ROLE OF HIV-1 VPR ON HOST-CELLULAR FUNCTIONS: CELL-SPECIFIC ANALYSIS IN PRODUCTIVELY-INFECTED MACROPHAGES

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ABSTRACT

Progression of human immunodeficiency virus type 1 (HIV-1) pathogenesis impedes on the patient's ability to combat foreign pathogens by infecting immune cells, a disease commonly referred to as acquired immunodeficiency syndrome (AIDS). In addition to the onset of AIDS, HIV-1 is known to cause other health issues over time, including cardiovascular disease, premature aging, neurocognitive impairment, and dementia. The quality of life for HIV-1-positive patients has drastically increased with the introduction of highly active anti-retroviral therapies (HAART). Though the use of HAART treatments lower the incidence of HIV-1-associated comorbidities, including dementia, more understanding on HIV-1-associated comorbidities is necessary to help further improve the quality of life for HIV-1-positive patients, which is of considerable public health significance.

This study focuses on the role of the HIV-1 accessory protein viral protein R (Vpr) in HIV-1 pathogenesis. Previous studies in our laboratory have established a role for Vpr in HIV-1 immunopathogenesis and neuropathogenesis, particularly in the modulation of cytokines and chemokines, including IL-8 and IL-1β, in monocyte-derived macrophages and dendritic cells. These proinflammatory cytokines were additionally found to cause immune dysregulation and neuronal injury. As a result, this study focuses on understanding the origin of these cytokines within subpopulations of monocyte-derived macrophages: productively-infected macrophages that are actively producing virions and non-productively-infected macrophages, which include latently-infected macrophages, uninfected macrophages, and macrophages exposed to virus

particles and viral proteins. In order to distinguish between the two groups, reporter viruses were created that are capable of infecting macrophages and expressing the enhanced green fluorescence protein (EGFP) upon replication. Productively-infected macrophages were successfully identified from non-productively-infected macrophages by utilizing EGFP expression, which allowed for cell-specific analysis of cytokine expression within the macrophage subpopulations. Productively-infected macrophages yielded a decrease in IL-8 and IL-1β when infected with Vpr-deficient (ΔVpr) reporter viruses, while non-productively-infected and exposed macrophages showed no noticeable difference. Similarly, a virion association defective mutant of Vpr (Vpr-A30L) resulted in lower amounts of IL-8 expression. These results elucidate the distinct role of Vpr in IL-1β and IL-8 expression in the virion-associated forms and during *de novo* synthesis in productively-infected macrophages.

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PREFACE

First and foremost, I would like to thank my advisor, Dr. Velpandi Ayyavoo, for all of her guidance, patience, and support that she has given me throughout my time in her lab. Even during times of frustration throughout my project, she patiently encouraged me to think critically and innovatively, to expand my knowledge, and to put 100% effort into my work. I always knew I could come to her with any question or problem that I had, which was so helpful to me during my lab work. Her zeal and dedication to her research has motivated me time and time again to become a better scientist. I would also like to thank my thesis committee members, Dr. Jeremy Martinson and Dr. Peter Di, for serving on my committee and providing invaluable advice and suggestions. Additionally, I would like to thank Mary White and the Gupta lab for running my p24 ELISA samples for each of my experiments. I'd also like to thank LuAnn and the rest of the IDM flow cytometry core staff for training me and tolerating my use of their flow cytometers.

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1.0   INTRODUCTION

The current estimation for people worldwide infected with HIV-1 (human immunodeficiency virus type 1) is 34 million in 2012 [1]. HIV has been isolated and characterized as the causative agent of acquired immune deficiency syndrome (AIDS), a disease which has caused a global pandemic that gained momentum throughout the early 1980's and peaked in the late 1990's [2-4]. HIV-1/AIDS-related research has led to crucial breakthroughs in prolonging the life expectancies of HIV-1 -positive individuals: production and access to highly-active antiretroviral therapies (HAART) has played a tremendous role in reducing the mortality rate associated with HIV/AIDS. Though preventative measures have been widely established through awareness and safer sexual behaviors, the creation of a successful vaccine or microbicide has yet to occur. [5] Since prevention of infection provides the most practical solution to the HIV/AIDS pandemic, continued efforts are needed for novel HIV preventative measures.

1.1 THE PATHOLOGY OF HIV/AIDS

1.1.1 HIV infection and clinical progression to AIDS

HIV targets the host immune cells to establish infection, causing a progressive depletion of the immune cells, specifically CD4+ T cells, necessary to fight infection, as well as viral propagation through the manipulation of cellular functions that comprise the immune system. HIV-1 primarily establishes infection in CD4+ T helper lymphocytes and macrophages. In the following weeks after initial infection, the patient enters acute infection. During this period, high amounts of virus are produced and CD4+ T cell apoptosis is abundant, thus diminishing the patient's CD4+ T cell count. Viral reservoirs in tissues and lymphoid organs are established during the acute phase. Once the immune response to infection begins, the viral load significantly drops to the "viral setpoint" and the CD4+ T cell count begins to rebound, but does not fully recover.

After the resolution of the acute phase, the patient enters clinical latency, in which the virus replicates at very low levels for 8 to 15 years depending on how well the patient respond to therapy. Currently, highly active antiretroviral therapy (HAART) is used to lower viral burden and facilitate the maintenance of HIV-1 latency. During this period, drug resistance mutations develop due to virus replication in less accessible sites and eventually, the viral load rises and the CD4+ T cell count drops. Once the patient reaches less than 200 CD4+ T cells/mm^3, the disease progression status is classified as AIDS. The progression to AIDS indicates that the immune system has been severely compromised. AIDS-related fatalities usually occur through opportunistic infections that would be cleared by a healthy immune system [6].

1.2   THE HIV LIFE CYCLE

1.2.1   Virus entry and establishment of infection

The human immunodeficiency virus-1 belongs to the *Lentivirus* genus of the Retroviridae family. HIV-1 virion includes two single-stranded RNA molecules, structural proteins and several enzymatic proteins necessary to establish infection. The capsid of the virion is enveloped with a lipid bilayer, which also harbors viral envelope proteins. HIV infects its target cells through the utilization of the surface viral envelope glycoprotein gp120. gp120 interacts with the CD4 antigen that is present on target cells, along with one of two potential chemokine co-receptors: CXCR4 or CCR5. The interactions between viral gp120 and host CD4 stabilize the virus-host binding to allow for the secondary interactions between gp120 and a co-receptor.   These interactions mediate a conformational change in the viral transmembrane protein gp41 that allows for membrane fusion and capsid entry into the cell [7-10].

Once the capsid has entered into the host cytosol, the preintegration complex (PIC) is formed. The PIC is comprised of capsid *gag* proteins (p24 and matrix proteins), the accessory protein viral protein R (Vpr), integrase, reverse transcriptase, and two copies of the single-stranded RNA viral genome. Release of the PIC into the host cytosol begins the process of reverse transcription, yielding a double-stranded proviral DNA product that is capable of integration. The PIC moves through the nuclear envelope and enters into the nucleus via active transport through the nuclear pore complex. Once the PIC enters the nucleus, the proviral DNA is integrated into the host genome, thus establishing infection and allowing for initiation of virion production [11-12].

1.2.2   The HIV-1 genome

The HIV genome is comprised of several essential structural genes (*gag, pol,* and *env*), two essential regulatory elements (*tat* and *rev*), and multiple accessory genes (*vif, vpr, vpu* and *nef*). These genes are flanked by long terminal repeats (LTRs), which contain the necessary promoters and signals to allow for viral gene expression. These signals include sequences that allow for transcription initiation, termination, and poly-adenylation, as well as elements that direct reverse transcription.
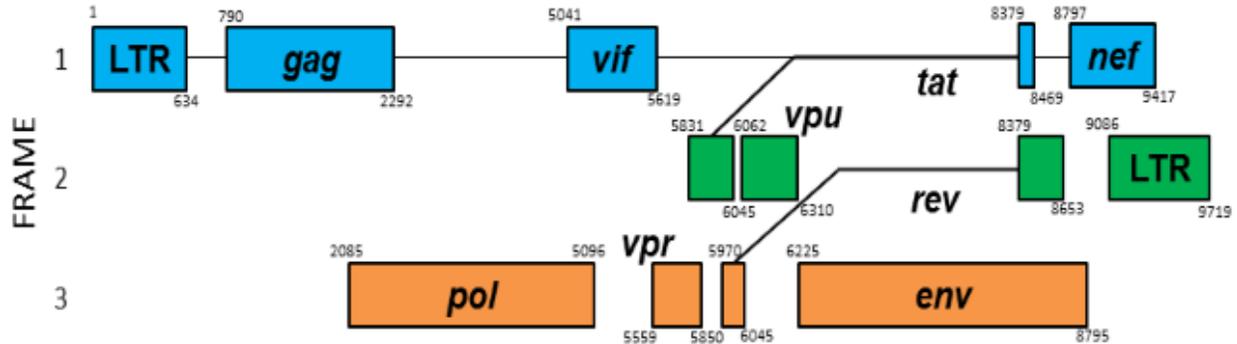
Figure 1. HIV gene organization.

The genomic organization schematic in Figure 1 depicts the reading frames that are necessary to produce each of the viral proteins [13]. The largest genes, *gag, pol*, and *env,* encode for the structural proteins in the virion. *gag* transcription results in the Gag polyprotein, which is cleaved to produce the capsid (CA), matrix (MA), and nucleocapsid (NC) proteins that encapsulate the virion. *pol* transcription yields an enzymes with multiple functions, including integrase (IN), protease (PR), and reverse transcriptase (RT) with RNase H activity. *env* transcription is responsible for the envelope protein that resides in the membrane; the transcribed proteins form a

heterotrimer that binds to the host CD4 glycoprotein and the chemokine co-receptor for viral entry. A *gag-pol* polyprotein is also produced in low levels, indicated by the presence of *pol* enzymes in the virion structure.

The regulatory elements, Tat and Rev, are necessary for viral transcription and protein translation. After viral integration, low levels of viral RNA transcripts are initially produced, which produces small numbers of Tat. Tat functions by enhancing the rate and efficiency of viral transcription through binding to a RNA hairpin structure at the 5' end of viral transcripts called the trans-activating response element (TAR). Once bound to TAR, Tat also interacts with RNA Polymerase II, thus upregulating polymerase efficiency [14]. The regulatory element Rev controls the nuclear export of unspliced and partially spliced viral transcripts, thus regulating the production of viral genomes and structural proteins. In the absence of Rev, viral transcripts remain in the nucleus and are fully spliced, only producing Rev, Tat, and the accessory protein Nef. Rev acts by binding to the Rev response element (RRE), a 351-nucleotide RNA sequence encoded within the unspliced *env* gene. Binding to the RRE exports unspliced and partially-spliced viral transcripts into the cytosol prior to complete splicing, allowing for translation of structural proteins and viral RNA genome for virion incorporation [15].

The HIV accessory genes, including *vpu, vpr, vif,* and *nef*, are not absolutely essential for infection but play a major role in augmenting infection and replication efficiency. Briefly, Vpu aids in the degradation of CD4 in infected cells; Vpr is present in the preintegration complex and regulates nuclear import; Vif interrupts the antiviral activity of the host restriction factor APOBEC3G; and Nef also aids in the degradation of CD4, as well as interrupts in cellular signal transductions [16].

1.2.3   Viral tropism

Tropism of HIV-1 limits infection to host cells that express both the CD4 glycoprotein and a chemokine co-receptor, CXCR4 or CCR5. Cells that express these receptors, and are susceptible to HIV-1 infection primarily include CD4+ T cells, monocytes, macrophages, and dendritic cells. Isolates of HIV-1 display different capabilities of tropism, which can be categorized into three groups: X4-tropic viruses, which are capable of infecting cells expressing the CXCR4 co-receptor; R5-tropic viruses, which infect CCR5-expressing cells; and dual-tropic viruses, which can infect both CXCR4-expressing and CCR5-expressing cells [17]. R5-tropic isolates are known to preferentially infect and establish infection in the asymptomatic phase of disease progression. These isolates are highly predominant during acute infection, denoting their importance in transmission [17-19].

The high mutation rate of reverse transcriptase during viral replication allows for the evolution toward CXCR4-tropic viruses as infection progresses. The affecting mutations primarily occur within the *env* gene, which yields structural changes in gp120 and gp41 that are involved in viral tropism evolution [19]. X4-tropic HIV-1 isolates begin to emerge after seroconversion, during the progress towards AIDS [20]. The emergence of X4-tropic isolates has been associated with higher rates of replication and faster disease progression, correlating with the onset of AIDS [21]. The tropism of HIV-1 isolates during *in vivo* infection does not necessarily utilize CXCR4 or CCR5 exclusively: Macrophage-tropic (M-tropic) and T cell-tropic (T-tropic) viruses are both capable of infecting both CD4+ T cells and macrophages, respectively [22]. Despite the dual-tropic nature of *in vivo* infection, co-receptor tropism can be utilized as a general indicator for patient disease progression, with approximately 50% of HIV patients progressing to AIDS through the emergence of X4-tropic quasispecies [17, 21].

1.3   HIV PATHOGENESIS IN MACROPHAGES

1.3.1   Role of macrophages in HIV-1 infection

Macrophages are monocyte-derived, mononuclear leukocytes that are primarily responsible for phagocytosis and antigen presentation, which provides a link between the innate and adaptive immune responses. These cells are not capable of proliferating and primarily reside in the tissues after terminal differentiation from monocytes [23]. Differentiation from a monocyte into a macrophage increases the cell's susceptibility to HIV-1 infection, particularly due to increased CCR5 expression on the cell surface. Macrophages play a crucial role in infection establishment, maintenance and latency by allowing for viral replication and virion release, thus termed "productive" infection [24].

Infection of macrophages does not lead to the cytopathic and apoptotic effects that are characteristic to CD4+ T cell infection, which facilitates a long-lived reservoir for virus production. Several factors are associated with sustained macrophage infection, which lead to the noticeable lack of cytopathic effects typically observed in HIV-infected CD4+ T cells [24]. HIV-infected macrophages express increased levels of NF-κB, a transcriptional regulator responsible for cytokine production and cell survival. Increased NF-κB levels abrogate the TNF-induced apoptosis, which is the archetypal signaling pathway for T cell apoptosis in HIV-1 infection [25]. Such modulations in NF-κB expression may be a direct causation of viral protein interactions in macrophages: Olivetta et al. (2003) concluded that soluble Nef may play a role in the activation of NF- κB in monocyte-derived macrophages through inhibitory interactions with the endosomal

V-ATPase [26], while Niederman et al. (1992) found that Nef participates in the downregulation of NF- κB expression in CD4+ T cells [27]. Similarly, Ayyavoo et al (1997) determined that Vpr regulates NF-κB activity in CD4+ T cells dependent on TCR-mediated activation [28]. Additionally, Swingler et al. (2007) found that gp120 interactions induced expression of macrophage colony-stimulating factor (M-CSF), a pro-survival cytokine that downregulates tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and thus TNF-induced apoptosis [29]. The prevention of apoptosis in macrophages results in the formation of a long-lasting viral reservoir capable of producing infectious virions and facilitates persistent infection *in vivo*.

Infected macrophages also contribute to the persistence of HIV-1 by enhancing viral spread to bystander cells, as well as increasing viral permissiveness through viral protein expression. Macrophages infected with HIV-1 release higher levels of CC-chemokines, particularly MIP-1α and MIP-1β, which attract CD4+ and CD8+ T lymphocytes near infection via chemotaxis [30]. Similarly, macrophages are involved in targeting CD4+ T lymphocytes for viral infection: Swingler et al. (2003) determined that the Nef-dependent secretion of soluble CD23 and ICAM from HIV-infected macrophages resulted in increased B-cell— T-cell interactions, leading to T-cell activation and elevated viral permissiveness [31]. Other viral proteins are also capable of increasing viral susceptibility: soluble Vpr has been associated with host permissiveness through its interactions with cellular proteins, including the glucocorticoid receptor and the p300/transcription factor initiation complex [32]. Such interactions modulate cellular genes, which increase the host cell's susceptibility to HIV.

Additionally, macrophages play an important role in the maintenance of infection during the course of antiviral therapy. Tissue-resident macrophages are capable of obscuring HIV-1 replication in sanctuary sites that are poorly penetrated by current anti-HIV pharmacologic agents.

This allows for maintained levels of HIV replication and virion release in areas including the lymphoid tissues, testis, gut-associated lymphoid tissue, and brain. The lack of antiviral treatments capable of reaching infected sanctuary site-resident macrophages allow for the perpetuation of HIV infection in the host [33].

1.3.2 Importance of cytokine expression in HIV-infected macrophages

Cytokines are small molecules responsible for alterations in cellular signaling and function via autocrine and paracrine mechanisms. These signaling molecules function by binding to corresponding cytokine receptors on the cellular surface, which results in the induction of signal transduction pathways and thus results in modulations in gene transcription and activation. Cytokines are responsible for the regulation of both innate and adaptive immune responses, as well as the maturation, differentiation, and activation of T lymphocytes. These signaling molecules are highly redundant and multifunctional due to the structural similarities of cytokine receptor subunits, which allow multiple cytokines of the same family to bind to the same cytokine receptor. The term "cytokine" comprises a large group of molecules that are diverse in origin and function, including interferons, interleukins, mesenchymal growth factors, the tumor necrosis factor (TNF) family, and chemokines. Chemokines play a unique role in immune responses to an invading pathogen: they act as chemoattractants to induce migration of surrounding immune cells to the site of inflammation or infection [34].

HIV-1 infection modulates cytokine production in order to promote establishment of infection and viral replication. Since HIV-1 targets cells of the immune system and causes persistent infection, HIV infection results in immune activation and thus cytokine production. In

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particular, proinflammatory cytokines TNF-α, IL-1β, and IL-6 exhibit increased expression in HIV-1 infected PBMC populations [35]. Modulations in cytokines that regulate the T helper response have also been observed: increases in T_H2-related cytokines, such as IL-4, IL-5, and IL-10, and decreases in T_H1-related cytokines, including IL-2 and IFN-γ, suggest a role in a regulatory switch in immune response upon HIV infection [36-38].

Conversely, the production of cytokines as an immune response can also suppress or induce HIV-1 replication, which is dependent on the tightly-regulated balance of secretion within the cytokine network. Several cytokines are responsible for the modulation of HIV-1 replication and have distinct roles in different cell types. Cytokine effects on HIV replication in monocyte-derived macrophages can result in induction, suppression, or bi-functionality, as detailed in Table 1 [39, 40]. Due to the autocrine and paracrine nature of cytokines, the combination of expressed stimulatory or inhibitory cytokines can impact the susceptibility of bystander macrophages to infection. Thus, cytokine-related immune responses play an integral role in the establishment of HIV infection and disease progression.

Table 1. Effects of cytokines on HIV-1 replication in monocyte-derived macrophages.

Cytokine	Function	Effect on replication
M-CSF	Stimulatory	Increase
IL-1α, IL-1β	Stimulatory	Increase
IL-6	Stimulatory	Increase
TNF-α, TNF-β	Stimulatory	Increase
IFN-α, IFN-β	Inhibitory	Decrease
GM-CSF	Inhibitory	Variable
IL-10	Inhibitory	Decrease
MIP-1α, MIP-1β, RANTES	Inhibitory	Decrease
IL-4	Bifunctional	Variable
IFN-γ	Bifunctional	Variable

Compiled from Kedzierska et al, 2003 [39] and Koyangi et al, 1996 [40].

Stimulatory cytokines that facilitate HIV replication have been implicated in the activation of the NF-κB pathway, thus leading to the up-regulation of a multitude of cellular genes. Most notably, TNF-α activates NF-κB expression, which significantly increases viral replication via NF-κB binding sites within the LTR of the HIV-1 genome. TNF-α binds to two receptors, TNF-R1 and TNF-R2, which activates the signal transduction pathway responsible for NF-κB expression [25]. Increases in TNF-α levels have been observed both *in vitro*, with HIV-1-infected MDM cultures, and *in vivo*, with HIV-infected patient sera samples [41]. TNF-α is expressed constitutively upon HIV-1 infection and has been shown to perpetuate production through an autocrine positive feedback loop [42].

Similarly, IL-1β is a pro-inflammatory cytokine that also increases viral replication. IL-1β binds to the interleukin 1 receptor (IL-1R), which leads to the up-regulated transcription of TNF-α, IL-6, IFN-γ, and the neutrophil chemoattractants CXCL1 and CXCL2. IL-1β transcription is regulated by NF-κB, NF-IL6/CCAAT enhancer-binding protein (C/EBP), and cAMP response element-binding proteins (CREB), and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), which are activated by HIV infection [43, 44]. Because NF-κB is a regulatory factor of IL-1β production, expression of TNF-α and IL-1β forms a positive feedback loop, thus potently enhancing HIV replication. Studies have shown that blocking TNF-α and IL-1β with neutralizing antibodies, along with other stimulatory cytokines, leads to a decrease or complete suppression of viral replication [45, 46].

In contrast, inhibitory cytokines are necessary to compensate for the potent enhancement of HIV-1 replication by stimulatory cytokines. The beta-chemokines, MIP-1α, MIP-1β, and RANTES, are chemoattractants produced by CD8+ T lymphocytes and macrophages that utilize the CCR5 chemokine receptor. Binding of β chemokines to the CCR5 receptor blocks R5-tropic

HIV isolates from entering macrophages and establishing infection [30, 47]. Cocchi et al (1995) demonstrated that treatment of MDMs with the β chemokines prevents viral entry and uncoating in a dose-dependent manner [48]. In order to circumvent this suppression, viral proteins are known to counteract viral suppression through down-regulating inhibitory cytokine expression: soluble Vpr suppresses the production of β chemokines in primary lymphocytes and MDMs, which enhances viral replication [49]. Other factors, such as IL-10, primarily act through the direct inhibition of stimulatory cytokine production [50].

