interventions to reduce its production could improve patient outcomes. Given that HIV is a chronic infection, interventions to modulate MR in order to reduce infection are likely not feasible. Short term, targeted interventions, at the vaginal epithelium for example (Fanibunda et al., 2011; Greenhead et al., 2000), could be useful to inhibit transmission.

Our data in chapter 3 provide strong evidence that MR can enhance HIV infection of MDM, an unexpected result that warrants further investigation. Earlier reports indicated that MR mediates entry of HIV to astrocytes (Liu et al., 2004; Trujillo et al., 2007) and spermatozoa (Cardona-Maya et al., 2006). Of these, Trujillo et al. did not observe signs of productive infection, but the other two groups did. To our knowledge, our experiments provide the first conclusive evidence that MR promotes productive infection of MDM. The most likely mechanism for MR-enhanced infection is that Env's interaction with MR, which is highly expressed on macrophages (Stahl et al., 1980), brings HIV virions in close proximity to the plasma membrane and thus increases the odds that Env binds CD4, which is expressed at low density on macrophages (Joseph et al., 2014). This model is supported by an earlier report that fleeting association between virions and cell membranes reduced infection in cell culture systems (Platt et al., 2010). The first step to testing this model is to determine if MR-enhancement of infection is dependent on CD4 and CCR5/CXCR4, using competitively inhibitory antibodies and small molecules (e.g. AMD3100 and maraviroc). If this infection is mediated through the classical CD4 entry pathway, we would expect that MR-mediated enhancement is most beneficial on cells expressing low levels of surface CD4. This can be tested using affinofile cells, 293T cells that express CD4 and CCR5 under the control of inducible promoters (Chikere et al.,

2013). Cells can be induced to express low or high CD4 and then transiently transfected with MR to determine if MR-mediated enhancement is more important in conditions in which CD4 is limiting.

If these experiments confirm that high MR expression can enhance infection of cells expressing low CD4, conclusions about macrophage tropism may need to be re-evaluated. It has been demonstrated that Envs from different HIVs vary significantly in their affinity for CD4. In vitro, some Envs infect cells with high CD4 well and cells with low CD4 poorly or not at all. Other Envs infect cells with low CD4 only slightly less efficiently than cells with high CD4, indicating they have high CD4 affinity (Arrildt et al., 2015). Envs with high CD4 affinity are often found in the CNS (Martín-García et al., 2006; Schnell et al., 2011) and at late stages of infection (Arrildt et al., 2015). Because macrophages express relatively low amounts of CD4, this has led some to conclude that "macrophage tropic" HIV only emerges late in infection and at sites that are irrelevant for transmission. However, this is discordant with *in vivo* observations of infected macrophages throughout the course of HIV infection (Gartner et al., 1986; Schuitemaker et al., 1991; Smith et al., 2003), indicating that virus that is categorized as "not macrophage tropic" by *in vitro* assays readily infects macrophages *in vivo*.

Infection of macrophages by HIVs that are categorized as "not macrophage tropic" can be explained in several ways. This may be due to infection through virological synapses, which is much more efficient than transfer of cell-free virus (Duncan et al., 2014; Groot et al., 2008; Jolly et al., 2004), but intriguingly transfer from infected $CD4^+$ T cells to uninfected macrophages via a virological synapse has never been demonstrated (Baxter et al., 2014; Bracq et al., 2018). The reverse, HIV transfer from infected

macrophages to uninfected $CD4^+$ T cells, has been widely documented and characterized by many earlier reports (Bracq et al., 2018; Duncan et al., 2014; Gousset et al., 2008) and by experiments described in Chapter 2 of this dissertation. Simple experiments to determine if and under what conditions $CD4^+$ T cells transmit HIV via virological synapses to macrophages would determine if this method of spread is likely *in vivo*. Another possible route of infection of macrophages is through phagocytosis of infected $CD4^+$ T cells (Baxter et al., 2014). Finally, it is possible that in vitro assays which rely on MDM do not accurately reflect the ability of HIV to infect macrophages *in vivo*. This may be because tissue resident macrophages, i.e. those of non-monocytic origin, are more susceptible to infection than MDM. Due to the inaccessibility of tissue resident macrophages, they have not been characterized as completely as MDM and are not used for routine tropism assays. Additionally, the various stimulation conditions used to generate MDM affect expression of CD4 and CCR5 (Lee et al., 1999) and of MR (our own unpublished results), which presumably affects susceptibility to HIV, although this has not been formally demonstrated. The protocols of different laboratories vary in the cytokines added, concentrations of those cytokines, serum used and length of stimulation. A set of experiments to determine the degree to which these parameters affect HIV infection could help the field adopt a standard approach. Given the disconnect between *in vitro* assays, which report HIV rarely infects macrophages, and *in vivo* studies, which find numerous infected macrophages, MDM protocols that generate greater HIV infection may be more physiologically relevant.

