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Role of viral protein R in infection of human dendritic cells by primate lentiviruses

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ROLE OF VIRAL PROTEIN R IN INFECTION OF HUMAN DENDRITIC CELLS BY PRIMATE LENTIVIRUSES

by

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THE ROLE OF VIRAL PROTEIN R IN INFECTION

OF HUMAN DENDRITIC CELLS BY

PRIMATE LENTIVIRUSES

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ABSTRACT

Viral protein R (Vpr) is an evolutionarily conserved but poorly understood protein encoded by all primate lentiviruses, including the lineages that gave rise to both human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), the causative agents of AIDS in humans. In this work, I sought to define the contribution of primate lentiviral Vpr to viral replication and evasion from cell-intrinsic antiviral defenses. I found that HIV-1 infection of human dendritic cells (MDDCs) is substantially attenuated upon infection with Vpr-deficient (HIV-1/ΔVpr) virus compared to wild-type (WT) infection. This replication defect to HIV-1/ΔVpr is evident in a single round of infection, results in reduced levels of viral transcription, and is relieved upon complementation by virion-associated Vpr. The block to transcription is alleviated through Vpr-engagement with the Cul4A/DCAF/DDB1 ($DCAF^{CRL4}$) ubiquitin ligase complex and a yet-to-be identified host factor, hypothesized to induce the DNA damage response (DDR) in infected cells. MDDCs are critical immune cells that are poised to detect invading viruses through a variety of cell-intrinsic antiviral sensors, resulting in the production of type I interferon (IFN) and restriction of virus replication. Surprisingly, infection of MDDCs with Vpr-

deficient lentiviruses (HIV-2 or SIV_{mac}) resulted in production of type I IFN indicating that this pathway is targeted by Vpr. I determined that signaling cascades that induce NF-κB-dependent type I IFN production are triggered in response to lentiviral integration, an obligatory process in lentivirus life cycle that results in host DNA lesions and subsequent repair by cellular DNA repair machinery. I also demonstrated that mutations in SIV_{mac} Vpr that ablate the ability to initiate DDR are unable to counteract the antiviral type I IFN response. Together, our work suggests the existence of a novel host factor that detects lentiviral integration in MDDCs to trigger an innate immune response that blocks virus dissemination. I hypothesize that Vpr by overcoming this cell intrinsic block to integration would be a critical viral adaptation to facilitate cross-species transmission that resulted in the HIV pandemic.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab.....antibody