1.3.3 The role of HIV-1 Vpr accessory protein in macrophage infection

The Vpr accessory protein is a small, 96 amino acid protein that has a molecular weight of 14 kDa. Vpr is highly conserved among HIV-1, HIV-2, and simian immunodeficiency virus (SIV). As shown in Figure 2, the structure of Vpr contains an N-terminal domain (residues 1-16), three alpha helices (residues 17-33; 38-50; 55-77), and a C-terminal domain (residues 78-96), as determined by NMR analysis of monomeric Vpr [51, 52]. The folded tertiary structure of Vpr is moderately unstable, particularly due to the two patches of exposed hydrophobic residues on alpha helices 1 and 3. Therefore, Vpr undergoes extensive oligomerization and interacts with various proteins to obscure its hydrophobic residues from its aqueous surroundings [53, 54]. Vpr exists as dimers, trimers, or higher orders of oligomers depending on the concentration, plays a crucial role in its ability to function. Mutational analyses of Vpr have identified specific regions of the Vpr structure that are responsible for its numerous functions.
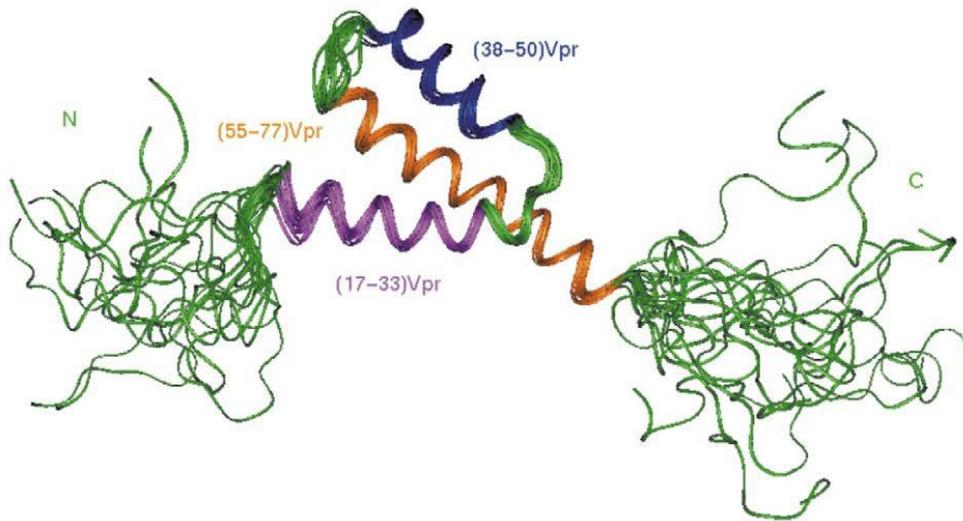
Figure 2. The structure of HIV-1 accessory protein Vpr with annotated domains.
NMR structure as determined by Morellet et al., published with permission [52].

Currently, there are several known functions of Vpr in the HIV-1 life cycle: association with entering and budding virions, nuclear localization with the pre-integration complex, G2 cell cycle arrest, and interactions with viral and host transcription factors for enhanced gene transcription. Vpr is also known to enhance the replication of HIV-1 in macrophages [35]. Studies have shown significant replication deficiencies in Vpr-deletion mutants of HIV-1 isolates, but not in infected PBMCs or isolated CD4+ T lymphocytes, as analyzed by p24 Gag subunit ELISA quantification [55]. Along with the tropic nature of Env, Vpr is an important viral determinant of macrophage infection. Vpr primarily enhances infection in macrophages through its role in nuclear transport of the pre-integration complex and viral gene transcriptional enrichment, as an early virion associated molecule.

Establishment of infection with retroviruses typically requires the mitotic breakdown of the nuclear envelope for viral integration, thus limiting infection to cells capable of mitotic division. In the case of HIV-1 and other lentiviruses, the viral genome and associated proteins are transported into the nuclear envelope for infection in non-dividing cells, such as macrophages.

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The active nuclear import of the pre-integration complex through the nuclear pore following viral entry is significantly mediated by the Vpr-host cellular protein interactions. Along with the matrix (MA) and integrase (IN) proteins, Vpr localizes to the nucleus and interacts with nuclear import proteins for nuclear entry. Unlike MA and IN, Vpr does not contain a canonical nuclear localization signal (NLS) and thus works independently of classical nuclear signaling pathways [56]. Fouchier et al (1998) has shown that Vpr acts in a karyophilic manner by directly interacting with the nuclear pore complex through nucleoporin phenylalanine-glycine repeat regions [57]. Therefore, Vpr bypasses classical signaling pathways by directly interacting with the nuclear pore complex. The interaction of importin-α with the leucine motif in the first α-helix of Vpr is also an important mechanism of Vpr-mediated nuclear translocation [58, 59]. However, Vpr is not essential for HIV-1 infection of non-dividing cells: in the absence of Vpr, expression of IN has been shown to be sufficient for nuclear import [57].

Vpr mediates the expression of host cellular genes, including NF-κB, survivin, and the cell cycle inhibitor p21/Waf1/Cip1. Regulation of these host cellular genes by Vpr facilitates enhanced viral transcription, mediation of apoptosis and cell survival, and G2 cell cycle arrest in CD4+ T cells [60]. Vpr also directly interacts with transcription factors such as the p300 coactivator and the transcription factor initiation complex. Vpr also has the capacity to recruit and bind to the glucocorticoid receptor (GR), further promoting cellular gene transcription favorable for infection [35].

Vpr may also utilize these interactions to enhance viral transcription via HIV-1 LTR activation. The LTR contains several elements responsible for transcriptional activation, including the glucocorticoid response element (GRE), NF-κB, NF-AT, and a TATA box [61]. Several studies have verified that Vpr mediates LTR transactivation by interacting with other activator

proteins yet does not directly interact with promoter sequences. Vpr mediates the activation of GRE by binding to p300 and the transcription factor initiation complex and recruiting these proteins to the LTR, thus functioning as an adaptor protein [35]. Ayyavoo et al (1997) also showed that Vpr-mediated viral replication enhancement in macrophages is partially mediated by the glucocorticoid pathway, which was further substantiated by experiments indicating that the GRE region within the LTR is transactivated by Vpr [62]. Similar to p300/transcription factor recruitment, Vpr-mediated activation of GRE results in enhanced viral expression.

Additionally, virion association of Vpr is significant for further infection of surrounding macrophages, particularly for PIC nuclear translocation and gene transactivation enhancement. Vpr binds to the p6 domain of the p55 Gag viral protein, a precursor to the p24 Gag capsid protein. This interaction allows for incorporation of Vpr into nascent virions. Venkatachari et al (2010) demonstrated that Vpr oligomerization was necessary for its incorporation into virions. Specifically, the mutation of alanine to leucine at the 30th amino acid in the 1st α-helix of Vpr (A30L) was shown to abrogate virion oligomerization, Gag p6 interaction, and virion incorporation, as indicated by BiFC complementation [63]. Similarly, Jacquot et al (2007) indicated that the A30L mutation occurred within the site necessary for nuclear localization, thus causing a disruption in Vpr nuclear translocation [64]. These studies corroborate the importance of virion association for the function of Vpr in macrophages and establishment of macrophage infection.

1.3.4   Macrophages and HIV-1-associated neurocognitive disorders (HAND)

Infiltration of infected macrophages into the brain is largely responsible for HIV-1-associated neurocognitive disorders (HAND). HAND primarily occurs as a result of neuronal damage and apoptosis, with a range of 18 to 50% loss of neuronal density occurring in patients with HAND. Early in the course of HIV-1 disease progression, infected macrophages may cross the blood-brain barrier (BBB) and establish infection within the brain. These macrophages are capable of increasing macrophage infiltration by producing elevated levels of TNF-α, which permeabilizes the BBB tissue [65, 66]. Infected infiltrating macrophages cause neuronal damage without direct infection of primary neurons and can perpetuate HIV infection by infecting resident microglia in the brain. Pro-inflammatory cytokines and chemokines are secreted from infected macrophages, which cause neuronal death and activate bystander microglia and astrocytes. As a result, an increased secretion of neurotoxic substances occurs, including platelet-activating factor (PAF), nitric oxide, quinolinic acid, and arachidonic acid. Soluble viral proteins, including gp120, Vpr, and Tat, are also known to be potent neurotoxins, which contribute to neuronal death and the onset of HAND in patients [67].

1.3.4.1   The role of macrophage-derived cytokines in HAND

HIV-induced cytokine expression is highly implicated in the pathogenesis of HIV-associated neurocognitive disorders, particularly TNF-α and IL-1β. As previously described, TNF-α is significantly responsible for the development of neurocognitive disorders through its role in the physical breakdown and permeabilization of the blood-brain barrier. TNF-α also plays a

role in the proliferation and activation of astrocytes, which further increases the permeability of the brain and influx of monocytes. Astrocytic activation also leads to the increased release of Ca^{2+} and glutamate, which facilitates neuronal apoptosis [68].

In addition to TNF-α expression, IL-1β levels in the cerebral spinal fluid of HIV-1 patients has been found to correlate with the presence of neurocognitive disorders, implicating a role for IL-1β in HIV-1 neuropathogenesis [46]. IL-1β has also been associated with the pathogenesis of other neurocognitive disorders, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and epilepsy [69]. Like TNF-α, IL-1β is thought to act by activating astrocytes and other glial cells, contributing to the increased permeability of the blood-brain barrier, and monocytic migration. Both IL-1β and TNF-α are known to be up-regulated by the neurotoxic substances released by infected microglia, including arachidonic acid and PAF [70].

Other cytokines have been implicated in the neuropathogenesis of HAND in both *in vivo* patient CSF samples and *in vitro* models: increased levels of IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), MIP-1α, MIP-1β, and CXCL10 correspond to the onset of HAND [71]. Increased expression of these cytokines additionally contribute to neuronal injury and the onset of neurocognitive dysfunction. Overexpression of CXCL10 and its receptor CXCR3 has been identified in SHIV-encephalitis rhesus macaque models, with CXCL10 co-localization occurring with the pro-apoptotic protein caspase-3. Furthermore, blocking CXCR3 has been shown to decrease neuronal injury [72]. Similarly, studies have shown that increased expression of IL-8 strongly correlated to viral load in CSF samples of patients with HAND. Expression of these cytokines moderately originates from TNF-α and IL-1β: stimulation of astrocytes with these

potent neurotoxic cytokines has been shown to dramatically increase production of IL-6, IL-8, M-CSF, and GM-CSF [73].

In addition to TNF-α/IL-1β induction, soluble viral proteins have been widely implicated in the stimulation of cytokine-related neuronal injury. Yeung et al (1995) demonstrated that IL-6 and TNF-α were upregulated in brain, astrocyte, and macrophage cultures when exposed to recombinant gp120, which resulted in neurotoxicity [74]. Shah et al (2010) similarly showed that astrocytes transfected with plasmids containing the gp120 gene also upregulated IL-8 expression via the NF-κB pathway [75], while Cheung et al (2008) showed IL-1β up-regulation through gp120 binding to the CCR5 co-receptor and activating the PI3K pathway [76]. HIV-1 Tat has also been associated with the up-regulation of neurotoxic cytokines, including TNF-α, IL-6, IL-1β, and IL-8. Tat can also directly cause neuronal injury through its interactions with the neuronal cell membrane, which causes a significant increase in intracellular Ca^{2+} levels and the induction of caspase-mediated neuronal apoptosis [43]. Similarly, Vpr has also been strongly implicated in the indirect mechanisms of neuronal apoptosis. Vpr is found in significant quantities in HAND-positive brain tissue samples, specifically within macrophages and neurons [77].

Recently, our laboratory (Guha et al, 2012) has identified Vpr as an important modulator of neuronal injury through its contribution to neurotoxic cytokine production in macrophages. Specifically, Vpr plays a role in the upregulation of the proinflammatory cytokines IL-8 and IL-1β, which directly contribute to neuronal apoptosis and progression of HAND. This mediation was found to be involved in the activation of the SAPK/JNK pathway in macrophages. Additionally, unpublished data from this study suggests that the chemokine CXCL10 also is partially regulated by the presence of Vpr in macrophages [44]. Roux et al (2000) have similarly found that Vpr modulates the expression of IL-8 through the activation of NF-κB and NF-IL-6

promoters in both T-cell and monocytic cell lines [78]. These results suggest that blocking or reducing the expression of Vpr may be an approach to decreasing neuronal death and slowing HAND progression.

1.3.5 Role of productively infected, exposed and bystander macrophages in HIV-1 infection

As previously stated, productive HIV-1 infection of macrophages is essential to viral pathogenesis and disease progression. However, not all macrophages become productively-infected *in vivo* or *in vitro*. A small fraction of macrophages are capable of producing nascent virions for further infection, while the remaining macrophage population may be non-productively infected, exposed to soluble viral proteins, or uninfected, as depicted in Figure 4. Previous studies have approximated that 3-15% of *in vitro*-infected monocyte-derived macrophages were found to be capable of producing virions [79]. This fraction of macrophages is substantially responsible for the maintenance of the HIV-1 viral reservoir and disease progression.
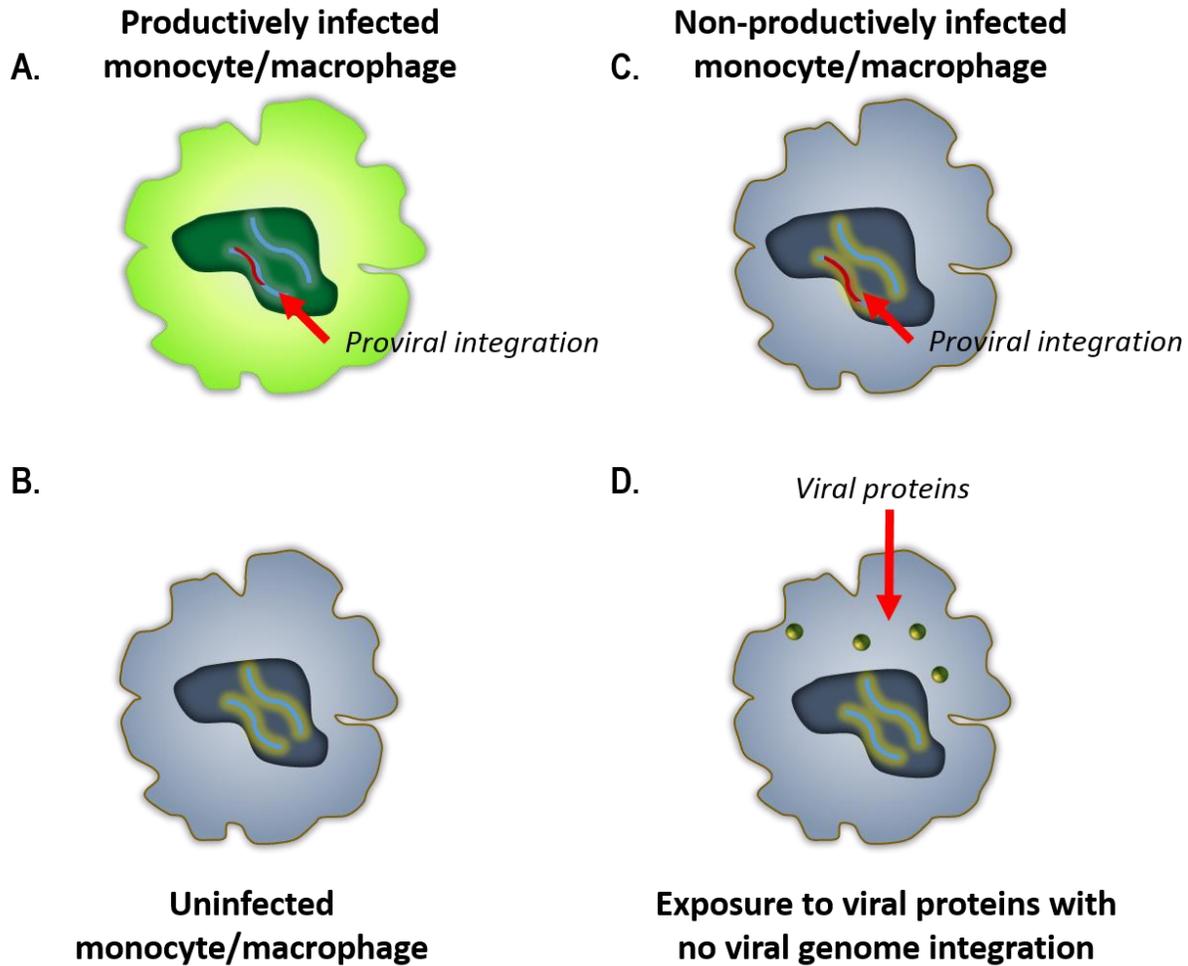
Figure 3. Schematic of potential outcomes in monocyte/macrophage populations after HIV-1 exposure.
A small fraction of macrophages are capable of viral replication (green; A). The rest of the macrophage population are uninfected (B), latently infected (C), or exposed to soluble viral proteins without viral genome integration (D).

Though the non-productively infected macrophage population does not contribute to viral load, macrophages that are latently infected or exposed to soluble proteins may contribute to the dysregulated expression of cytokines. As previously described, soluble viral proteins, including gp120, Tat, Nef, and Vpr, can modulate cytokine expression independent of viral genome integration [67]. Therefore, several types of viral exposure outcomes contribute to the cytokine expression in infected macrophage populations. This variability may confound or dilute the magnitude of cytokine production in overall macrophage populations. In order to elucidate the

role of productive infection in macrophages and to determine the exact percentage of infection, an *in vitro* modeling system in monocyte-derived macrophages is typically utilized. As described by Brown et al (2006), reporter viruses employing a fluorescent element allow for the rapid identification and separation of productively-infected cells from bystander cells [79]. Fluorescence-based separation for analysis of cytokine expression clarifies the roles of productively infected and non-productively infected macrophages at the single-cell level. Furthermore, cytokine profiling based on fluorescent expression may indicate the expression of cytokines by productively-infected macrophages otherwise diluted by total macrophage populations.

1.4   RATIONALE

In order to analyze the subpopulations of HIV-1-infected macrophages, an *in vitro* modeling system similar to Brown et al. (2006) was developed [79]. The *in vitro* modeling system utilized reporter viruses with an enhanced green fluorescent protein (EGFP) element as an indication of productive infection. Two macrophage-tropic HIV-1 isolates commonly used in our laboratory, YU-2 and AD8, were used in the development of reporter viruses. As previously described, there are several viral determinants of HIV tropism, such as Vpr and Env, which are required for establishment of infection in macrophages. Therefore, chimeric reporter viruses that specifically utilize the necessary R5-derived viral proteins are sufficient for macrophage productive infection and are capable of productive infection identification.

In this study, the *in vitro* modeling system for MDM infection is applied to the analysis of cytokine expression and the role of Vpr in cytokine modulation. As previously explained, our laboratory has found a Vpr-mediated upregulation of IL-1β and IL-8 in MDMs at both mRNA and protein levels. Similar data from our laboratory has indicated a Vpr-mediated upregulation of CXCL10 at the protein level by ELISA quantification. These analyses were executed by infecting MDMs with HIV-1 YU-2 and YU-2ΔVpr isolates for cytokine analysis [44]. The use of wild-type isolates did not allow for the quantification of productively-infected macrophages or identification of subpopulations within the infected macrophage culture. Therefore, the *in vitro* modeling system will be constructed and characterized for use in the identification of subpopulation cytokine expression. This system will also be used to verify the role of Vpr in modulating the expression of IL-1β, IL-8, and CXCL10.

Additionally, due to the important role that Vpr plays in macrophage-tropic viral replication, YU-2 ΔVpr infection is significantly replication-deficient in comparison to wild-type

YU-2 infection. This may confound the analysis of Vpr-mediated cytokine expression due to the overall low rate of viral replication. The utilization of functional Vpr mutants capable of maintaining viral replication kinetics similar to wild-type Vpr will both confirm the role of Vpr in cytokine modulation and identify which Vpr function causes the modulations. In this study, we will analyze the effects of the previously-described Vpr-A30L mutation, which is deficient in oligomerization and virion association [63]. The Vpr-A30L mutant does not associate with budding virions, but is expressed within infected cells. Therefore, Vpr-A30L can act as a transcriptional regulator upon expression within infected macrophages but is not present during early infection events as a virion associated molecules and does not involve in translocation of the pre-integration complex after viral entry of non-dividing cells. Vpr-A30L was chosen based on its minimal effects on replication deficiencies in macrophage infection, as well as its functional deficiencies that allow for the identification of Vpr-mediated cytokine expression.

1.5   HYPOTHESIS

We hypothesize that the characterization of the YU-2 and AD8 *in vitro* modeling systems for MDM infection will result in infection patterns similar to that of wild-type MDM infections. The use of these *in vitro* modeling systems will accurately identify the subpopulations within infected MDM cultures based on fluorescence expression. Furthermore, we hypothesize that the analysis of these subpopulations of infected MDMs for cytokine expression will clarify the roles of contribution among exposed bystander and productively-infected macrophages in the expression of IL-1β, IL-8, and CXCL10. The role of Vpr in the expression of these cytokines will also be further elucidated based on the analysis of wild-type, ΔVpr, and A30L mutant infections.