## Conclusions

In combination, the findings presented in this dissertation provide significant insight into the role of macrophages in HIV infection. It establishes MR as an unusual hybrid of entry factor and restriction factor, which the virus uses to establish infection but must disable to permit egress. The importance of efficient viral egress is underscored by the finding that HIV encodes two accessory proteins, Vpr and Nef, to counteract MR-mediated restriction. Perhaps most importantly, these findings demonstrate that although Vpr counteracts a restriction factor that is only expressed in macrophages, this function boosts infection of CD4+ T cells as well. By demonstrating that Vpr has a robust and immediate effect on T cell infection, this work provides a rationale for conservation of Vpr. It has identified new targets for future research and potential interventions that may reduce the pathogenesis and transmission of HIV.

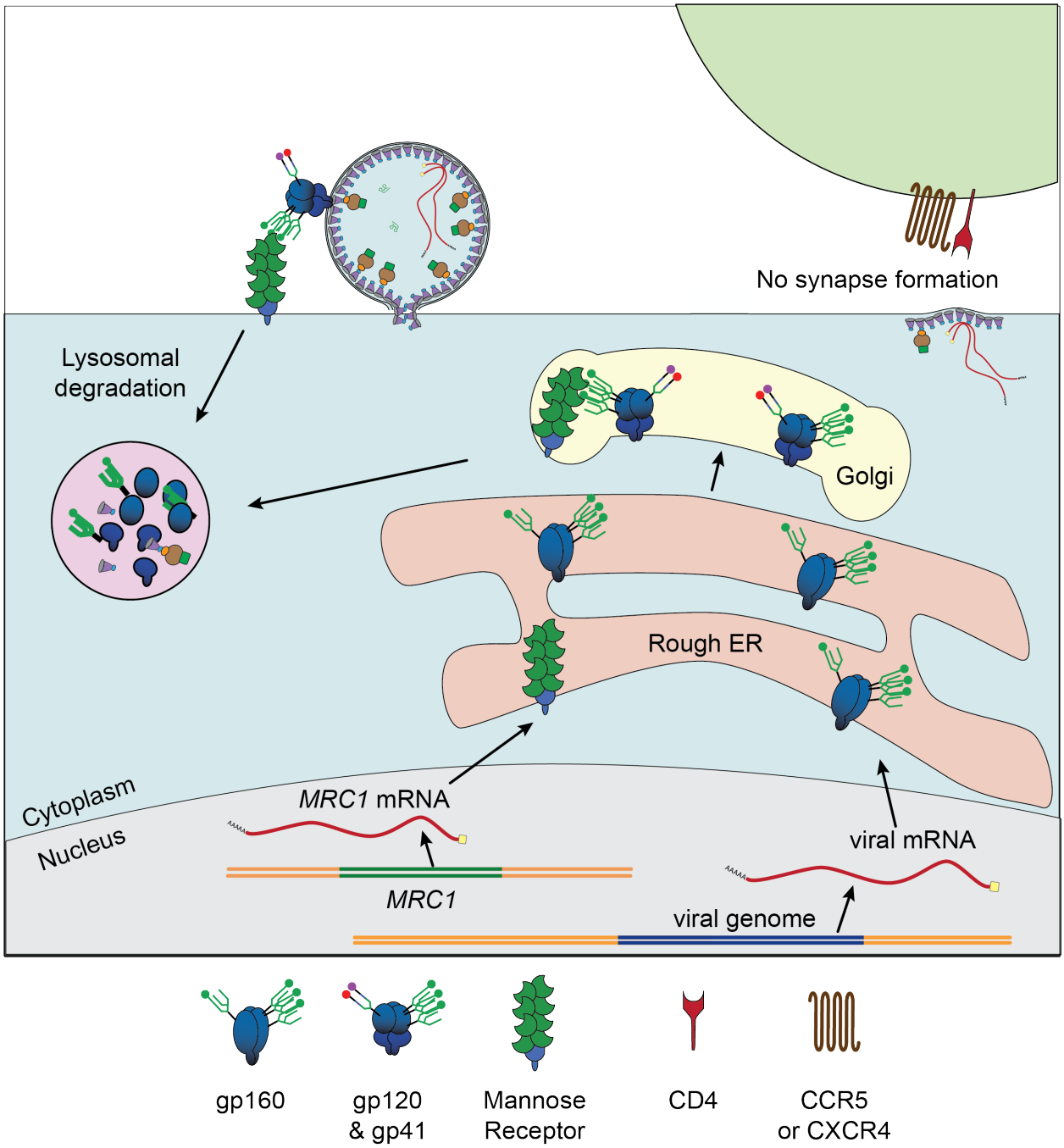


**Figure 4.1: HIV restriction by MR[1].** Graphical depiction of our proposed model for HIV restriction by MR in macrophages. In this model MR binds Env directly via high mannose glycans in Env. This causes Env and Env-associated virions to be degraded in the lysosome. Due to low levels of Env on the cell surface, virological synapses do not form, which inhibits spread to $CD4^+$ T cells.