AIDS .....acquired immunodeficiency virus

APC..... antigen presenting cell

APOBEC.....apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

APS .....ammonium persulfate

ATM.....ataxia-telangiectasia mutated

ATR.....ataxia-telangiectasia mutated and Rad3 related

AZT.....zidovudine

BAF.....barrier to autointegration

BMDCs ..... bone marrow derived DCs

BMDMs ..... bone marrow derived macrophages

bNAb..... broadly neutralizing antibody

ºC.....degree(s) Celsius

CCR..... C-C chemokine receptor

CD .....cluster of differentiation

CDC ..... Centers of Disease Control and Prevention

cDNA ..... complementary deoxyribonucleic acid

cGas..... cycle-GMP-AMP synthase

CMV .....cytomegalovirus

Cul4A.....Cullin 4A

CXCR.....C-X-C chemokine receptor

DC ..... dendritic cell

DCAF ..... DDB1 and CUL4-associated factors

$DCAF^{CRL4}$ ..... DCAF-Cul4A complex

DC-SIGN ..... dendritic cell-specific ICAM-3 grabbing nonintegrin

DDB1 ..... damage-specific DNA binding protein 1

DDR ..... DNA-damage response

dH_2O ..... distilled water

DMEM ..... Dulbecco's Modified Eagle's Medium

DMSO ..... dimethyl sulphoxide

DNA ..... deoxyribonucleic acid

dNTP ..... deoxynucleotide triphosphate

dNTPase ..... deoxynucleotide triphosphatase

dsRNA ..... double stranded RNA

EDTA ..... ethylenediaminetetraacetic acid

eGFP ..... enhanced green fluorescent protein

EFV ..... efavirenz

ELISA ..... enzyme-linked immunosorbent assay

EME1 ..... essential meiotic structure-specific endonuclease 1

Env ..... envelope

ER ..... endoplasmic reticulum

FA ..... Fanconi Anemia

FACS ..... fluorescence-activated cell scanning

FBS .....fetal bovine serum

FITC.....fluorescein isothiocyanate

FTC ..... emtricitabine

g..... gram

Gag.....group-specific antigen

GALT..... gut-associated lymphoid tissue

GFP .....green fluorescent protein

GM-CSF.....granulocyte/monocyte colony stimulating factor

gp..... glycoprotein

GSL .....glycosphingolipid

HAART.....highly active antiretroviral therapy

HANA .....HIV-associated non-AIDS condition

HDAC .....histone deacetylase

HEK .....human embryonic kidney

HIV .....human immunodeficiency virus

HIV-1 .....human immunodeficiency virus type 1

HIV-2 .....human immunodeficiency virus type 2

HLTF.....helicase-like transcription factor

HR .....homologous recombination

ICAM ..... intercellular adhesion molecule

IFN .....interferon

IL..... interleukin

IN/INT.....integrase

IP-10..... interferon-γ inducible protein 10

IRF .....interferon regulatory factor

ISG .....interferon stimulated gene

ISRE..... interferon stimulated response element

J .....Joule

k..... kilo

kDa..... kiloDalton

LEDGF.....lens epithelium-derived growth factor

LPS..... lipopolysaccharide

LRA..... latency reversing agent

LTNP.....long term non-progressor

LTR..... long terminal repeat

M .....Molar

MA .....matrix protein

MDA-5.....melanoma differentiation-associated protein 5

mDC..... myeloid dendritic cell

MDDC..... monocyte-derived dendritic cell

MDM..... monocyte derived macrophage

MHC ..... major histocompatibility complex

mL .....milliliter

mM.....millimolar

MOI ..... multiplicity of infection

mRNA ..... messenger RNA

MSM ..... men who have sex with men

MUS81 ..... MU81 structure specific endonuclease subunit

NC ..... nucleocapsid

NCS ..... newborn calf serum

nef ..... negative infectivity factor

NF-κB ..... nuclear factor κ of B cells

ng ..... nanogram

NHEJ ..... non-homologous end joining

NIH ..... National Institute of Health

nm ..... nanometer

nM ..... Nano Molar

NNRTI ..... non-nucleoside RT inhibitor

NRTI ..... nucleoside/nucleotide RT inhibitor

NTP ..... nucleotide triphosphate

NLRP3 ..... NACHT, LRR and PYD domains-containing protein 3

ORF ..... open reading frame

OWM ..... Old World monkeys

PAGE ..... polyacrylamide gel electrophoresis

PAMP ..... pathogen associated molecular pattern

PBMC ..... peripheral blood mononuclear cell

PBS ..... phosphate buffered saline

PBS-T..... PBS-Tween 20

PCR.....polymerase chain reaction

pDC.....plasmacytoid DC

PE..... phycoerythrin

PEB ..... PBS + EDTA + BSA

PD-1 .....cell death protein 1

PFA ..... paraformaldehyde

PHA..... phytohemagglutinin

PIC .....pre-integration complex

PMA.....phorbol-12-myristate-13-acetate

pmol .....picomole

Pol ..... polymerase

PPT.....polyproline tract

PrEP .....pre-exposure prophylaxis

PRR..... pattern recognition receptor

P/S.....penicillin, streptomycin

Ral.....raltegravir

Rev ..... regulator of viral expression

RIG-I.....retinoic acid-inducible gene

RNAse..... ribonuclease

RNA .....ribonucleic acid

RRE.....rev responsive element

RT .....reverse transcriptase

RT-PCR.....reverse transcription-PCR

SAMHD1 ..... SAM domain and HD containing protein 1

SDS ..... sodium dodecyl sulfate

SEM ..... standard error of the mean

SERINC ..... serine incorporator

SIV .....simian immunodeficiency virus

SIV_{amg} .....African green monkey simian immunodeficiency virus

SIV_{cpz} .....chimpanzee simian immunodeficiency virus

SIV_{gor}..... gorilla simian immunodeficiency virus

SIV_{mac}.....macaque simian immunodeficiency virus

SIV_{sm}.....sooty mangabey simian immunodeficiency virus

SLX4.....structure specific endonuclease subunit 4

ssRNA.....single strand RNA

TAR.....trans-activation response element

Tat .....trans-activator of transcription