2.0 SPECIFIC AIMS

The following are the specific aims toward elucidating the role of Vpr on host-cellular interactions in monocyte-derived macrophages.

Aim 1: Construct and characterize a macrophage-tropic chimeric reporter virus for cell-specific analysis in MDMs.

    A. Construct an R5-tropic reporter virus that utilizes fluorescent expression during productive infection.

    B. Confirm the tropism and functionality of the reporter viruses through replication kinetics and viral protein production.

    C. Characterize the chimeric reporter viruses in relation to parental, non-reporter strains through parallel replication kinetics in infected MDMs.

    D. Construct and verify two Vpr mutations within the chimeric reporter viruses: deletion of Vpr production (ΔVpr) and production of Vpr-A30L, which is deficient in virion-association function.

Aim 2: Identify the effects of Vpr mutations in host cellular gene expression.

    A. Verify effects of Vpr mutants on inflammatory cytokine/chemokine expression at the protein level in harvested supernatants from infected macrophages

    B. Analyze effects of Vpr mutants on cell-specific cytokine/chemokine expression in infected and bystander macrophages

3.0   MATERIALS AND METHODS

3.1.1   Construction of reporter chimeric viruses

This study aimed to construct a reporter virus capable of infecting macrophages and expressing EGFP, which will be utilized for the separation of productively-infected macrophages. In order to achieve this, two cloning strategies were devised in the construction of an EGFP-expressing, R5-tropic virus:

1.   Clone the *env*-containing region from the neurotropic YU-2 isolate onto the NL4-3-EGFP-IRES X4-tropic laboratory strain via restriction sites EcoRI and BamHI.

2.   Clone the EGFP-containing region from NL4-3-EGFP-IRES onto the chimeric construct pNL(AD8), which contains the R5-tropic AD8 gp120 region within a NL4-3 background, via restriction sites BamHI and XhoI.
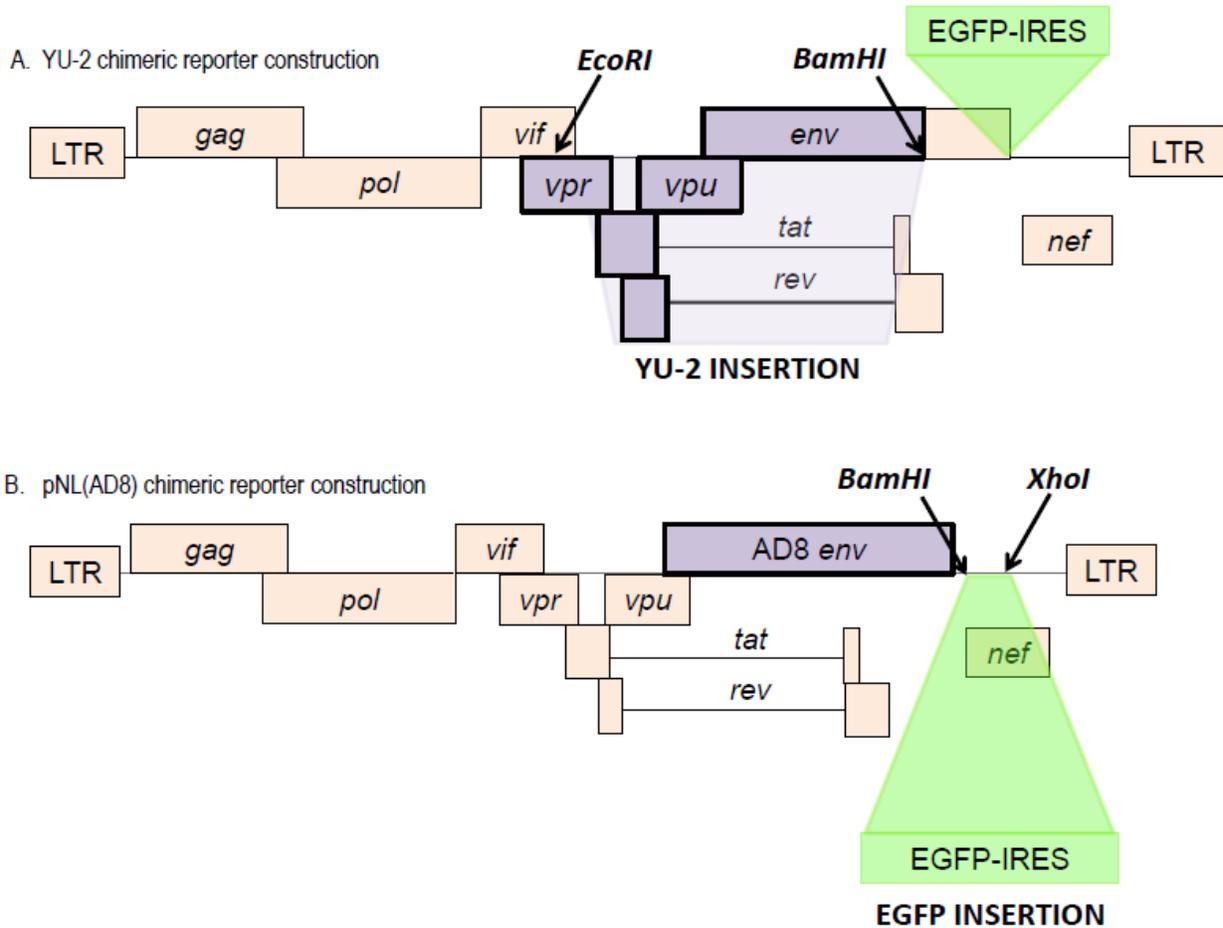
Figure 4. Schematic detailing the construction of chimeric reporter proviral plasmids pNL(YU-2) and pNL(AD8).

Strategies 1 and 2, as depicted in Figure 4, herein are referred to as pNL(YU-2)-EGFP and pNL(AD8)-EGFP, respectively. In order to construct pNL(YU-2) in Figure 4A, two restriction sites were selected at approximately the same location on both pNL4-3-EGFP and YU-2 WT within the *env* region. *EcoRI*, a unique restriction site located at 5746 bp on the YU-2 proviral plasmid and 5707 bp on the NL4-3-EGFP proviral plasmid, was chosen as the 5' site of insertion. Primers were designed to amplify the *env* region, including the *EcoRI* restriction site, and to introduce a 3' site of insertion as the *BamHI* restriction site. The *BamHI* site was introduced at 8435 bp on the YU-2 insert and previously existed at 8465 bp on the NL4-3-EGFP proviral backbone. The primer sequences utilized for amplification are as follows:

Forward: 5'-CTATGAAACTTATGGAGATACTTGGGCAGGAG-3'

Reverse: 5'-GATAATTGCTAA<u>GgAtCC</u>ATCCACTAATGGACCGG-3'

The underlined portion of the reverse primer sequence indicates *BamHI* restriction site. Mismatched nucleotides for BamHI site introduction are shown by lower-case letters. The YU-2 *env* region was amplified by PCR using these primers, ligated into a shuttle vector using pcDNA™3.1/V5-His TOPO® TA Expression Kit (Invitrogen), and sequenced by the Sanger method for verification. The YU-2 *env* insert containing both *EcoRI* and *BamHI* restriction sites was subcloned into pNL4-3-EGFP. The resulting proviral construct pNL(YU-2)-EGFP was Sanger or capillary sequenced and digested for verification.

In order to construct a reporter chimeric virus utilizing a second R5-tropic *env* region, pNL(AD8), shown in Figure 4B, a HIV-1 AD8 macrophage-tropic R5 clone, was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Eric O. Reed. The EGFP region from pNL4-3-EGFP was subcloned onto pNL(AD8) using the *BamHI* restriction site, located at 8465 bp on both pNL(AD8) and pNL4-3-EGFP, and *XhoI* restriction site, located at 8936 bp on both pNL(AD8) and pNL4-3-EGFP. The resulting proviral construct pNL(AD8)-EGFP was Sanger sequenced and digested for verification.

3.1.2   Construction of Vpr mutants in chimeric reporter viruses

In order to analyze the role of Vpr in HAND-related cytokine expression, two mutations were separately introduced onto both R5-utilizing reporter viruses: ΔVpr and A30L-Vpr, as shown in Figure 5. The ΔVpr mutation was subcloned from the existing pNL4-3ΔVpr, which contains a frameshift mutation in the unique *AflII* restriction site, yielding a truncated Vpr that lacks expression. The fragment excised using restriction sites *SphI* and *EcoRI* was used to subclone the

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ΔVpr mutation onto both pNL(AD8)-EGFP and pNL(YU-2)-EGFP. Similarly, the A30L-Vpr mutation was subcloned from the existing pVpr-A30L, which was constructed as previously described [63]. Vpr-A30L is an amino acid substitution from alanine to leucine at 30 a.a., which causes a functional defect in Vpr virion association. Restriction sites *SphI* and *EcoRI* were also used to subclone A30L-Vpr onto both pNL(AD8)-EGFP and pNL(YU-2)-EGFP.
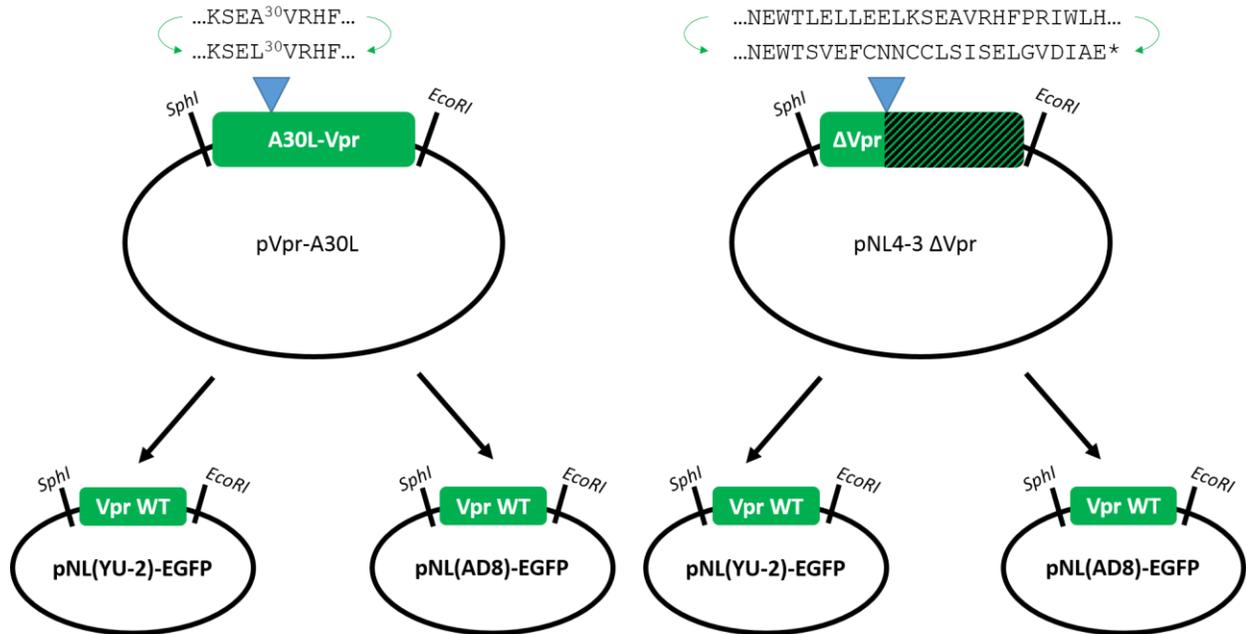
Figure 5. Schematic of Vpr-A30L and ΔVpr mutation cloning in reporter chimeric viruses.

3.1.3 Cells

HEK-293T and TZM-bl cells were maintained at 37°C, 5% CO_2 in DMEM (Gibco) containing 10% fetal bovine serum (HyClone), 1% L-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). U87-CXCR4 and U87-CCR5 cells were similarly maintained in DMEM with 15% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin.

Monocyte-derived macrophages: Monocyte (CD14+) cells were isolated from healthy donors using the Ficoll separation method: fresh donor blood was layered onto lymphocyte separation media and centrifuged at 1800 g at 4°C for 30 minutes. Peripheral blood mononuclear cells (PBMCs) were isolated from the gradient, counted, and washed for further separation. CD14 MicroBeads (Miltenyl Biotec) were added to the PBMCs suspended in MACS buffer (PBS pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA) at a ratio of 100 µl CD14 beads: 100 million PBMCs and incubated at 4°C for 30 minutes with occasional mixing. CD14+ cells were then separated from the PBMC suspension using a MACS column and MACS Separator (Miltenyl Biotec). Isolated CD14+ cells were counted and maintained in DMEM containing 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, 1 ng/µl sargramostim (recombinant granulocyte macrophage colony-stimulating factor [GM-CSF]; Genzyme), and 1 ng/µl macrophage colony-stimulating factor (M-CSF; NIH). CD14+ cells were seeded at 2.0×10^6 cells per well in a 6-well tissue culture plate and differentiated for 7 days prior to infection. Media and growth factors were replenished every three days throughout the course of infection.

3.1.4   Virus production and titration

HEK-293T cells were transfected by seeding 3.0×10^6 cells in 10 ml media on 10 cm^2 tissue culture-grade plates, which were grown for 24 hours prior to transfection to 75% confluence. Thirty minutes prior to transfection, the media was changed to 5 ml per plate. The cells were then transfected with 5 µg of purified plasmid DNA and 15 µl of PolyJet^{TM} transfection reagent (SignaGen) diluted in 500 µl DMEM, which was added drop-wise to the plate. 16 hours post-transfection, the transfected media was removed and 7 ml of fresh media was added for incubation for the following 60 hours. 72 hours post-transfection, the supernatants were harvested,

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centrifuged at 800 g to remove cell debris, and purified using a 0.22µm Steriflip® filter unit (Millipore).

Virus infectivity was quantified by a TZM-bl assay using the transfected supernatants. 3.0 x 10^4 TZM-bl cells were seeded in triplicate in a 96-well plate for 24 hours. Once the cultures reached 90% confluence, the cells were infected with 1:10, 1:100, 1:1000, and undiluted quantities of virus and incubated for 6 hours. After 6 hours, the media was changed and the plate was incubated for another 48 hours. The infected cells were then washed, fixed, and stained with β-galactosidase substrate for 3-4 hours. The stained cells were then counted in the optimal well (containing 50-150 stained cells) for each replicate and averaged to yield the measure infectivity in infectious particles per ml.

3.1.5 Viral infections and replication kinetics

U87-CXCR4 and U87-CCR5 infections

U87-CXCR4 and U87-CXCR5 cells were seeded at 1.5 x 10^4 cells per well in a 96-well plate and incubated for 24 hours prior to infection. Once the cultures reached 75% confluence, the cells were infected with 1:10, 1:100, 1:1000, and undiluted quantities of virus and incubated for 15-18 hours. After initial infection, the media was changed and the plated was incubated for another 54-57 hours. 72 hours post-infection, the fluorescent cells were then counted in the optimal well (50-150 fluorescent cells) for each replicate and averaged to yield the number of productively-infected cells per well.

MDM infections and replication kinetics

7 days post-differentiation, monocyte-derived macrophages (MDMs) were washed twice with PBS and replenished with cell media. Purified virus was added based on p24 concentration (500 ng p24) or TZM-bl assay results (0.1 MOI) to each well. The plates were incubated for 15-18 hours and washed twice with PBS to remove virus inoculum before replenishing with fresh media. MDM cultures were maintained for a 15-day infection course. Supernatants were harvested and centrifuged at 1800 *g* for 10 minutes on days 0, 3, 6, 9, 12, and 15 post-infection. Remaining monocytes were resuspended in 2 ml cell media for media replenishment. Cumulative replication kinetics were established by quantification of p24 viral capsid protein at each time point by p24 ELISA (NCI). At day 15, MDMs were washed twice with PBS and used for intracellular staining or immunoblotting.

3.1.6   Virus and cell lysate immunoblotting

Infected MDMs were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP40, 1% Triton-X 100, and phenylmethanesulfonyl fluoride protease inhibitor [PMSF; Cell Signaling Technology, Inc]) for 2 hours at 4°C with shaking. Cell debris were pelleted by centrifugation at 1800 *g* at 4°C for 10 minutes. Protein quantification was done via bicinchoninic acid colormetric detection using BCA Protein Assay Kit (ThermoScientific). In order to analyze virus replication by immunoblotting, MDM virus supernatant was concentrated. At day 15 of the MDM infection course, 1 ml of MDM supernatant was harvested, purified, and concentrated by high-speed centrifugation at 22,000 rpm for 1 hour at 4°C. Concentrated virus was resuspended in 50 µl sample buffer for use in immunoblotting.

Forty (40) μg of cell lysate sample and 50 μl of concentrated virus were electrophoresed by SDS-PAGE under reducing conditions at 70V for 3.5 hours and transferred to a PVDF membrane (Millipore) at 300 mA for 1 hour. The membrane was blocked for 1 hour at room temperature in 5% milk in PBS-T (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Tween 20). Following blocking, primary antibody diluted in 5% milk in PBS-T was added to the membrane for overnight incubation at 4°C with shaking. The following primary antibodies were used in MDM cell lysate immunoblotting: anti-Vpr (1:2000, NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Vpr (1-50) Antiserum from Dr. Jeffrey Kopp, Cat # 11836), anti-Nef (1:200, NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH), anti-p24 (1:500, NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Monoclonal Antibody to HIV-1 p24 (AG3.0) from Dr. Jonathan Allan), and anti-actin (1:3000, Sigma). The membranes were washed three times in PBS-T for 20 minutes each and incubated with the appropriate conjugated secondary antibody diluted in 5% milk in PBS-T for 1 hour at room temperature with shaking. The following secondary antibodies were used: goat-anti-mouse HRP-conjugated secondary antibody (1:3000, Cell Signaling) and goat-anti-rabbit HRP-conjugated secondary antibody (1:5000, Cell Signaling). The membranes were then washed with PBS-T three times for 20 minutes each and then developed using WesternBright ECL HRP substrate kit (Advansta).

3.1.7   Intracellular staining and flow cytometry

At day 15 post-infection, MDMs were washed twice with PBS and detached by adding pre-warmed CellStripper™ (CellGro) to MDMs for incubation on ice for 10 minutes. The MDMs were then incubated at 37°C in CellStripper for 1-2 hours with occasional agitation for complete detachment. Cells were collected into round-bottom tubes, centrifuged at 1200 g for 15 minutes,

and resuspended in 250 µl FACS buffer (PBS, 1% bovine serum albumin). 1 µl of GolgiPlug[TM] protein transport inhibitor (BD Biosciences) was added to the cell suspension for incubation at 37°C for 6 hours. 2 mM EDTA was added for incubation at room temperature for 10 minutes. MDMs were then fixed with Cytofix/Cytoperm[TM] solution (BD Biosciences) at 4°C for 20 minutes and washed twice with FACS buffer. Cell permeabilization was done by washing MDMs twice in 1X Perm/Wash[TM] buffer (BD Biosciences). MDMs were resuspended in 50 µl 1X Perm/Wash buffer and the following antibodies were added: 4 µl Pacific Blue[TM] anti-human IL-1β antibody (BioLegend), 5 µl AlexaFluor 700[TM] anti-human TNF-α antibody (eBioscience), 5 µl APC anti-human CXCL10 antibody (BioLegend), and 20 µl PE anti-human IL-8 antibody (BioLegend). The cell suspensions were mixed thoroughly and incubated for 45 minutes at room temperate in the dark. MDMs were then washed twice with 1X Perm/Wash buffer and stored in FACS buffer/1% paraformaldehyde solution until analysis.

Flow cytometric analysis was conducted on the LSRFortessa[TM] cell analyzer (BD Biosciences) using FACSDiva® software (BD Biosciences). Cells were analyzed based on expression of EGFP, and subsequently analyzed by expression of IL-8, IL-1β, CXCL10, and TNF-α. Further analysis for polyfunctionality was carried out using FlowJo (Tree Star, Inc.) and Simplified Presentation of Incredibly Complex Evaluations (SPICE; NIH).

3.1.8   Cytokine ELISA quantification

Harvested supernatants from infected MDMs were evaluated for CXCL10, IL-8, IL-1β, and TNF-α production using Human IL-1β/IL-1F2 DuoSet ELISA kit (R&D Systems), Human CXCL8/IL-8 DuoSet ELISA kit (R&D Systems), Human CXCL10/IP-10 DuoSet ELISA kit (R&D Systems), and Human TNF Elisa kit (BD OptEIA[TM]). 100 µl of capture antibody diluted

to 2.0 µg/mL in PBS was added to each well of a 96-well plate and incubated overnight at room temperature. The plate was then washed with wash buffer (0.05% Tween®20 in PBS) three times and thoroughly dried. 100 µl of reagent diluent (1% BSA in PBS, 0.2 µm filter sterilized) was added to each well as a blocking agent and incubated at room temperature for 1 hour. Following incubation, the plate was washed three times and dried thoroughly, as before.

Harvested supernatants were diluted to the appropriate amount and added in 100µl volumes to the 96-well plate. Samples for IL-1β analysis were diluted to 1:10, CXCL10 to 1:10, IL-8 to 1:1000, and TNF-α samples were undiluted. A seven-point standard curve for each cytokine standard was made and added in 100µl volumes to the 96-well plate. The highest standard concentration for each cytokine were as follows: 250 pg/mL for IL-1β, 2000 pg/mL for CXCL10, 2000 pg/mL for IL-8, and 500 pg/mL for TNF-α. 2-fold serial dilutions from the highest standard concentration were completed to establish a seven-point standard curve. The samples and standards were incubated at room temperature for 2 hours.