[1] This figure was generated by Jay Lubow.

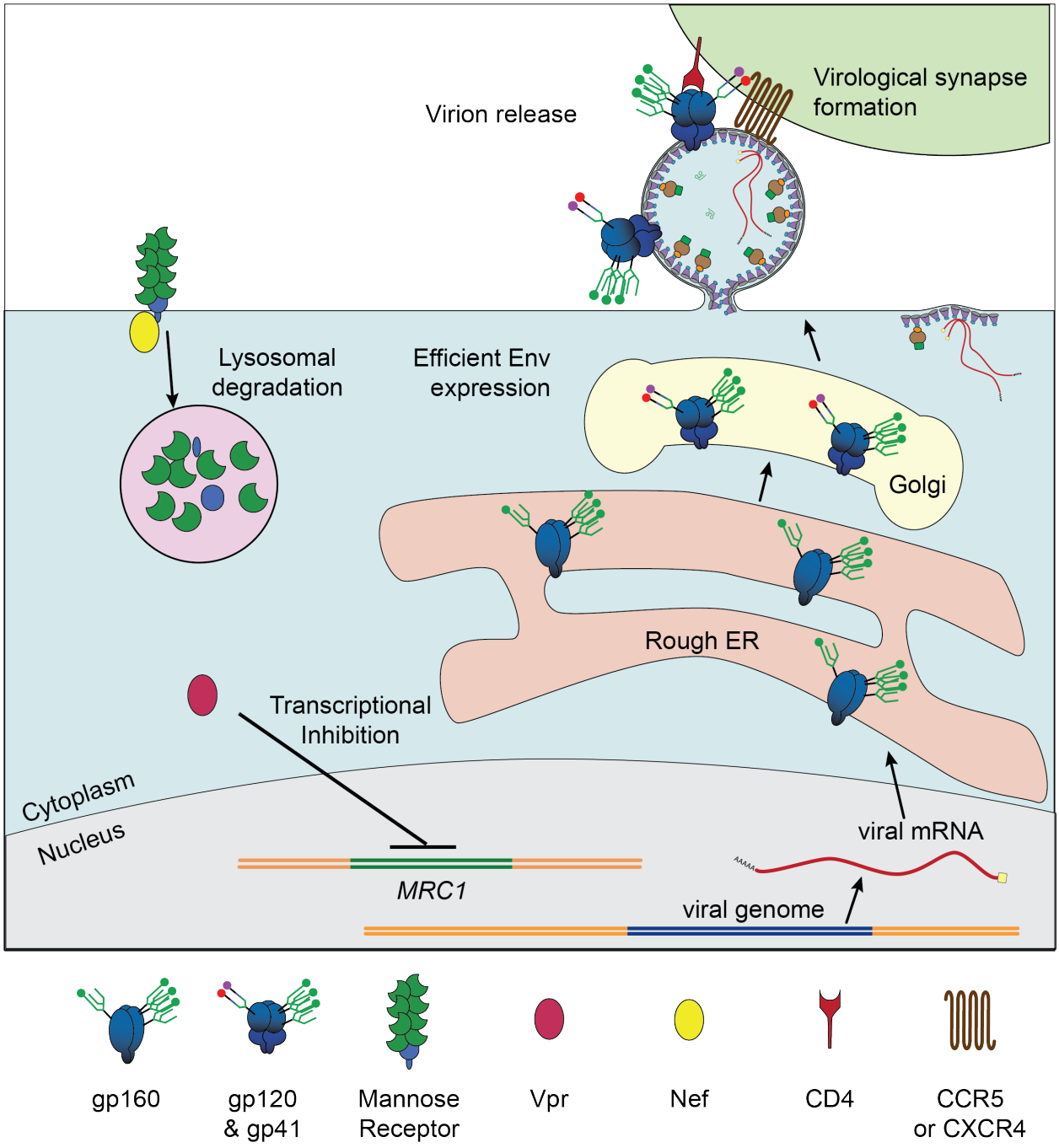


**Figure 4.2: Vpr and Nef mediated downmodulation of MR lifts restriction of HIV in macrophages[2].** Graphical depiction of our proposed model in which Vpr reduces transcription of *MRC1*, the gene that produces MR, and Nef binds to MR to remove it from the cell surface. Low MR expression allows for high Env expression, efficient virion release, and formation of virological synapses between infected macrophages and uninfected $CD4^+$ T cells.

[2] This figure was generated by Jay Lubow.

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