The plate was then washed three times and dried thoroughly, as before. Detection antibody was diluted to 20 ng/mL in reagent diluent, added to each well in 100µl volumes, and incubated for 2 hours at room temperature. The plate was then washed three times and dried thoroughly, as before. Streptavidin conjugated to horseradish-peroxidase (HRP) was diluted 1:200 in reagent diluent and added in 100µl volumes to each well. The plates were incubated for 20 minutes at room temperature in the dark, washed three times, and dried thoroughly.

3,3',5,5'-tetramethylbenzidine (TMB) substrate was prepared using the TMB Substrate Reagent kit (BD OptEIA™) by combining equal amounts of Substrate Reagent A and Substrate Reagent B. 100µl volumes were added to each well and incubated for approximately 20 minutes at room temperature in the dark, or until the appropriate level of color detection has occurred. 50µl

of 2 N H_2SO_4 was added to stop the reaction. Color intensity was measured using the ELx800 Absorbance Microplate Reader (Bio-Tek) and KCJunior^{TM} analysis software (Bio-Tek). Seven-point standard curves were assembled based on known concentrations and experimental absorbances. Linear trend lines were used to create an equation for calculating cytokine amounts in pg/mL ($R^2 \geq 0.900$). Experiments were repeated across four separate donors, excluding TNF-α (due to undetectable levels in collected supernatants).

3.1.9   Microscopy

Cell-specific tropism fluorescence microscopy

Coverslips were added to a 6-well tissue culture plate and seeded with 1.5 x 10^4 U87MG-CD4/CCR5 or U87MG-CD4/CXCR4 cells. Cultures were incubated for 15-18 hours to 90% confluency, then infected with 0.1 MOI virus. 15-18 hours after infection, the cells were washed twice with PBS and replenished with media. After 72 hours of infection, the cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, washed twice with PBS, and stained with DAPI for 5 minutes. The cells were washed twice with PBS and mounted onto slides with gelvatol, then imaged with Olympus IX71 inverted fluorescent microscope. Overlay images were composited using GNU Image Manipulation Program (GIMP).

MDM infection fluorescence microscopy

15 days post-infection, MDMs were washed twice with PBS and imaged with Olympus IX71 inverted fluorescent microscope for brightfield and EGFP expression analysis. Overlay brightfield/green fluorescence images were composited using SPOT Advanced Modular Imaging Software for Microscopy (Diagnostic Instruments, Inc.).

3.1.10   Statistical Analysis

Statistics were performed using Graphpad Prism® software (GraphPad Software, Inc.). Comparisons of samples were attempted using a two-sided Student's t-test using a significance level of $p < 0.05$.

4.0 RESULTS

4.1 AIM 1: CONSTRUCTION AND CHARACTERIZATION OF A MACROPHAGE-TROPIC CHIMERIC REPORTER VIRUS FOR CELL-SPECIFIC ANALYSIS IN MONOCYTE-DERIVED MACROPHAGES (MDM)

4.1.1 Verification of sequence homology in proviral chimeric reporter constructs

As previously discussed, the utilization of reporter viruses for *in vitro* MDM infection provides a clear analysis of the subpopulations within infected MDM cultures. Two reporter viruses were chosen for analyses and construction, as described in Section 3.1.1: pNL(YU-2)-EGFP and pNL(AD8)-EGFP. Both constructs require restriction site-based fragment insertion that could interrupt viral protein sequences. To ensure that the R5 *env* cloning would not shift or interfere with the reading frame of downstream viral proteins, sequences were compared between the parental and reporter chimeric virus plasmids. The viral proteins that could be interrupted by reporter cloning included Vpr, Vif, gp41, Rev, Tat, and Nef. The sequence of each viral protein was assessed for potential reading frame interferences using SerialCloner 2.6.1 (SerialBasics), as represented in Figure 6.

The sequences of viral proteins were also compared among pYU-2, pNL4-3-EGFP, and pNL(AD8) to determine the degree of variation between HIV-1 isolates. The analyzed sequences were compiled from sequencing data previously collected in our laboratory. As expected, Env has a high degree of variability between YU-2, AD8, and NL4-3 isolates, which is primarily due to its inherent diversity to infect multiple target cell types. Vpu was also highly variable among the

selected isolates since it is notably non-functional in the YU-2 isolate.   In order to retain the

highest degree of similarity to wild-type R5-tropic isolates, the non-functional *vpu* sequence was

included in the R5-*env* cloning of pNL(YU-2)-EGFP.

Figure 6. Sequence comparisons show no interruption in chimeric viral proteins in reporter virus cloning.
Representative sequence comparison of two viral proteins interrupted during proviral cloning, Vpr (*A*) by pNL(YU-2)-EGFP cloning and Nef (*B*) by pNL(AD8)-EGFP cloning.

The chimeric Vpr protein assembled in the pNL(YU-2)-EGFP construct contains a portion

of pNL4-3-EGFP *vpr* (amino acids 1 to 62) and a portion of YU-2 *vpr* (amino acids 63 to 96), as

shown in Figure 6A. Sequence analysis was performed in order to confirm that the NL4-3 portion

of chimeric *vpr* did not drastically differ from YU-2 *vpr*, which is known to function correctly in

macrophage infection. Four differences in amino acids were found prior to the start of chimeric

reporter virus cloning: H15Y, R28S, P37I, and G41N. None of these amino acid substitutions are

associated with significant changes in Vpr function, as determined from the Los Alamos National

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Laboratory HIV Epitope Variant and Escape Mutation Database (www.hiv.lanl.gov). Additionally, no frameshift changes were identified prior to chimeric cloning. Similarly, the *nef* region within the pNL(AD8)-EGFP construct was analyzed for changes in amino acids or frameshift alterations occurring after the site of the EGFP-IRES-Nef element insertion. Chimeric Nef contains a portion from pNL(AD8) (amino acids 1 to 9) and a portion from pNL4-3-EGFP (amino acids 10 to 206). No changes were identified in the sequence analysis between pNL4-3 and pNL(AD8) within the *nef* region.

4.1.2   Confirmation of the chimeric reporter virus construction

In order to generate R5-tropic reporter viruses for further experiments, two cloning strategies were utilized: YU-2 *env* region insertion into the X4-tropic pNL4-3-EGFP reporter construct and EGFP insertion into the R5-tropic pNL(AD8) construct (see Figure 4 for illustration). Briefly, the *EcoRI – BamHI* region of pYU-2 was amplified and ligated into pNL4-3-EGFP for pNL(YU-2)-EGFP construction. This insertion was confirmed by restriction digest with *BamHI* and *EcoRI* for insertion release from the proviral reporter construct, as shown in Figure 7A. The expected band sizes of the subsequent fragments were approximately 2.7 kb and 13.4 kb for the insert and proviral fragment, respectively.
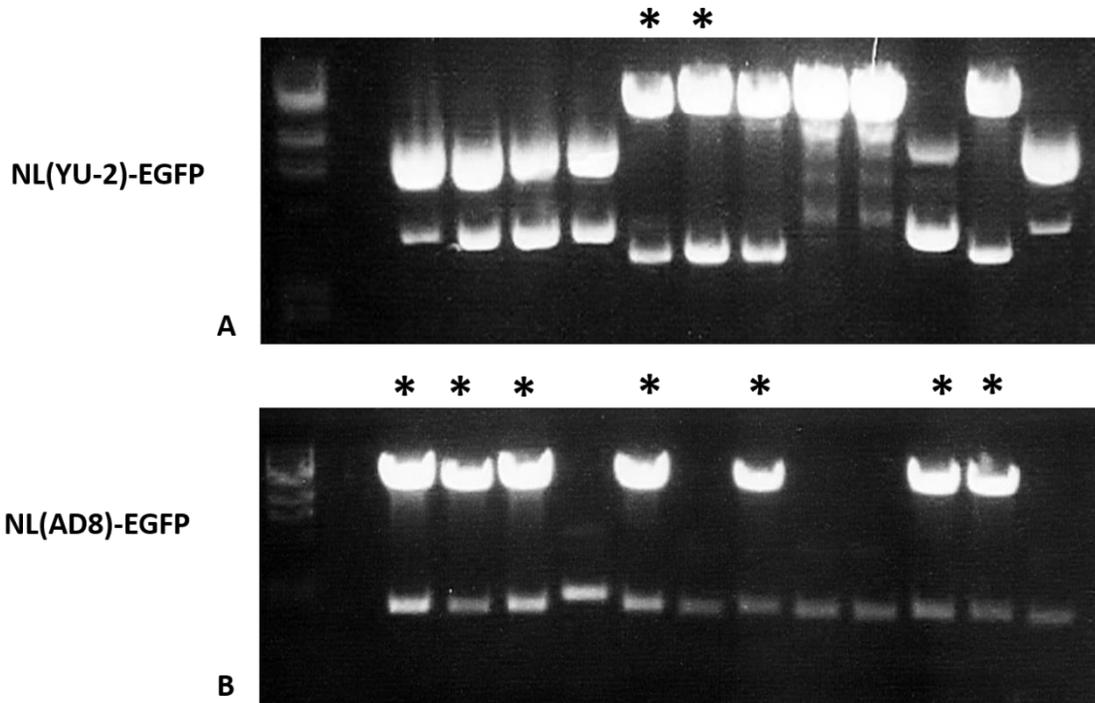
Figure 7. pNL(YU-2)-EGFP and pNL(AD8)-EGFP cloning yielded correct isolates.
Restriction digests were used to confirm the insertion of YU-2 *env* into NL4-3-EGFP (*A)* or the insertion of the EGFP-IRES reporter element into pNL(AD8) (*B*). Asterisks indicate the isolate was positively verified by both restriction digest and sequencing.

Similarly, the *BamHI – XhoI* region of pNL4-3-EGFP was isolated and ligated into pNL(AD8) for pNL(AD8)-EGFP construction. Restriction digest analysis with *BamHI* and *XhoI* yielded a release of the ligated insert, as shown in Figure 7B. The released insert was measured at approximately 1.7 kb and the proviral fragment was measured at 14.4 kb, as expected. Restriction digest analysis revealed four correct clones for pNL(YU-2)-EGFP construction and seven clones for pNL(AD8)-EGFP. Further confirmation with Sanger sequencing determined that two pNL(YU-2)-EGFP isolates contained the R5-tropic *env* insert, while all seven pNL(AD8)-EGFP isolates contained the correct EGFP element insertion. Two isolates from each construct were chosen for further verification: pNL(YU-2)-EGFP clones 5A and 7B, as well as pNL(AD8)-EGFP clones 2 and 11.

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4.1.3   Characterization of chimeric reporter virus tropism in U87 cell lines

Following the successful construction of chimeric reporter viruses, the resulting constructs were evaluated for EGFP expression in the producer cell line, HEK 293T cells, by transient transfection. All reporter virus clones were capable of EGFP expression upon transfection, as observed by fluorescence microscopy (data not shown). However, the capacity to express the EGFP reporter element upon infection, rather than transfection, of cells must be established. In order to verify that the constructed reporter viruses contained a functional Env region capable of R5-tropic infection, CCR5 co-receptor utilization was examined. U87 glioblastoma cell lines expressing one of the two HIV-1 co-receptors (U87-CD4-CCR5 and U87-CD4-CXCR4) were infected with reporter virus for viral tropism identification, as depicted in Figure 8.

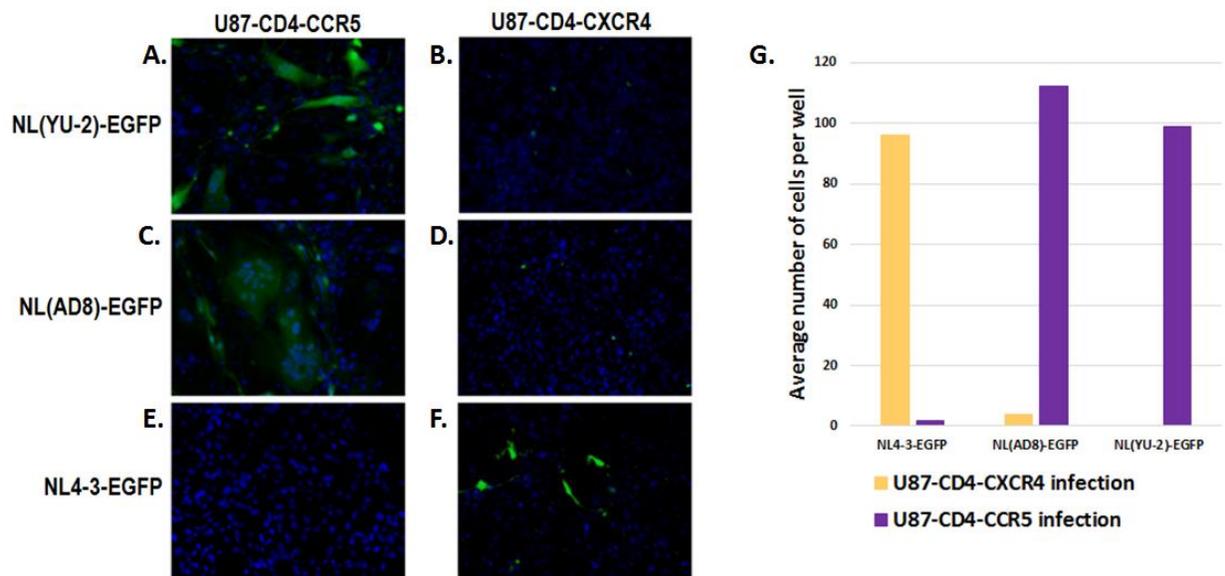**Figure 8. Chimeric reporter viruses NL(YU-2)-EGFP and NL(AD8)-EGFP preferentially utilize the CCR5 co-receptor for HIV-1 infection.**
U87-CCR5 and U87-CXCR4 cells were infected with NL(YU-2)-EGFP (*A, B*), NL(AD8)-EGFP (*C, D*), and NL4-3-EGFP (*E, F*). EGFP expression is indicated by green fluorescence, DAPI nuclei staining is indicated by blue fluorescence. The average number of fluorescent cells per well are shown in (*G*).

Upon infection in U87-CD4-CCR5 cells, both NL(YU-2)-EGFP and NL(AD8)-EGFP showed high levels of fluorescent expression, indicative of successful productive infection (Figures 8A, 8C). In contrast, infection in U87-CD4-CXCR4 cells, both reporter viruses yielded minimal to no EGFP expression (Figures 8B, 8D). As expected, the X4-tropic NL4-3-EGFP laboratory strain, which was used as the proviral backbone for reporter cloning, yielded high levels of productive infection in U87-CD4-CXCR4 cells and no infection in U87-CD4-CCR5 cells (Figures 8E, 8F). Collectively, this indicates that the chimeric reporter viruses were successfully altered from the X4 tropism of the original NL4-3-EGFP isolate to R5 tropism (Figure 8G). Additionally, EGFP production from infected U87 cells, rather than transiently transfected 293T cells, confirms that expression occurs upon viral integration. Therefore, successful EGFP expression indicates a true productive infection.

4.1.4 Confirmation of chimeric reporter virus functionality in primary MDMs

After confirming tropism and EGFP reporter expression as an indicator for productive infection, chimeric reporter virus infections were characterized in primary MDMs. Reporter viruses were assessed for EGFP expression, viral protein expression, and replication competence. As previously described, production of EGFP is crucial for MDM subpopulation analysis and quantification of infected cells. Differentiated macrophages from a healthy donor were infected with four separate clones of reporter viruses: NL(YU-2)-EGFP 5A, NL(YU-2)-EGFP 7B, NL(AD8)-EGFP 2, and NL(AD8)-EGFP 11. Reporter virus infections were assessed for the maintenance of EGFP expression after virus exposure.
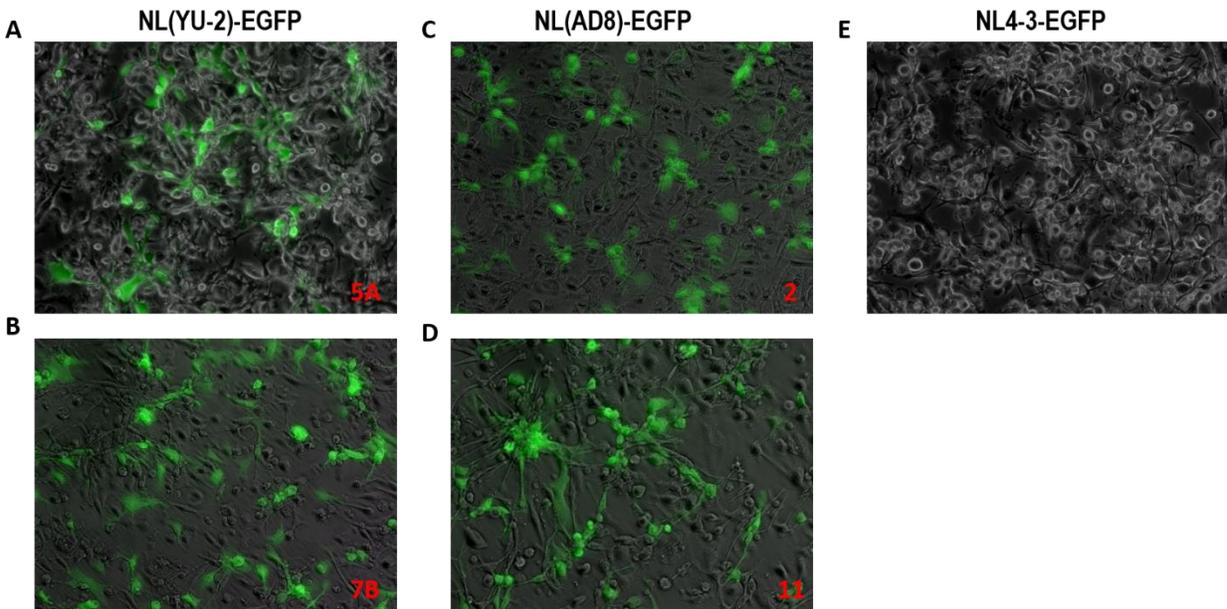
Figure 9. Chimeric reporter viruses infect monocyte-derived macrophages via CCR5 co-receptor utilization and express EGFP upon viral replication.
Differentiated MDMs were infected with NL(YU-2)-EGFP 5A and 7B (*A, B*), as well as NL(AD8)-EGFP 2 and 11 (*C, D*), with NL4-3 exposure (*E*) as a control. Infected and exposed MDMs were imaged at 7 d.p.i.

As shown in Figure 9 (A through D), the selected reporter clones successfully infected primary MDMs. EGFP expression was observed by three days post-infection; amplification of EGFP over time provided a crude marker for the spread of infection to surrounding macrophages. As expected, exposure of primary MDMs to the X4-tropic NL4-3-EGFP laboratory virus yielded no EGFP with no effects on cellular morphology (Figure 9E). EGFP expression allowed for a rapid visual estimation of infectivity, which was used to determine which reporter clone better reflected the true infection rates in macrophages. Overlays between fluorescent imaging (green) and brightfield imaging provided an assessment for cell morphology and EGFP expression. As such, NL(YU-2)-EGFP 7B (Figure 9B), and NL(AD8)-EGFP 11 (Figure 9D) were chosen for further experimentation.

In addition to EGFP expression, sufficient levels of viral proteins within infected macrophages is also indicative of successful MDM infection. Furthermore, the insertion of the YU-2 *env* region in NL(YU-2)-EGFP and the insertion of the EGFP element in NL(AD8)-EGFP may alter viral protein production. The viral protein Nef was of particular interest, as its gene is located downstream of cloning sites in both constructs, as shown by the schematic depicted in Figure 10A.
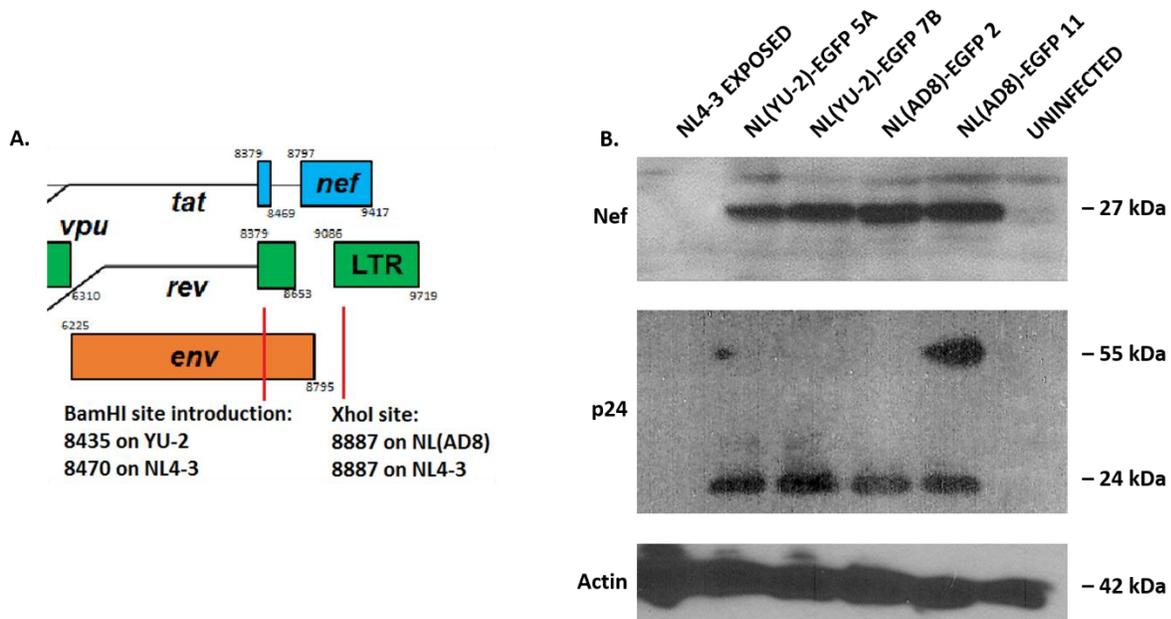
Figure 10. Verification of viral proteins expression from chimeric reporter viruses in infected MDMs. The production of viral protein Nef was assessed due to its downstream location from both chimeric reporter cloning sites (A). Immunoblotting of lysed primary MDMs collected 14 d.p.i. for HIV-1 Nef, p24, and actin as a loading control (B).

In order to ensure that cloning did not introduce frameshift mutations that would cause issues in downstream production, viral protein expression was measured by immunoblots from lysed infected primary MDMs, as shown in Figure 10B. The Gag capsid protein p24 serves as a measure of viral particle production. Its precursor protein, p55, is also detected by the anti-p24 antibody. The results indicate that each clone from both reporter constructs successfully produce

the 27 kDa viral protein Nef and the 24 kDa capsid protein p24. The NL4-3-exposed and uninfected MDMs expectedly produced no detectable levels of either viral protein, despite the presence of sufficient amounts of cellular protein. Collectively, these results demonstrate that the chimeric cloning in both reporter viruses had no effect on downstream viral protein expression. Both NL(YU-2)-EGFP and NL(AD8)-EGFP are capable of viral protein expression necessary for viral replication.

The expression of secreted p24 capsid protein in released viral particles was also analyzed by ELISA quantification. Secreted p24 from infected cells serves as a measurement of viral replication. Increasing p24 levels over time, denoted as replication kinetics, indicate that macrophages are continuously producing and releasing new virus particles. Viral replication is a crucial attribute of true productive infection, which is capable of promoting viral spread. HIV-1 is capable of infecting macrophages by macropinocytosis and endocytosis of surrounding virus particles, including X4-tropic viruses [80]. However, this type of infection is inefficient and does not reflect the *in vivo* infection patterns. Similarly, endocytosis-based infections do not yield equivalent amounts of p24 production as productively-infected cells. In order to ensure that the macrophages are replication-competent and productively-infected, replication kinetics were assessed for the chimeric reporter viruses in primary MDMs.
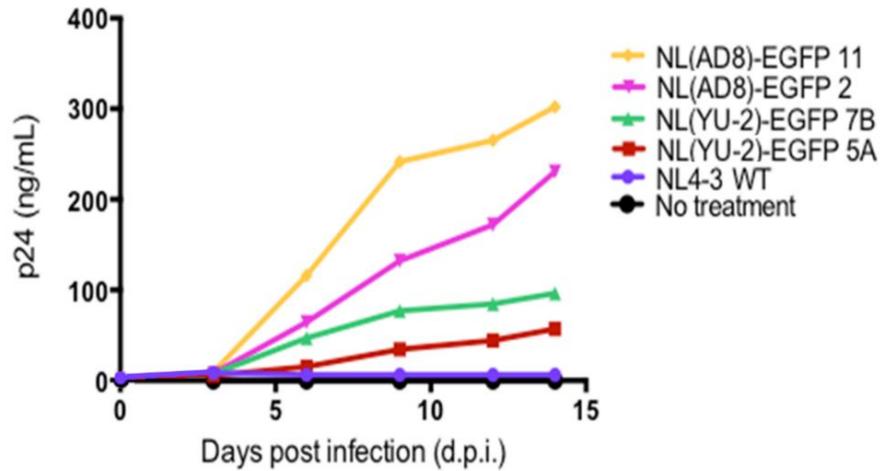
Figure 11. Chimeric reporter viruses NL(YU-2)-EGFP and NL(AD8)-EGFP are replication-competent in MDMs.
Cumulative p24 replication kinetic curves were assembled based on p24 ELISA quantifications of harvested supernatants from infected MDMs at days 0, 3, 6, 9, 12, and 14.

As shown in Figure 11, each clone from both reporter constructs were capable of infecting and producing continuous amounts of p24 over the course of 14 days. Particularly, each reporter virus replicates at a higher level than the X4-tropic NL4-3-EGFP. Since X4-tropic viruses are capable of low levels of replication in MDMs via endocytosis-based infection, the higher levels of p24 production from the reporter viruses indicate a productive infection through CD4/CCR5 receptor utilization. Though both reporter constructs are replication-competent, both NL(AD8)-EGFP isolates produce higher levels of virion production than the NL(YU-2)-EGFP isolates. Similar to the fluorescence analysis, isolates NL(AD8)-EGFP 11 (yellow line) and NL(YU-2)-EGFP 7B (green line) were chosen for further experimentation based on their efficient virion production rates. From this donor, NL(AD8)-EGFP 11 maximally accumulated 306.1 pg/mL of p24 capsid protein by 14 days post-infection, while NL(YU-2)-EGFP 7B accumulated 238.6 pg/mL p24 protein by 14 days post-infection.

4.1.5　　Characterization of chimeric reporter viruses in comparison to parental strains

Since the chimeric reporter viruses are partially derived from R5-tropic viruses that have been well-characterized in macrophage infection, p24 replication kinetics were compared between the chimeric reporter viruses and their respective parental strains. For the YU-2-enveloped reporter virus, the YU-2 wild-type isolate was used as a comparison. For the AD8-enveloped reporter virus, the NL(AD8) clone was used for comparison. Similar replication kinetics between chimeric reporter isolates and parental strains would strongly suggest that the infectivity and replication efficiency of these viruses are also similar.
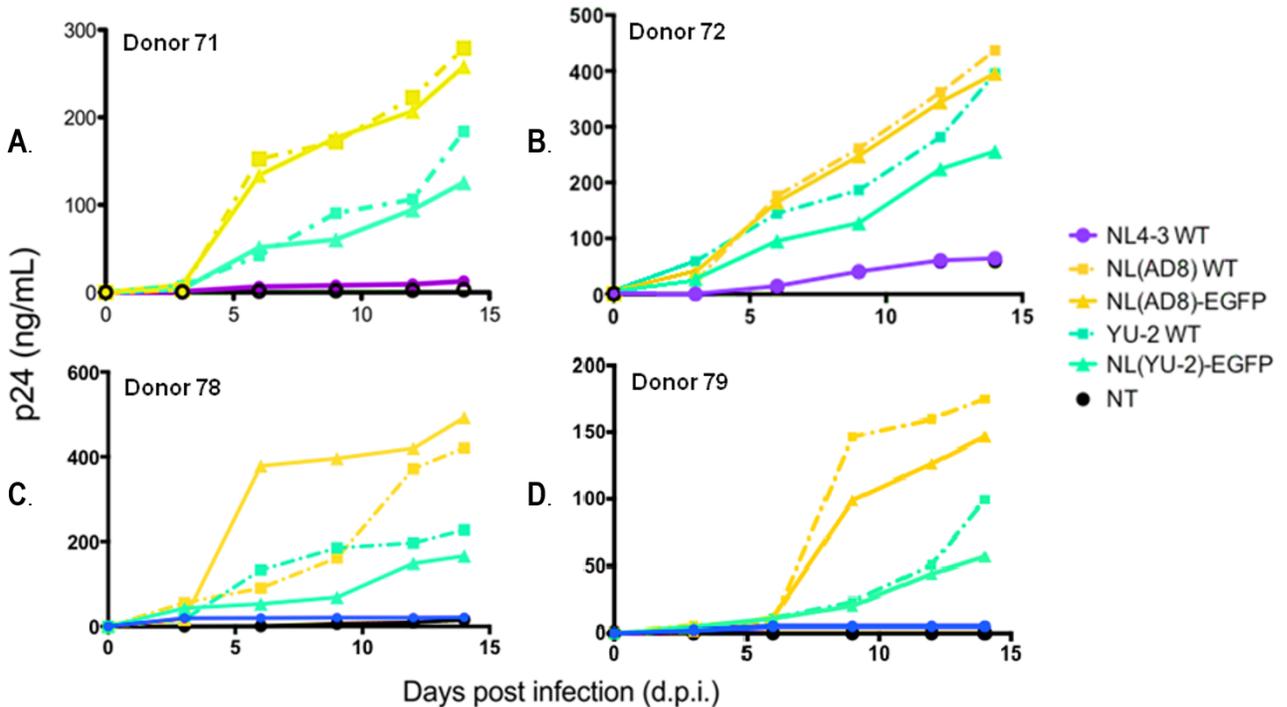
Figure 12. Chimeric reporter viruses NL(YU-2)-EGFP and NL(AD8)-EGFP replicate similarly to their respective parental isolates in MDMs.
Cumulative p24 replication kinetic curves were assembled based on p24 ELISA quantifications of harvested supernatants from infected MDMs at days 0, 3, 6, 9, 12, and 15.

The results demonstrated that both chimeric reporter viruses replicate at similar rates to their respective parental strains, as shown in Figure 12. In all four donors, the wild-type YU-2 isolate (light blue, dashed line) replicated at a slightly higher rate than NL(YU-2)-EGFP (light blue, solid line). Similarly, the NL(AD8) parental clone (yellow, dashed line) had marginally higher replication rates than NL(AD8)-EGFP (yellow, solid line) in three of the four donors. However, these differences are negligible and do not suggest any deficiencies in viral replication.

As previously observed in reporter virus replication kinetics, the AD8-enveloped viruses consistently replicated more efficiently than the YU-2-enveloped viruses. By 14 days post-infection, AD8-enveloped viruses show up to a two-fold increase in p24 production in comparison to YU-2-enveloped viruses, as represented in Donor #79 (12C). However, minimal differences were seen in Donor #72 (12B) at 14 days post-infection. The maximum p24 accumulation during the course of infection was averaged at 334.35 pg/mL for NL(AD8)-EGFP and 226.9 pg/mL for NL(YU-2)-EGFP.

Though NL(AD8)-EGFP could be selected for further experimentation based on its replication efficiency, the NL(YU-2)-EGFP reporter clone contains a larger portion of R5-tropic viral genes, which maintains its significance by reflecting a true macrophage infection. Therefore, both constructs are chosen for further Vpr analysis: NL(AD8)-EGFP, which contains Vpr from the NL4-3 laboratory strain, and NL(YU-2)-EGFP, which contains a chimeric Vpr primarily reflecting the YU-2 isolate.

4.1.6 Construction and characterization of Vpr mutants in chimeric reporter viruses

As previously stated, two mutations were separately chosen for Vpr analysis using chimeric reporter viruses: Delta (Δ) Vpr, which is a non-functional truncation of Vpr that does not contribute to macrophage infection, and Vpr-A30L, which is a structurally and functionally deficient form of Vpr that results in a loss of virion association and oligomerization, respectively. The HIV-1ΔVpr virus results in a functional and structural loss of Vpr, which leads to a loss in Vpr-mediated enhanced viral replication in macrophages. As previously described, Vpr-deficient strains of R5-tropic HIV-1 isolates result in significant decreases in viral replication [55]. In order to circumvent replication deficiencies of HIV-1ΔVpr, a Vpr mutant was chosen that resulted in a structural association and functional deficit yet expressed in the infected cells only. . Since the Vpr-A30L mutation still results in the expression of Vpr, this mutant is not limited in the infected cell replication yet lacks the functions associated during early phase of infection as virion associated molecule.

A. NL(YU-2)-EGFP WT

B. NL(YU-2)-EGFP ΔVpr

C. NL(YU-2)-EGFP A30L

D. NL(AD8)-EGFP WT

E. NL(AD8)-EGFP ΔVpr
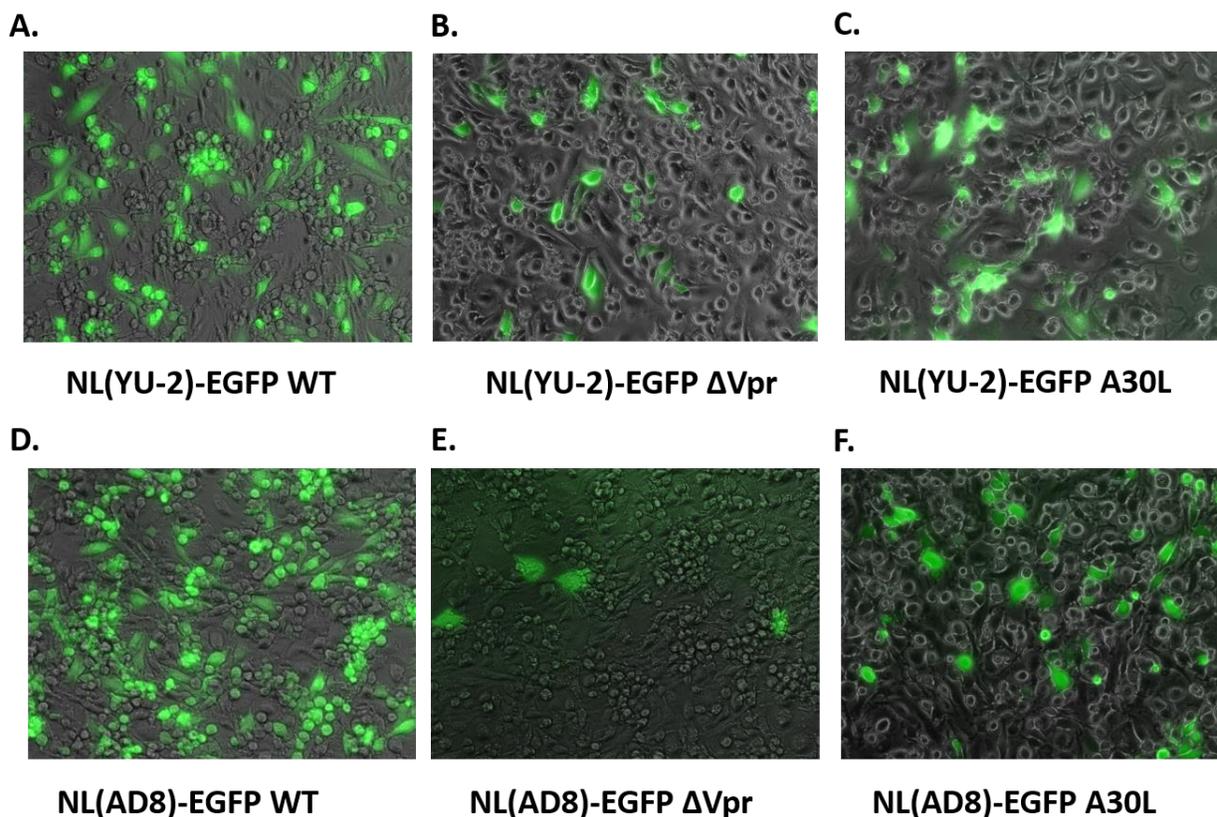
F. NL(AD8)-EGFP A30L

Figure 13. EGFP expression in primary MDMs reveal distinct productive infection patterns among Vpr mutants.
Differentiated MDMs from Donor 78 were infected with NL(YU-2)-EGFP WT (*A*), ΔVpr (*B*), and Vpr-A30L (*C*), as well as NL(AD8)-EGFP WT (*A*), ΔVpr (*B*), and Vpr-A30L (*C*). Infected MDMs were imaged at 14 d.p.i. Overlay images were compiled from green fluorescence imaging and brightfield imaging.

Both Vpr mutants were separately cloned onto pNL(YU-2)-EGFP and pNL(AD8)-EGFP background, as described in Section 3.1.2 (see Figure 5 for illustration). The resulting four mutants, pNL(YU-2)-EGFPΔVpr, pNL(YU-2)-EGFP-VprA30L, pNL(AD8)-EGFPΔVpr, and pNL(AD8)-EGFP-VprA30L, were packaged in HEK 293T cells for virus production using transfection. All mutants were capable of EGFP expression in HEK 293T cells, with approximately 90% transfection efficiency (data not shown).

Wild-type and mutant viruses from both constructs were used for infections in primary MDMs for analysis of EGFP expression patterns in Donor 78, as shown in Figure 13. Both NL(YU-2)-EGFP (Figures 13A-13C) and NL(AD8)-EGFP (Figures 13D-13F) shown similar

patterns between wild-type, ΔVpr, and A30L constructs. Wild-type reporter viruses seemed to yield the highest amount of productive infection, while the ΔVpr viruses expectedly resulted in minimal levels of productive infection. Interestingly, the A30L mutant viruses showed a near recovery in EGFP expression, indicating an increased efficiency in productive infection in comparison to the ΔVpr mutants. Collectively, these comparisons indicate that the A30L mutation successfully circumvents the replication deficiencies seen in the ΔVpr mutation.

NL(YU-2)-EGFP MUTANTS   **NL(AD8)-EGFP MUTANTS**

A. 6.2%   D. 9.0%   WILD-TYPE

B. 1.7%   E. 0.1%   Δ VPR

C. 11.4%   F. 6.3%   A30L-VPR

G.

Figure 14. Quantification of EGFP expression confirms productive infection patterns of wild-type, ΔVpr, and Vpr-A30L constructs.
EGFP expression was further confirmed and quantified by flow cytometry, as shown by Donor 79 as a representative figure (A through F). The EGFP quantification of all donors (G) confirmed the productive infection patterns previously seen. NL4-3-exposed and uninfected MDMs were used as controls for auto-fluorescence.

The expression of EGFP similarly allows for direct quantification of productively-infected cells, as depicted in Figure 14 (also see Appendix). Each reporter construct was used in macrophage infections and analyzed for the proportion of infected macrophages that express EGFP, as represented in 14A-14F for Donor #79. Interestingly, NL(YU-2)-EGFP A30L had a higher percentage of productively-infected cells than wild-type NL(YU-2)-EGFP in Donor #79. These inconsistencies suggest that there is a high level of donor variability in infection patterns between wild-type and A30L replication efficiencies in MDMs. WT/A30L replication patterns seemingly rely on a multitude of host factors, which vary from donor to donor. However, mean expression levels of EGFP expression across four donors (Figure 14G) indicate that wild-type reporter constructs generally have a higher infectivity (8.1% EGFP+ in NL(YU-2)-EGFP WT, 9.3% EGFP+ in NL(AD8)-EGFP WT) in comparison to A30L-Vpr reporter constructs (7.7% EGFP+ in NL(YU-2)-EGFP A30L, 6.2% EGFP+ in NL(AD8)-EGFP A30L). Though EGFP quantification does accurately represent infection patterns among reporter constructs, it does not fully verify the functionality of Vpr mutants.

In order to further confirm that both the ΔVpr and A30L constructs properly function, viral protein expression was evaluated by immunoblotting. Because the Vpr-A30L mutation is deficient in virion association, infected cells will produce Vpr but it will not associate with budding virions for further infection. Therefore, Vpr should be present within infected cells, but not within released virions. The ΔVpr mutation, which does not produce Vpr, should result in a lack of Vpr in both infected cells and released virions.

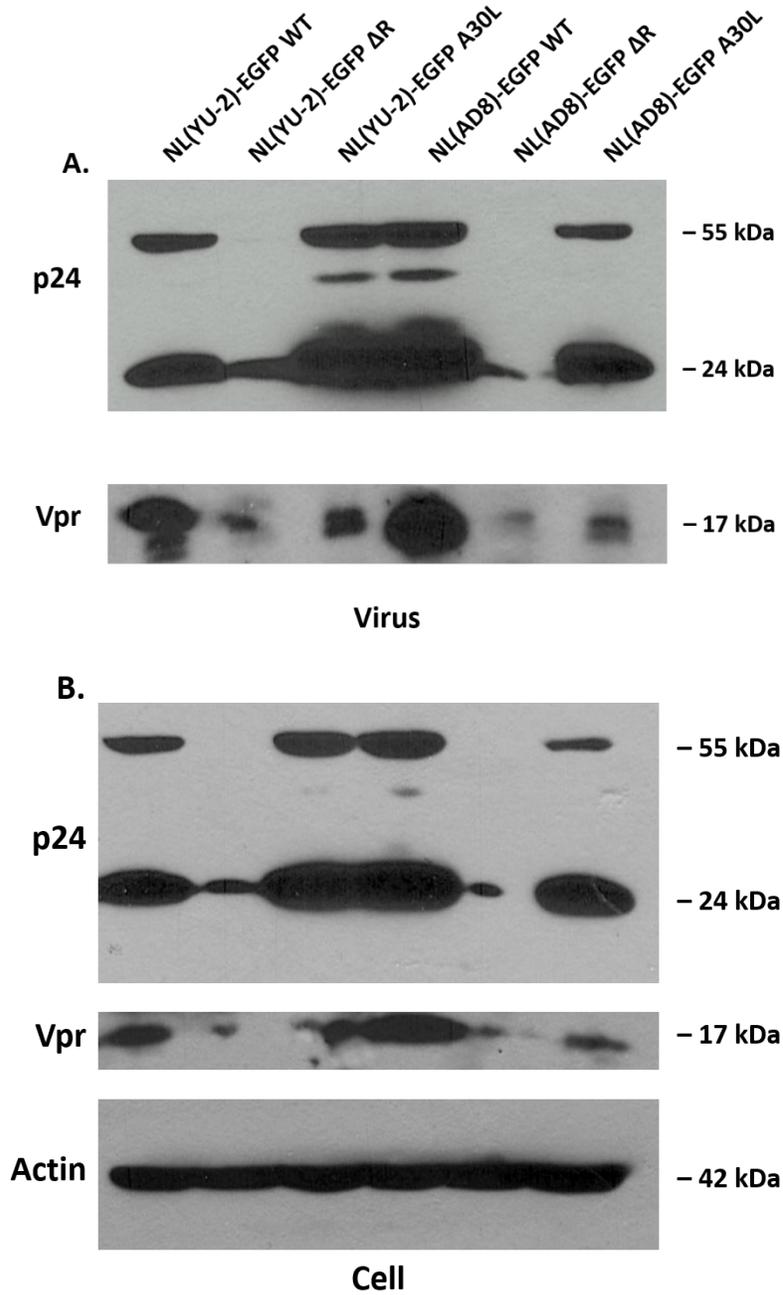**Figure 15. Expression of viral proteins in virus particles and infected cells from infected MDM cultures.**
p24 and Vpr expression levels were analyzed in virus particles from harvested supernatants and in cell lysates from infected MDMs.

As depicted in Figure 15, immunoblot comparisons of reporter virus constructs indicate that both the ΔVpr and A30L mutations produce the expected viral protein expression patterns in primary MDMs. Constructs were assessed for expression of the p24 capsid protein and Vpr. As expected, both wild-type clones (lanes 1 and 4; Figures A and B) produced sufficient amounts of p24 and Vpr in harvested supernatants and cell lysates. In contrast, the ΔVpr mutants (lanes 2 and 5; Figures A and B) yielded levels of p24 in both released virions and infected cells. This is corroborated by the minimal fluorescent expression found in infected MDMs and well-characterized replication deficiencies in macrophages. Vpr production was minimal in both released virions and infected cells, particularly in comparison to wild-type expression.

Remarkably, the A30L mutants (lanes 3 and 6; Figures A and B) produced similar levels of p24 to wild-type reporter constructs in both virus and cell lysates, indicative of a full recovery in replication kinetics. Immunoblotting revealed that Vpr production within cells infected with the A30L mutants was present, but reduced in comparison to wild-type production levels. Vpr was minimally present in harvested supernatants. As such, both ΔVpr and Vpr-A30L mutants express viral proteins as expected, which confirms their functionality in primary MDM infections.

Furthermore, the replication kinetics of each reporter virus was evaluated for additional verification of replication patterns, as shown in Figure 16. As previously described, replication kinetics curves were assembled based on p24 ELISA quantifications for each reporter construct. Primary MDMs were isolated from four independent donors to characterize the replication kinetics of NL(AD8)-EGFP viruses (yellow) and NL(YU-2)-EGFP viruses (blue).
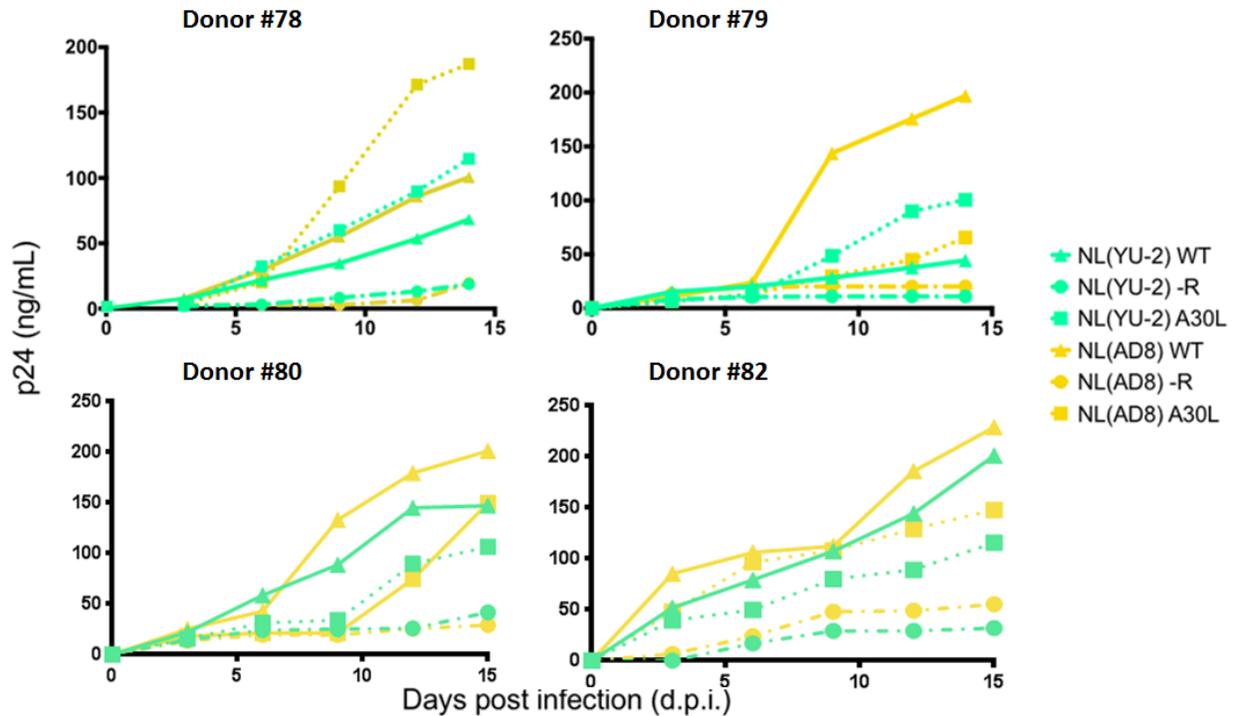
Figure 16. Vpr mutations in chimeric reporter viruses demonstrate distinct replication kinetics patterns in primary MDMs.
Cumulative p24 replication kinetic curves were assembled based on p24 ELISA quantifications of harvested supernatants from infected MDMs at days 0, 3, 6, 9, 12, and 14. NL(AD8)-EGFP (yellow) and NL(YU-2)-EGFP (green) are compared among wild-type (solid line), ΔVpr (dashed line), and Vpr-A30L (dotted line).

In each donor, the ΔVpr mutant viruses in each reporter construct (dashed lines) replicated at a consistently lower rate than the wild-type and Vpr-A30L viruses. However, the comparison of replication rates between Vpr-A30L and wild-type viruses varied across donors, which is consistent with the productive infection quantification seen in Figure 14G. As shown in Donor #78, the Vpr-A30L viruses in both constructs replicated at a noticeably higher rate than their respective wild-type counterparts. However, NL(YU-2)-EGFP WT displayed a higher replication rate in comparison to NL(YU-2)-EGFP A30L in Donor #79, while the NL(AD8) constructs remained consistent from Donor #78. Donors #80 and #82 depicted both wild-type constructs

with higher replication rates than both Vpr-A30L constructs. The donor-specific variations in A30L and wild-type replication patterns mimic the results found in quantification of EGFP production, thus supporting the fact that replication rates correlate to infectivity.

4.1.7 Aim 1 Summary

The focus in this aim was to construct and characterize a macrophage-tropic reporter reporter virus for use in the cell-specific analysis of macrophage subpopulations that are exposed or productively-infected for further analyses. Two chimeric constructs were used in this study: NL(YU-2)-EGFP, which utilizes the R5-tropic YU-2 envelope region, and NL(AD8)-EGFP, which utilizes the AD8 *gp120*. Both of these constructs are capable of expressing EGFP upon productive infection in MDMs and provide an accurate assessment for infectivity. NL(YU-2)-EGFP and NL(AD8)-EGFP allow for expression of viral proteins and can replicate efficiently in macrophages. These chimeric reporter viruses also replicate similarly to their parental strains, indicating a similar pathogenesis to their wild-type counterparts in macrophages.

In order to study the effects of Vpr on host-cellular function and cytokine expression, two Vpr mutants were separately introduced onto the reporter viruses: ΔVpr and Vpr-A30L. Both mutations were verified by sequence analysis, restriction digest analysis, and Vpr immunoblotting. As expected, the ΔVpr mutation caused significant drops in replication efficiency, as corroborated by the low amounts of p24 detected by immunoblotting. The Vpr-A30L mutation yielded surprising yet inconsistent results: in some donors, the Vpr-A30L constructs replicated at higher rates than the wild-type constructs, though donor variability seems to play a significant role in the determination of these replication rates.

4.2   AIM 2:   EFFECTS OF VPR ON HOST CELLULAR GENE EXPRESSION

In the previous aim, an *in vitro* modeling system was constructed and established for further analysis in infected macrophage subpopulations. The construction of chimeric reporter viruses were utilized as markers for productive infection based on fluorescently-tagged viral constructs. Thus, the subpopulations separated based on this modeling system include productively-infected macrophages, through the presence of green fluorescence, and non-productively-infected macrophages, through the absence of fluorescence. The non-productively-infected macrophage subpopulation includes uninfected, latently-infected, and exposed cells. Fluorescence-based separation allows for a multitude of analyses, including cytokine production, transcriptome profiling, and microRNA analysis.

As previously discussed, our laboratory recently identified Vpr as a modulator for IL-1β and IL-8 expression in macrophages, which directly induces neuronal injury. Guha et al. (2012) utilized wild-type R5-tropic viruses (YU-2) for macrophage infections and analyzed for cytokine and chemokine production in harvested supernatants. Results from that study indicate that YU-2 ΔVpr-infected macrophages yielded significantly lower IL-8 and IL-1β levels than YU-2 wild-type-infected macrophages, which was further confirmed by the analysis of signaling pathways responsible for IL-8 and IL-1β production. In addition to IL-8 and IL-1β modulation, the previous study also determined that CXCL10 may also be regulated by Vpr. However, significance in CXCL10 expression was only identified at the protein level, but not the RNA level. Though it was not found to be significantly modulated by Vpr in the previous study, TNF-α will also be analyzed due to its implication in neuronal injury.

In Aim 2, we intend to verify that the fluorescently-tagged chimeric reporter viruses are capable of producing similar patterns of cytokine expression among wild-type and ΔVpr constructs

as were established by Guha, et al. Similarly, A30L-Vpr is used for a replication-competent, functional-deficient analysis of the impact of Vpr on cytokine analysis. Furthermore, production of IL-1β, IL-8, CXCL10, and TNF-α will be analyzed at the protein level as a comparison between productively-infected macrophages and non-productively-infected macrophages, as separated by EGFP expression from reporter viruses.

4.2.1   Verification of the role of Vpr on cytokine expression in chimeric reporter viruses

In order to verify that the Vpr mutant reporter viruses behaved similarly to the wild-type and ΔVpr viruses utilized in the study previously described, ELISAs for IL-1β, IL-8, CXCL10, and TNF-α were performed on harvested MDM supernatants infected with mutant reporter viruses. In particular, IL-1β, IL-8, and CXCL10 expression was previously found to be significantly decreased during HIV-1 infection using YU-2 ΔVpr in comparison to YU-2 wild-type. Similar patterns in cytokine expression for chimeric reporter virus infections are verified using NL(YU-2)-EGFP wild-type and ΔVpr, as well as NL(AD8)-EGFP wild-type and ΔVpr. Additionally, A30L reporter constructs were investigated for cytokine expression patterns in relation to wild-type and ΔVpr levels.

As depicted in Figures 17 through 20, cytokine expression levels reveal distinct patterns among wild-type, ΔVpr, and Vpr-A30L constructs. Uninfected macrophages were also analyzed for basal-level expression of cytokines. Cytokine expression levels were evaluated in four separate donors infected with each chimeric reporter virus. Each donor is shown individually due to the high variability in numerical quantifications across donors. Cytokine production was evaluated at days 3, 6, 9, 12, and 15 for Donors 78 and 79. An additional timepoint at day 0, indicating the

time at which virus-containing media was removed, was taken for Donors 80 and 82 to evaluate for cytokine expression during viral exposure.

As described earlier, CXCL10 expression was previously found to be upregulated by Vpr through protein quantifications, but not RNA analysis. CXCL10 expression patterns among wild-type, ΔVpr, and Vpr-A30L constructs were variable, as shown in Figure 17. Donor 78 showed a slight decrease in CXCL10 expression in both ΔVpr constructs (top two panels), with no difference noticeable difference between wild-type, Vpr-A30L, and uninfected macrophages. Donor 79 revealed the most visible difference among Vpr mutants, with ΔVpr and uninfected secretions yielding similarly low levels. Again, wild-type and Vpr-A30L infections secreted similar amounts of CXCL10 during the course of infection. Donors 80 and 82 yielded similar results, with moderate downregulation of CXCL10 in ΔVpr mutants. However, wild-type and Vpr-A30L infections showed mixed patterns, with Vpr-A30L occasionally reaching higher CXCL10 concentrations than wild-type, as seen in the NL(YU-2)-EGFP constructs for Donor 80. Generally, CXCL10 production among reporter virus infections peaked at 9 days post-infection, with maximal production levels peaking at 30,000 pg/mL in Donors 78 and 79.

Figure 17. CXCL10 production by chimeric reporter virus infections, including wild-type (WT), ΔVpr (-R), and A30L, over time.
CXCL10 secretion levels were quantified by ELISA (in pg/mL) from harvested supernatants at days 0, 3, 6, 9, and 15 post-infection. Macrophages were isolated from four healthy donors (Donors 78, 79, 80, and 82) and infected with NL(YU-2)-EGFP WT, ΔVpr, and A30L (left column) or NL(AD8)-EGFP WT, ΔVpr, and A30L (right column). Wild-type production levels are marked in red, ΔVpr production levels in blue, and A30L production levels in green. Uninfected macrophages were evaluated for basal-level CXCL10 production (NT, in black).

Similarly, IL-1β and IL-8 production levels were analyzed for expression patterns among wild-type, ΔVpr, and Vpr-A30L constructs. As previously determined, ΔVpr results in a drastic decrease in both IL-1β and IL-8 expression, as shown in Figures 18 and 19. IL-1β expression was particularly downregulated in ΔVpr constructs, which largely follows the basal-level expression of uninfected macrophages (Figure 18). Across all four donors, ΔVpr maximally reaches 166.48 pg/mL of IL-1β at 3 days post-infection in the NL(YU-2)-EGFP construct, in comparison to 1212.62 pg/mL and 1033.73 pg/mL in wild-type and Vpr-A30L constructs, respectively. IL-8 expression also follows moderate patterns of downregulation among ΔVpr constructs (Figure 19). This strongly suggests that the role of Vpr in IL-8 and IL-1β is verified in the chimeric reporter virus constructs.

However, the use of A30L-Vpr constructs in IL-8 and IL-1β cytokine analysis yields variable results in comparison to wild-type, as seen in CXCL10 expression patterns. Surprisingly, Vpr-A30L infections generally peaked at higher levels in IL-1β expression than wild-type infections, excluding Donors 79 and 82 in the NL(AD8)-EGFP constructs. In contrast, IL-8 levels in Vpr-A30L were generally lower than wild-type expression levels (as seen in Donors 79, 80, and 82 from Figure 19).
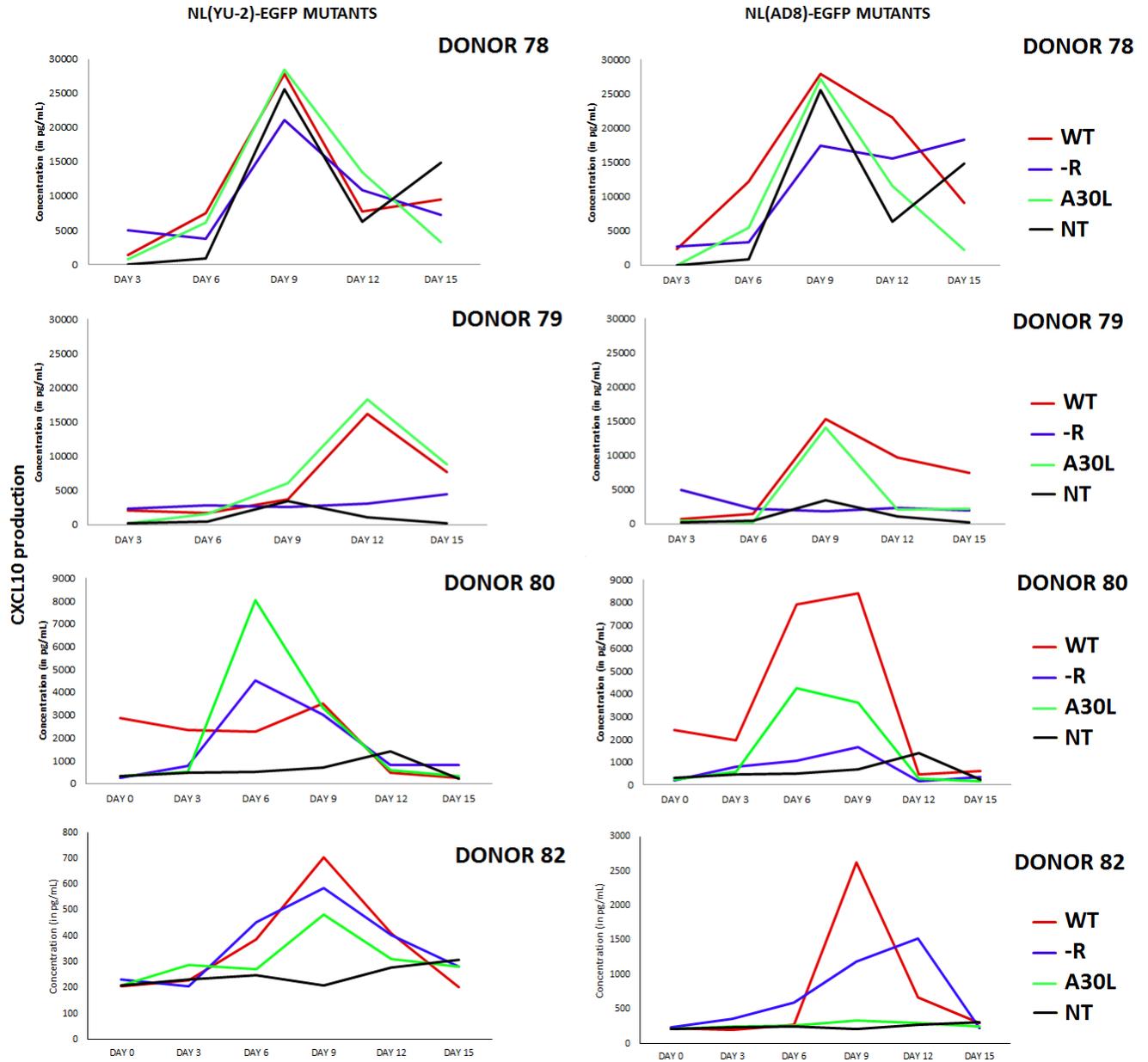
Figure 18. IL-1β production by chimeric reporter virus infections, including wild-type (WT), ΔVpr (-R), and A30L, over time.

IL-1β secretion levels were quantified by ELISA (in pg/mL) from harvested supernatants at days 0, 3, 6, 9, and 15 post-infection. Macrophages were isolated from four healthy donors (Donors 78, 79, 80, and 82) and infected with NL(YU-2)-EGFP WT, ΔVpr, and A30L (left column) or NL(AD8)-EGFP WT, ΔVpr, and A30L (right column). Wild-type production levels are marked in red, ΔVpr production levels in blue, and A30L production levels in green. Uninfected macrophages were evaluated for basal-level IL-1β production (NT, in black).

Figure 19. IL-8 production by chimeric reporter virus infections, including wild-type (WT), ΔVpr (-R), and A30L, over time.
IL-8 secretion levels were quantified by ELISA (in pg/mL) from harvested supernatants at days 0, 3, 6, 9, and 15 post-infection. Macrophages were isolated from four healthy donors (Donors 78, 79, 80, and 82) and infected with NL(YU-2)-EGFP WT, ΔVpr, and A30L (left column) or NL(AD8)-EGFP WT, ΔVpr, and A30L (right column). Wild-type production levels are marked in red, ΔVpr production levels in blue, and A30L production levels in green. Uninfected macrophages were evaluated for basal-level IL-8 production (NT, in black).
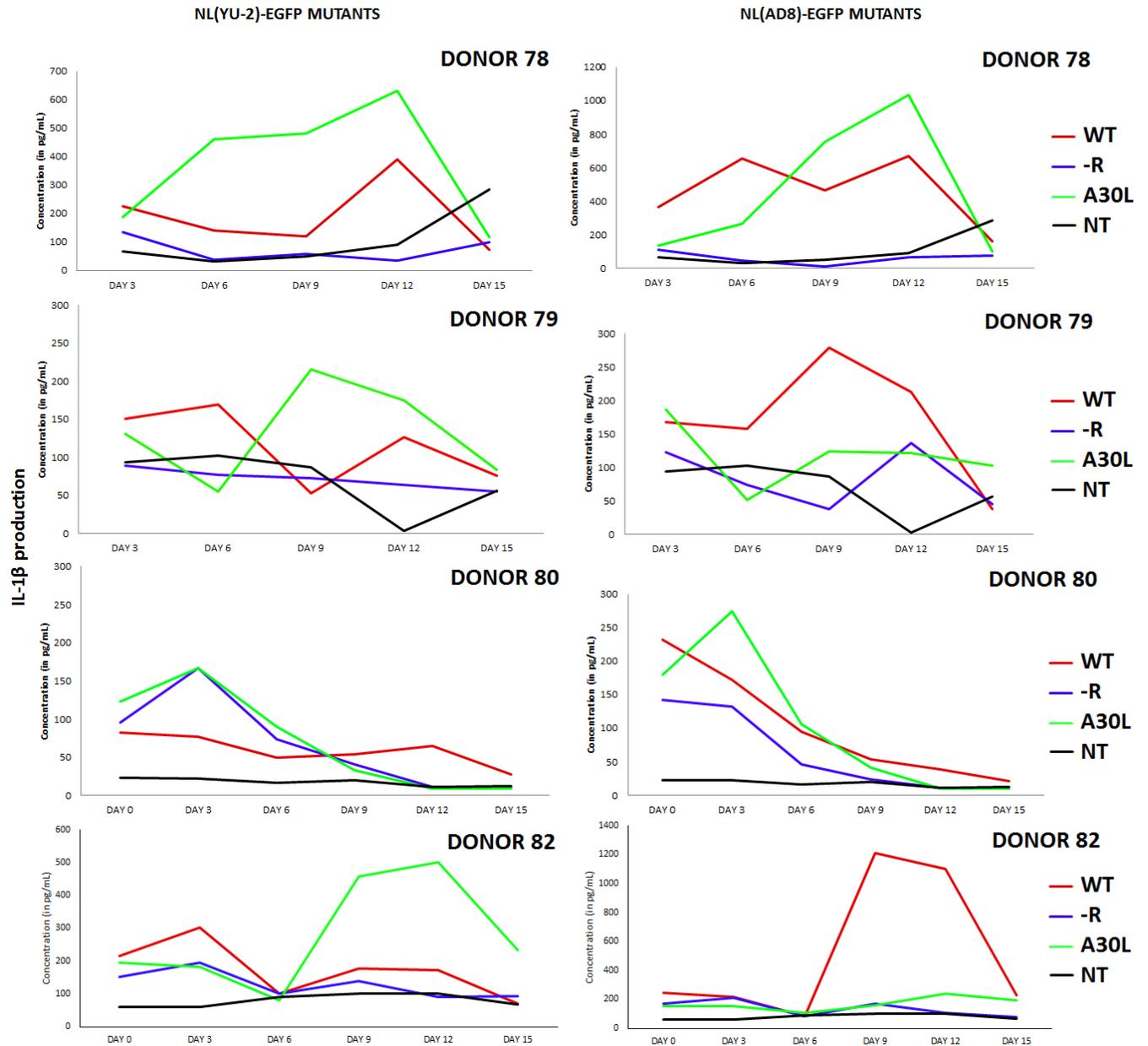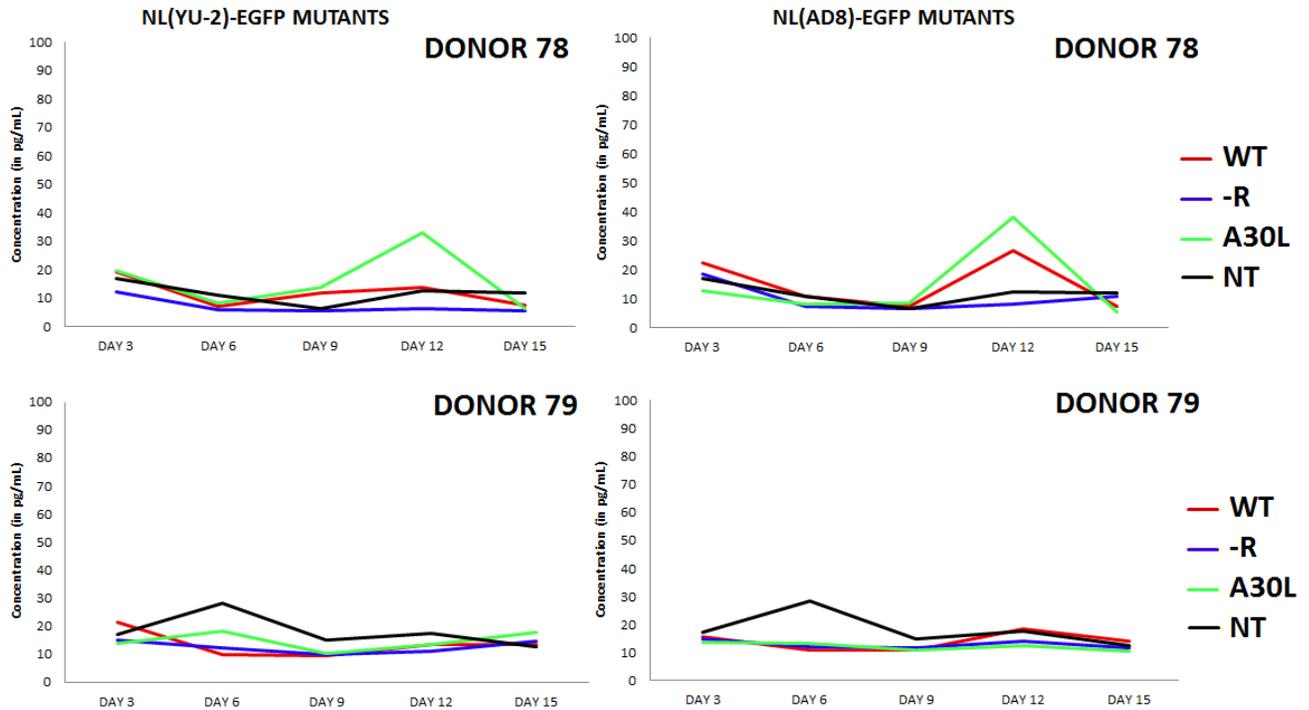
Figure 20. TNF production by chimeric reporter virus infections, including wild-type (WT), ΔVpr (-R), and A30L, over time.
TNF secretion levels were quantified by ELISA (in pg/mL) from harvested supernatants at days 0, 3, 6, 9, and 15 post-infection. Macrophages were isolated from four healthy donors (Donors 78, 79, 80, and 82) and infected with NL(YU-2)-EGFP WT, ΔVpr, and A30L (left column) or NL(AD8)-EGFP WT, ΔVpr, and A30L (right column). Wild-type production levels are marked in red, ΔVpr production levels in blue, and A30L production levels in green. Uninfected macrophages were evaluated for basal-level TNF production (NT, in black).
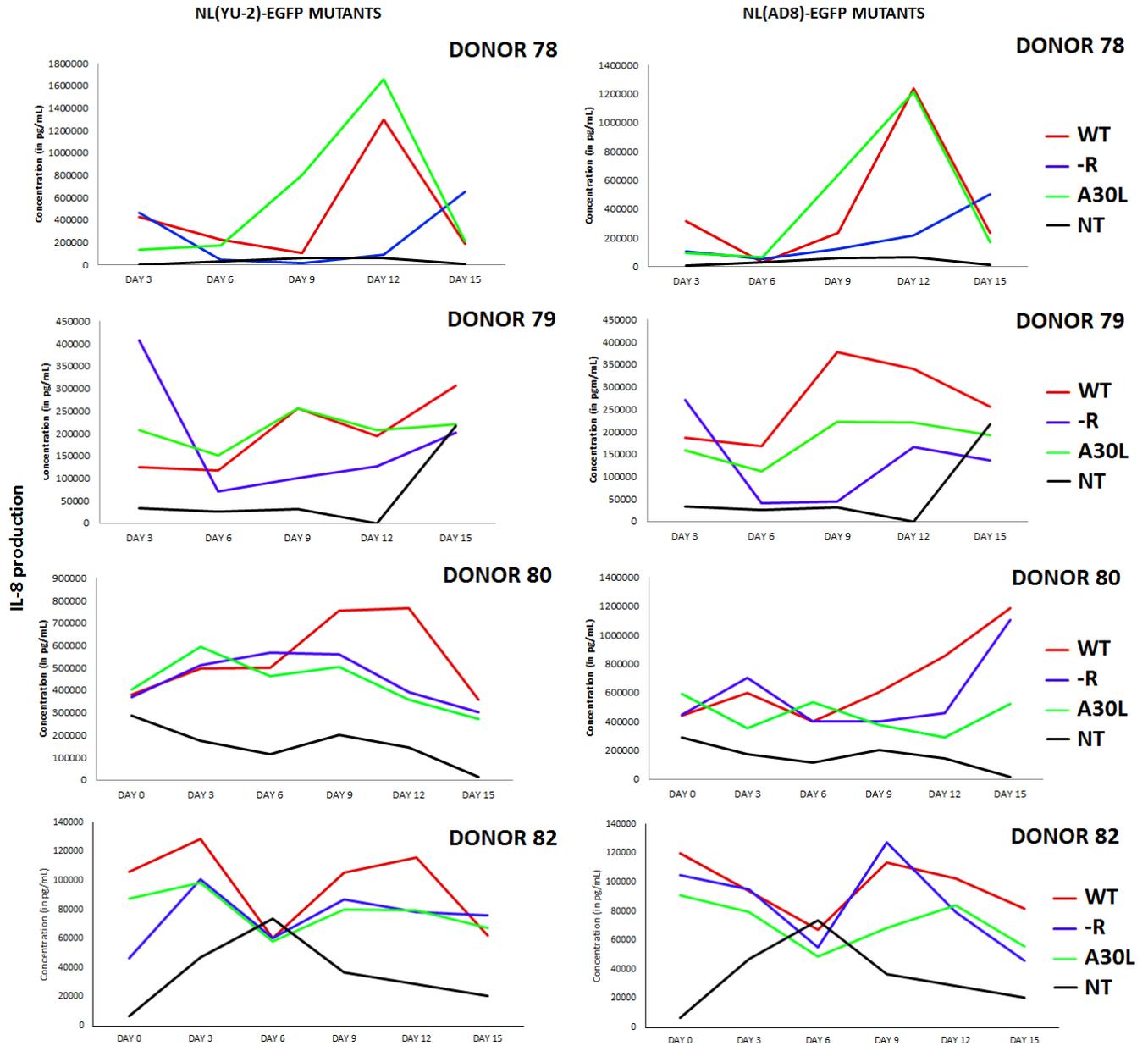
Expression levels of TNF-α were also analyzed in the Vpr mutant chimeric reporter viruses for Donors 78 and 79, depicted in Figure 20. As previously found by Guha et al. (2012), Vpr does not regulate TNF-α expression in protein or RNA production in the absence of a secondary signal. Therefore, TNF-α serves as an appropriate negative control for Vpr-mediated cytokine analysis in the context of viral infection. As expected, no differences in TNF-α levels were detected among wild-type, ΔVpr, and Vpr-A30L infections. Furthermore, low levels of TNF-α were produced among infected macrophages at the assessed time points, with maximal levels only reaching approximately 40 pg/mL. Both donors showed similar expression patterns between infected and

uninfected macrophage supernatants. Similar results have been previously seen in our laboratory using monocyte-derived macrophages. As such, earlier time points may be necessary to detect increased, HIV-1-mediated TNF-α production.

4.2.2   Cell-specific analysis of productively-infected and non-productively-infected monocyte-derived macrophages for expression of CXCL10, IL-8, IL-1β, and TNF-α

Following the analysis of secreted cytokines in macrophage supernatants, the contribution of cytokine production of different macrophage subpopulations in cytokine production was assessed.   Specifically, productively-infected macrophages and non-productively-infected macrophages were assessed for their individual cytokine expression patterns. In order to analyze macrophage subpopulations for expression, both EGFP expression and intracellular cytokine staining were utilized. In contrast to the overall production of cytokine secretion as determined by ELISA, this method allows for cell-specific analysis of cytokine production.

As represented in Figure 21 by the gating strategy, macrophages were run through a flow cytometer and gated using a forward scatter vs. side scatter plot (FSC vs SSC). Since macrophages are large cells with characteristic pseudopodia, the expected forward scatter values for true macrophages are also large, as represented in the top panel of Figure 21. The gated macrophage population was then further separated based on EGFP expression, as shown by the FITC vs "Count" histogram in Figure 21. Two peaks were separated based on EGFP expression: a peak below 10^3 FITC intensity, termed EGFP-negative, and a peak above 10^3 FITC intensity, termed EGFP-positive.
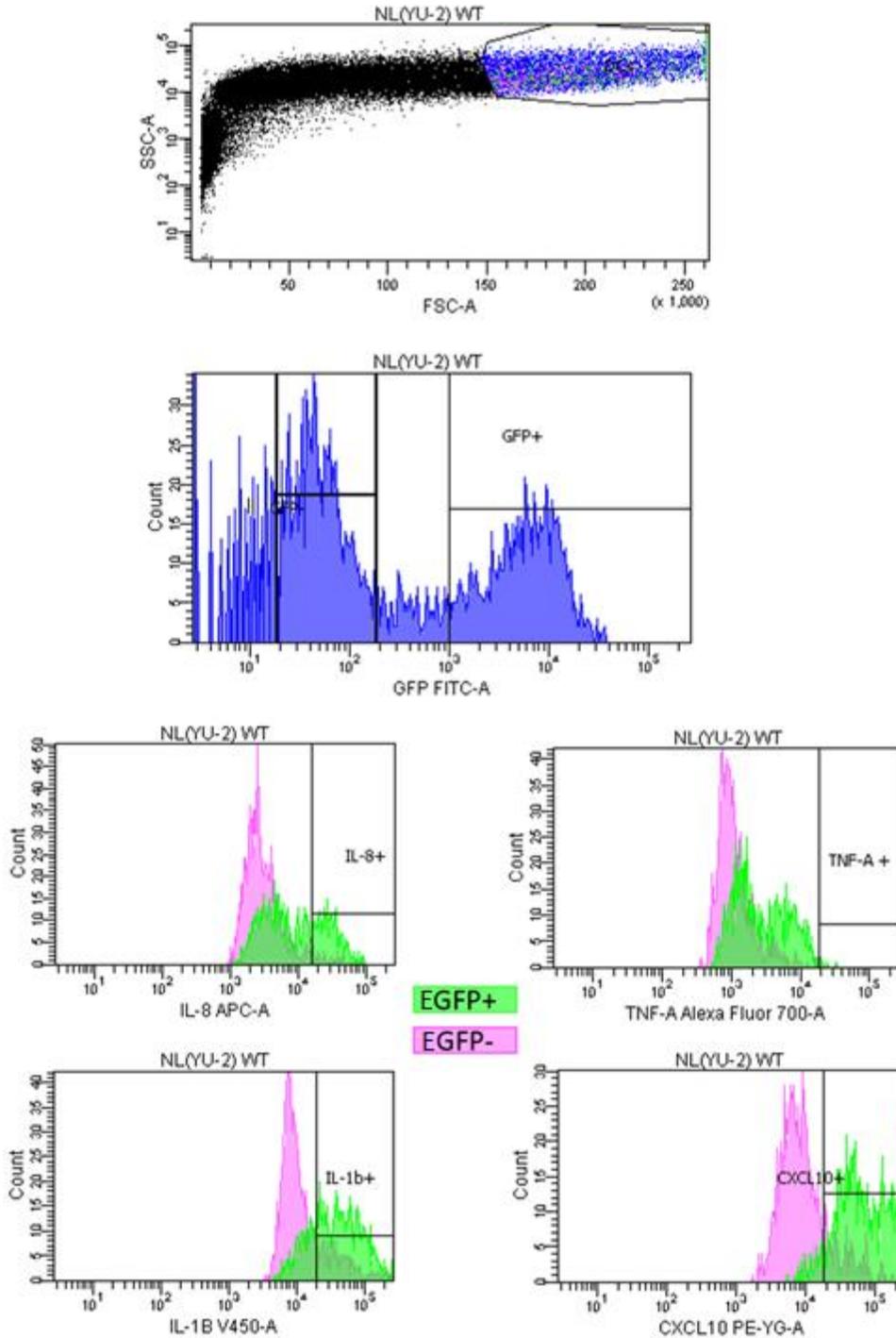
Figure 21. Representative gating strategy for cell-specific cytokine analysis of productively-infected and non-productively-infected MDMs.
After 14 days post-infection, infected macrophages were detached and stained for intracellular IL-8, IL-1β, CXCL10, and TNF-α. EGFP production, as analyzed by FITC quantification, was used as a marker for productive infection. Macrophage populations were separated based on EGFP expression and separately analyzed for cytokine expression (pink = EGFP-negative, green = EGFP-positive).

Following EGFP expression-based gating, each subpopulation (EGFP-negative and EGFP-positive) were further analyzed for cytokine expression, as represented in the bottom panel of Figure 21. Both subpopulations were analyzed for expression of CXCL10, IL-8, IL-1β, and TNF-α simultaneously. A stringent gating strategy (above 10^4 intensity) was utilized to minimize basal-level expression patterns, especially in the non-productively-infected macrophage subpopulation. As depicted, the EGFP-positive histogram (green) and EGFP-negative histogram (pink) were used to differentiate between subpopulations. From this analysis, the percentage of cytokine-positive cells for each cytokine were assessed. In order to ensure an accurate measurement of cytokine expression, a minimum of 100 cytokine-positive cells were necessary for percentage calculations. Due to the inefficient infectivity of NL(AD8)-EGFP ΔVpr, EGFP-positive subpopulation cytokine expression could not be assessed.

In order to assess modulations in cytokine expression among reporter virus constructs, the percentage of expression-positive cells for each cytokine were assessed for three donors in both NL(YU-2)-EGFP and NL(AD8)-EGFP constructs. Cell-specific analyses were classified into EGFP-positive, or productively-infected macrophages, and EGFP-negative, or non-productively-infected macrophages, as demonstrated in Figures 22 and 23, respectively. However, high levels of donor variability, as previously seen in infectivity and ELISA quantification results, restrict statistical analyses in cytokine regulation. More donors are needed for statistical relevance.

As shown in Figure 22, the EGFP-positive subpopulation was analyzed for the expression of CXCL10, IL-8, IL-1β, and TNF-α in both NL(YU-2)-EGFP and NL(AD8)-EGFP constructs. Among productively-infected macrophages, NL(YU-2)-EGFP ΔVpr shows a mean decrease in the number of cytokine-positive cells (27.5%) from NL(YU-2)-EGFP wild-type (43.8%) in IL-8.

Similarly, a slight decrease was also seen in IL-1β, 67.9%-positive in wild-type and 43.2%-positive in ΔVpr. However, very little differences were seen in CXCL10 and TNF-α production between wild-type and ΔVpr. Comparisons among NL(AD8)-EGFP wild-type and ΔVpr could not be determined due to the low number of cytokine-positive cells in each donor.

Figure 22. Cell-specific analysis of cytokine expression levels of IL-8, IL-1β, CXCL10, and TNF-α for productively-infected macrophage subpopulations.
Macrophages were infected with NL(YU-2)-EGFP wild-type (WT), NL(YU-2)-EGFP ΔVpr (–R), NL(YU-2)-EGFP A30L, NL(AD8)-EGFP wild-type (WT), NL(AD8)-EGFP ΔVpr (–R), or NL(AD8)-EGFP A30L and cultured for 14 days. Cytokine expression levels were analyzed using intracellular staining and flow cytometry in macrophages subpopulations capable of EGFP expression. Percentage of cells positive for cytokine expression were used for the calculation of standard deviation and median. Expression was analyzed across three donors: Donor #79 (red), Donor #80 (purple), and Donor #82 (blue).

The comparison between Vpr-A30L and wild-type in both NL(YU-2)-EGFP and NL(AD8)-EGFP constructs yielded similar results. Among productively-infected cells, Vpr-A30L produced similar levels of IL-1β, CXCL10, and TNF-α when compared to wild-type infection in both constructs, revealing a recovery in cytokine expression. Productively-infected cells infected with Vpr-A30L virus (20.1% IL-8-positive in YU-2, 23.4% IL-8-positive in AD8) expressed lower levels of IL-8 than wild-type infections (43.8% IL-8-positive in YU-2, 38.6% IL-8-positive in AD8). When compared to NL(YU-2)-EGFP ΔVpr infection (27.5% IL-8-positive), the NL(YU-2)-EGFP A30L infection (20.1% IL-8-positive) yielded lower amounts of IL-8 production, as well. However, this pattern of expression is also revealed in ELISA quantifications, as shown in Figure 19.

Additionally, cytokine expression was also analyzed in the non-productively-infected macrophage subpopulations in Figure 23. As expected, the median percentages of cytokine-positive cells in the non-productively-infected macrophage group (Figure 23) are typically lower than those of the productively-infected macrophage group (Figure 22). However, there are no noticeable variations among ΔVpr mutant infections in the EGFP-negative subpopulation in comparison to wild-type infections. Similarly, there are no noticeable trends among Vpr-A30L mutant infections in comparison to wild-type infections. Interestingly, the comparison of NL4-3-exposed macrophage populations to uninfected macrophage populations revealed that viral exposure results in a slight increase in cytokine expression, despite the absence of productive infection and viral replication by 15 days-post infection.
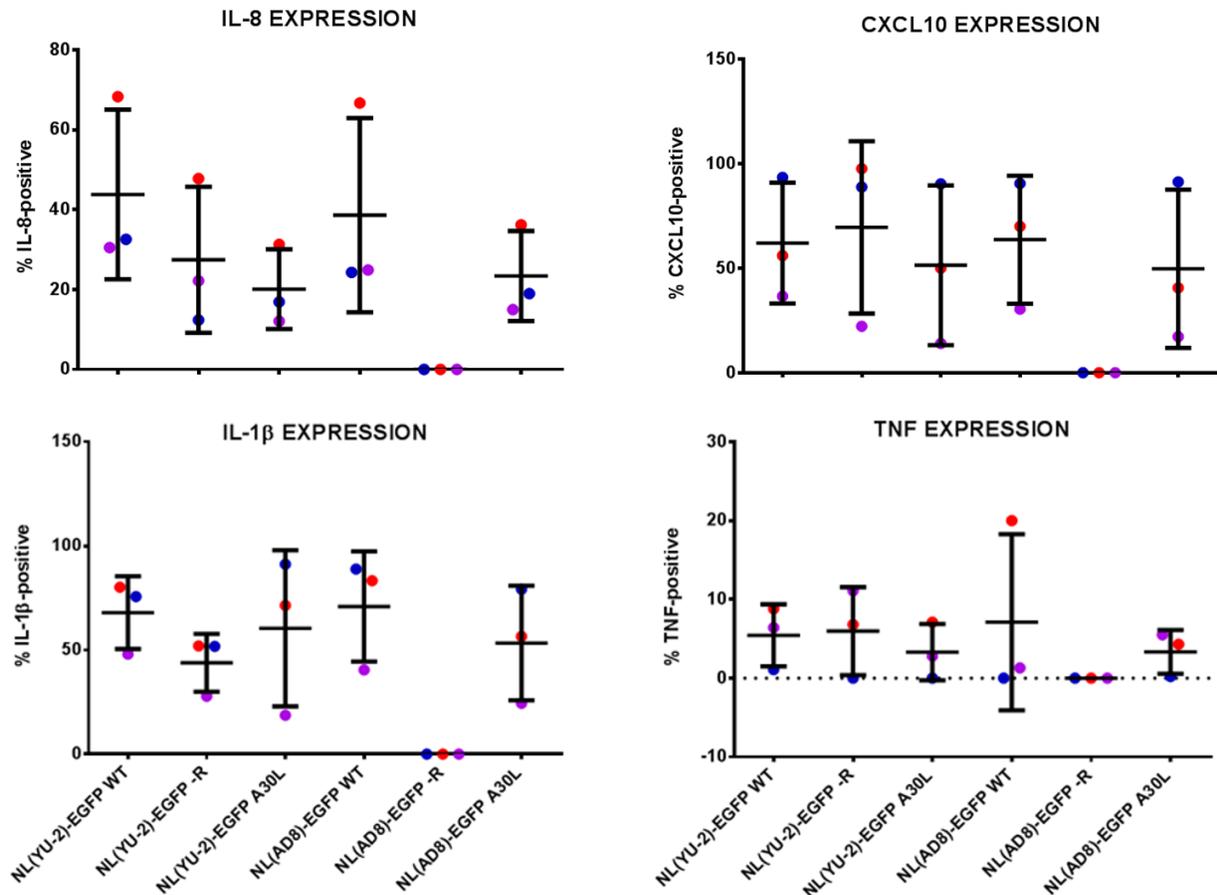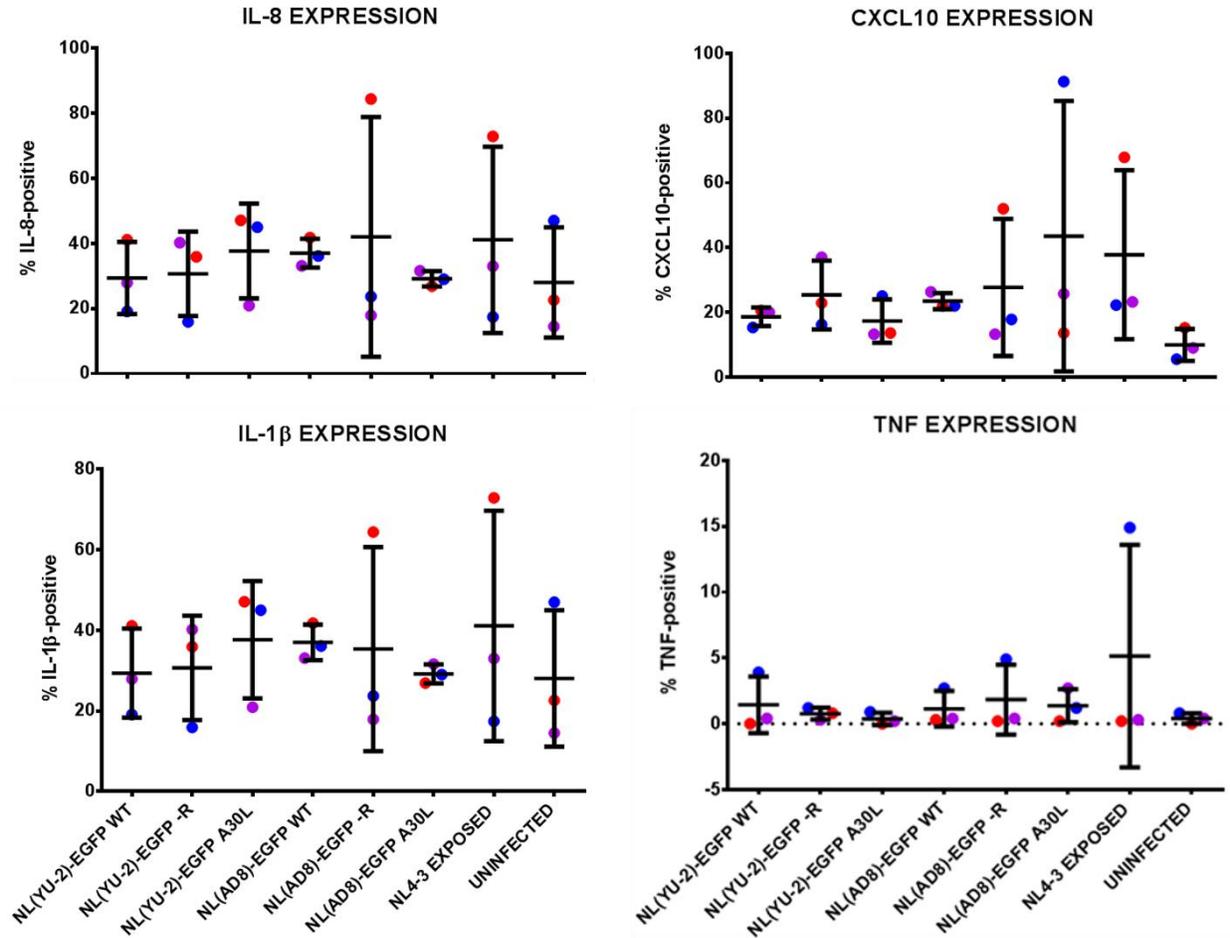
Figure 23. Cell-specific analysis of cytokine expression levels of IL-8, IL-1β, CXCL10, and TNF-α for non-productively-infected/exposed macrophage subpopulations.
Macrophages were infected with NL(YU-2)-EGFP wild-type (WT), NL(YU-2)-EGFP ΔVpr (–R), NL(YU-2)-EGFP A30L, NL(AD8)-EGFP wild-type (WT), NL(AD8)-EGFP ΔVpr (–R), or NL(AD8)-EGFP A30L and cultured for 14 days. Cytokine expression levels were analyzed using intracellular staining and flow cytometry in macrophages subpopulations without EGFP expression. Percentage of cells positive for cytokine expression were used for the calculation of standard deviation and median. Expression was analyzed across three donors: Donor #79 (red), Donor #80 (purple), and Donor #82 (blue).

4.2.3   Aim 2 Summary

The focus of this aim is to utilize the constructed chimeric reporter viruses and their Vpr mutants, ΔVpr and Vpr-A30L, for inflammatory factors expression analysis. Specifically, the cytokines analyzed were chosen based on a previous study related to Vpr-mediated cytokine expression in neuronal injury: CXCL10, IL-8, IL-1β, and TNF-α. In this study, ΔVpr mutants within the NL(YU-2)-EGFP and NL(AD8)-EGFP constructs had lower expression patterns in IL-8, IL-1β, and CXCL10, which is consistent with previous data. This successfully verifies that the chimeric reporter constructs produce similar expression patterns in relation to Vpr-regulated cytokine modulation. Additionally, infections with the Vpr-A30L mutant virus generally yielded a recovery or increase in cytokine production in comparison to wild-type, which indicates that the Vpr-A30L virion association mutation is not sufficient for the downregulation of Vpr-mediated expression of cytokines.

Furthermore, cytokine expression was analyzed by utilizing the separation of productively-infected macrophages and non-productively-infected macrophages. Analysis of productively-infected macrophages revealed that the productively-infected macrophage population of NL(YU-2)-EGFP ΔVpr infections yielded lower levels of IL-8 and IL-1β production in comparison to NL(YU-2)-EGFP wild-type. This confirms the function of Vpr in IL-8 and IL-1β expression and further suggests that Vpr-regulated cytokine expression is not replication-dependent. Moreover, productively-infected macrophages infected with Vpr-A30L viruses depict lower expression patterns of IL-8, which may indicate a role for virion association in IL-8 upregulation.

5.0   DISCUSSION

HIV-1 macrophage infection plays a crucial role in the establishment of a viral reservoir and the maintenance of infection. One of the mechanisms attributed to HIV-1 induced immune dysregulation is selective inhibition of the cytokine network in monocytes, macrophages, and dendritic cells, which eventually alters immune function. HIV-1-infected dendritic cells, subjected to exposure of different types of viral factors, differentially regulate Th1 and Th2 cytokine production to impair host protective antiviral immunity and facilitate viral replication. Studies have shown that Vpr selectively suppresses the production of cytokine IL-12 but not IL-6 upon CD40L stimulation [82-84]. The downregulation of inflammatory cytokines by HIV-1 Vpr[+] virus-exposed or -infected dendritic cells and upregulation of IL-10 found in our recent studies further confirm the role of this protein in host cytokine dysfunction [81]. Although signaling pathways directing the maturation of dendritic cells have been delineated by different groups, there is no clear indication which particular cascade is predominantly deregulated during HIV-1 induced dendritic cell function.

Different studies showed different patterns of alteration of cytokine profiling during HIV-1 infection. Although not much work has been done to detail the role of different HIV-1-derived factors in the regulation of cytokine production, studies from our laboratory and others have shown that Vpr as a soluble protein or as a virion-associated molecule inhibits IL-12 production from both DC and PBMC upon specific stimulation conditions [93-95]. Interestingly, TNF-α production was shown to enhance in the presence of Vpr upon a second signal by primary myeloid-derived target cells [96-98]. In case of plasmacytoid dendritic cells (pDCs), Vpr diminishes IFN-γ

production [99]. It will be interesting to see in the future how different viral proteins interact to regulate the cytokine network during HIV-1 infection and influence the course of infection.

Among a multitude of functions in disease progression, macrophages and microglia contribute to the viral invasion and neuronal injury associated with HIV-1-associated neurocognitive disorders (HAND). Recently, our laboratory has determined that the HIV-1 accessory protein viral protein R (Vpr) mediates the expression of IL-1β and IL-8, which in turn caused neuronal injury and contributed to HIV-1-associated neurocognitive disorders. Together these studies further support the notion that HIV-1 Vpr alters the host cellular cytokine/chemokine expression in host cells. However, it is not clear whether *de novo* synthesized and/or virion-associated Vpr contribute to host cellular transcription in a synergistic or additive manner.

This study aims to further elucidate the role of Vpr in cytokine expression in monocyte-derived macrophages through the use of cell-specific analysis. The utilization of cell-specific analysis in this study is based on the construction of a fluorescent-tagged reporter virus capable of macrophage infection. Fluorescence utilization allows for the separation of productively-infected cells from non-productively-infected cells. This modeling system for infection identification has been successfully implemented in several other studies [79, 100]. Furthermore, these studies utilized chimeric reporter viruses with macrophage-tropic envelope regions, thus substantiating the reliability and likeness of the constructed reporter viruses to wild-type isolate infections. Though fluorescent-tagged wild-type isolates would be the most desirable reporter virus, several HIV-1 isolates, including HIV-1 YU-2, are particularly difficult to manipulate via cloning due to the flanking genomic DNA sequences, as well as a lack of proper sequence information of the parental clone.

Ideally, productively-infected cells and non-productively-infected cells each behave similarly to the establishment of infection, which creates a homogenous subpopulation of cells with similar responses to infection upon fluorescent separation. Therefore, cell-specific analysis of these subpopulations allows for the elucidation of pathogenesis in productively-infected cells and the understanding of exposure in bystander cells. As previously described, bystander cells are known to be affected by surrounding soluble viral proteins, including gp120, Tat, and Vpr. These soluble viral proteins can transactivate host transcriptional factors, thus altering the transcriptional profile of non-productively-infected macrophages. Specifically, Vpr is known to transactivate a wide array of host promoters and transcription factors, such as SAPK/JNK, NF-κB, and p300 [28, 101]. Studies have shown that soluble Vpr are capable of host transcriptional activation, which influence bystander macrophage characterization. However, the data collected in this study showed little visible difference among non-productively-infected macrophages exposed to wild-type and ΔVpr viruses. The overall exposure of non-productively-infected macrophages to soluble proteins may not have been uniformly substantial enough to detect any variability across mutants.

In this study, two particular Vpr mutations were used in the analysis of Vpr-mediated cytokine expression in relation to HAND development: ΔVpr, which results in both a functional and structural deficiency of Vpr in pathogenesis, and Vpr-A30L, which results in de novo synthesis in the infected cells and deficiency in virion-association function. The ΔVpr mutation was previously used by Guha et al. (2012) in the YU-2 ΔR isolate for similar functional analyses [44]. The use of YU-2 ΔR resulted in a significant drop in viral replication, which further complicated the functional analysis of Vpr. Though ΔVpr showed a significant downregulation of IL-1β and IL-8 expression in relation to the wild-type isolate, the lower replication kinetics of ΔVpr raised a

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question: is the downregulation of IL-8 and IL-1β in ΔVpr infection a result of deficient Vpr function, or is it a result of the lower viral replication associated with ΔVpr infection? This study aimed to clarify this issue by specifically isolating productively-infected macrophages in ΔVpr infections for cytokine production analysis.

As earlier described, NL(YU-2)-EGFP ΔVpr infections supported the previous findings: Vpr mediates the expression of IL-1β and IL-8 in productively-infected macrophages. However, the previous study also determined that CXCL10 may be Vpr-mediated at the protein level, though no significant modulations were found at the RNA level. Further analyses in this study determined that though there was a moderate downregulation of CXCL10 in overall cytokine quantifications for ΔVpr infections, there was no visible difference in CXCL10 production in productively-infected macrophages. This suggests that CXCL10 may be downregulated in ΔVpr infections due to the deficiency in replication kinetics and not the deficiency in functionality. Although no previous studies exist on the correlation between Vpr and CXCL10 expression, Si et al. (2002) determined that CXCL10 expression was dependent on viral replication in microglial cells, thus suggesting a confounding issue for CXCL10 expression in ΔVpr infection analyses [102].

Furthermore, TNF-α expression was measured as a negative control in this study, particularly because previous studies in our laboratory have found no significant Vpr mediation for TNF-α. However, diminutive amounts of TNF-α were observed in each donor. Expression of TNF-α for *in vitro* experiments typically requires induction from external sources outside of viral infection, such as LPS-TLR ligand stimulation. Because this study is solely interested in virus-host interactions without external induction, LPS stimulation was never conducted. TNF-α amounts are found in high amounts during *in vivo* infection, primarily thought to be induced by surrounding stimulants. Some increases in TNF-α expression may occur during entry and

establishment of infection in monocyte-derived macrophages; however, this would require hourly timepoints that were not the focus of this study.

In addition to confirming the reliability of ΔVpr infections, the Vpr-A30L virion association defective mutant was also analyzed for any role in cytokine expression. Vpr-A30L viruses cannot associate with budding virions, thus not allowing for effect during early infection phase (before de novo synthesis) or association with the pre-integration complex in further infections. Other studies utilizing A30L have determined that Vpr-A30L fails to oligomerize, does not concentrate at the nuclear envelope, and cannot function in G2 cell cycle arrest in proliferating CD4+ T cells [63, 64]. However, these studies were completed using transfection methods with Vpr mutant plasmids to understand Vpr-A30L function. This study aimed to understand the effects of virion association mutations in Vpr during the different phases of infection life cycle.

Interestingly, the Vpr-A30L mutation resulted in replication kinetics that were similar to wild-type viruses in monocyte-derived macrophages. Though Vpr-A30L lacks important functions for macrophage infection, including virion/PIC association, nuclear envelope concentration, and oligomerization, it remains competent in viral replication. This may be explained by the fact that Vpr-A30L remains within macrophages throughout the course of infection, leading to a more concentrated amount of Vpr within the cell than in wild-type infections. Therefore, Vpr-A30L may interact more extensively with viral and host transcription factors within the nucleus, due to its absence in virion packaging. Theoretically, this would result in an enhanced level of viral copies and would lead to a higher infectivity, despite deficiencies in Vpr functionality. More analyses of Vpr-A30L subcellular localization and transactivation during the course of infection would be needed to substantiate this observation.

Because infections with Vpr-A30L mutant viruses have not previously been carried out, its effects in HIV-1 viral pathogenesis are generally unknown. In this study, we aimed to understand if virion association was necessary for the expression of cytokines that were previously established to be Vpr-mediated. Such mutational analyses have previously utilized to understand the structure-function relationship of Vpr, and have previously elucidated key protein-protein interactions responsible for Vpr functionality [103]. Therefore, the expression of IL-8, IL-1β, and CXCL10 were analyzed in Vpr-A30L infections. As corroborated by ELISA cytokine quantifications and cell-specific analysis in monocyte-derived macrophages, virion association may play a role in the Vpr mediation of IL-8 expression. This narrows the potential functions of Vpr that may affect IL-8 expression to virion association, nuclear envelope concentration, or oligomerization. Because Vpr-mediated IL-8 expression has been previously shown to occur through p38 and SAPK/JNK signaling pathways, virion association may play a role in the activation of this pathway and may be structurally involved in Vpr-mediated binding to the IL-8 promoter region [44, 104].

This study additionally sought to identify the macrophage subpopulation responsible for the expression of Vpr-mediated cytokines, including IL-8 and IL-1β. Though non-productively-infected macrophages did produce moderate amounts of these cytokines, Vpr-mediated expression was particularly evident in productively-infected macrophages. This strongly implicates Vpr in the pathogenesis and development of HAND, since IL-8 and IL-1β are well-known proinflammatory mediators involved in neurocognitive disorders. Furthermore, IL-8 and IL-1β, along with several other cytokines, have been previously found to have common transcription factor binding sites within their promoters, potentially resulting in a Vpr-transcription factor interaction to mediate the expression of these cytokines [44].

5.1   CONCLUSIONS

This study sought to elucidate the role of Vpr in cytokine expression in monocyte-derived macrophages, in relation to HIV-1-associated pathogenesis, disease progression and comorbidities. In order to further analyze the effects of Vpr on HIV-1 macrophage pathogenesis, reporter viruses were constructed in order to analyze macrophage subpopulations based on HIV-1 productive infection through the utilization of EGFP expression. Results from this study suggest that Vpr does play a role in the expression of IL-8 and IL-1β in productively-infected macrophages, which leads to immune dysfunction and neuronal injury. Furthermore, the virion association function of Vpr may specifically be involved in the Vpr-mediated expression of IL-8.

6.0   FUTURE DIRECTIONS

The current study utilized macrophage-tropic reporter viruses capable of EGFP expression, which was constructed by cloning the R5-tropic *env* region from YU-2 and AD8 HIV-1 isolates onto a EGFP-tagged, X4-tropic laboratory virus.   Comprehensive confirmation and characterization was completed to ensure that viral replication in primary macrophages with reporter viruses were similar to wild-type isolate infections. Additional portions of macrophage-tropic viral genes could be cloned onto the chimeric reporter viruses to further strengthen its likeness to wild-type isolates. Though EGFP reporter cloning into the wild-type YU-2 isolate has already been extensively attempted, this method could be repeated in order to obtain the most accurate macrophage infection and pathogenesis. An emphasis on stabilizing the proviral construct during cloning in competent *E. coli* cells, particularly during the transformation of large and unstable plasmids, is necessary.

The Vpr-A30L mutant used in Aim 2 was chosen for this study based on its relevance in macrophage infections, particularly in virion association functions. Further structure-function mutations may be chosen to identify specific Vpr interactions and its role in cytokine production. Previous findings in our laboratory have found that mutations, such as ΔQ44, also affect Vpr's ability to associate with budding virions [63]. Similarly, this study has also defined other Vpr structure-function mutations that would be significant in macrophage infection: nuclear localization mutants, such as R36W, may elucidate the correlation between the Vpr structure-function relationship and Vpr-mediated cytokine expression [103].

As previously stated, donor variation remains an important issue within this study. In order to reach statistical significance in cytokine measurement, particularly in cell-specific analysis,

more donor replicates are needed. Typically, using primary cells from individual donors, approximately 8 donors are used in order to accurately determine significance. Variable infectivity quantifications, as seen by EGFP expression across four donors, correlate to the discrepancies observed across donors in ELISA quantifications. Additionally, the primary cells from each donor respond differently to infection and thus express different levels of cytokines, as seen by intracellular staining and ELISA quantifications.

APPENDIX

INFECTIVITY OF REPORTER CONSTRUCTS BASED ON EGFP EXPRESSION

		REPORTER CONSTRUCT							
		NL(YU-2)-EGFP WT	NL(YU-2)-EGFP ΔR	NL(YU-2)-EGFP A30L	NL(AD8)-EGFP WT	NL(AD8)-EGFP ΔR	NL(AD8)-EGFP A30L	NL4-3 EXPOSED	UNINFE-CTED
DONOR	#78	14.1%	9.3%	11.0%	15.8%	2.0%	12.0%	0.0%	2.6%
	#79	6.2%	1.7%	11.4%	9.0%	0.1%	6.3%	0.3%	0.0%
	#80	7.4%	1.2%	1.4%	6.0%	0.8%	2.4%	2.1%	0.7%
	#82	4.4%	0.6%	6.8%	7.4%	0.1%	2.7%	0.0%	0.2%

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