TCR..... T cell receptor

TDF ..... tenofovir disoproxil fumerate

TEMED.....tetramethylethlenediamine

TLR.....toll-like receptor

TNFα..... tumor necrosis factor α

TRIM.....tripartite motif

tRNA.....transfer RNA

UNG2.....uracil DNA glycosylase 2

V.....volts

V1, V2, V3.....gp120 variable loop 1, 2, or 3

Vif.....viral infectivity factor

VLP.....virus like particle

Vpr.....viral protein R

Vpu.....viral protein U

Vpx.....viral protein X

VSV.....vesicular stomatitis virus

VSV-G.....vesicular stomatitis virus glycoprotein

WHO.....World Health Organization

WT.....wild type

X-gal.....5-bromo-4-chloro-3-indolyl-P-D- galactopyranoside

µ.....micron

µg.....microgram

µL.....microliter

µm.....micrometer

µM.....microMolar

INTRODUCTION

History

The first reported cases of HIV infection occurred in the US in Los Angeles, San Francisco and New York in mid-1981 (1, 2). The unusual disease was characterized as a severe immunodeficiency that resulted in death from normally non-pathogenic bacteria and fungi or rare forms of cancer like Kaposi's Sarcoma (2–5). At the time, HIV had only been observed in gay men, resulting in it initially being called "gay cancer" and later that year GRID or gay-related immunodeficiency (2). It wasn't until the fall of 1982 that the CDC renamed the disease acquired immunodeficiency syndrome, or AIDS (2, 6). At the time, it had also been documented in female sexual partners of AIDS patients, injection drug users, recipients of blood transfusions and hemophiliacs and was suggested to be the result of an unidentified infectious agent (1, 2). The virus itself was isolated by two separate labs, one by Dr. Françoise Barré-Sinoussi and colleagues at the Pasteur Institute and another at the US National Cancer Institute by Dr. Robert Gallo in spring of 1983 (7–9). Dr. Gallo's group also developed the first diagnostic blood test for the virus which allowed for screening for infected individuals (10). By the end of 1985, AIDS cases had been reported in every region of the world, totaling to over 20,000 reported cases (1, 2). The first treatment for HIV infection, a reverse transcription inhibitor called zidovudine (AZT) was released in the US in March of 1987 (11). While AZT is able to help control infection, it is not a cure and resistance mutations occur rapidly in infected individuals (12, 13). Combination therapy, in which several inhibitors are used in a cocktail that target at least two different steps of the viral life cycle was not developed for another 8

years (11). In 1995, highly active antiretroviral therapy (HAART) was released, which resulted in a 60-80% reduction in AIDS-related deaths in the coming years in countries that could afford the medication (1, 11). At this time, close to 5 million people worldwide had been diagnosed with the virus (1). Despite continued drug development, by the end of the 1990s, 33 million people had become infected with HIV and 14 million people had died from AIDS-related disease (1, 2).

According to most recent estimates, 36.7 million people are still living with HIV and 35 million people have died from the disease since the start of the epidemic (14). Due to initiatives since the 1990s, globally 18.2 million people have access to HAART medication that is able to keep their infection under control (15). Currently, the World Health Organization (WHO) has a goal of having 90% of people with HIV identified, 90% of that population on antiretroviral treatment and 90% of these individuals virally suppressed (undetectable viral load in the plasma) by 2020 (16). While there still needs to be improvement to meet these goals, global initiatives to lower the cost of medication and provide access to developing countries, particularly in Sub-Saharan Africa where disease burden is highest, has helped tremendously (16).

Human disease- HIV-1 and HIV-2

It is now understood that the HIV pandemic originated in central Africa due to several cross-species transmission events from non-human primates to humans that resulted in two distinct viruses, HIV-1 and HIV-2 (17, 18). It is thought that these zoonotic transmissions occurred in the early 1900s, around 1920, though little information exists about disease transmission within human populations pre-1980s (19–

22). The oldest identified infections were discovered in frozen clinical samples from Kinshasa, Democratic Republic of Congo obtained in 1959 and 1960 (23, 24). These clinical samples were used to help determine the evolutionary clock for HIV in order to estimate the amount of time since divergence from the most recent common ancestor (17, 18). It is also likely that Kinshasa, which at the time was still part of Zaire and referred to as Leopoldville, was a cradle for HIV-1 evolution (17). The origins of all cross-species transmissions have been traced to nearby areas in Western Africa and all sub-groups of HIV-1 have been discovered to still exist in Kinshasa as well as unique viral strains that have remained confined to the city (17, 23, 25). At the time, in early colonial Africa, urban populations were expanding and Kinshasa was the largest city in the region (23). Emergence of HIV-1 with it would allow for the virus to spread and diversify more easily, creating the pandemic that has plagued the world.

Both HIV-1 and HIV-2 are thought to have originated from a cross-species jump of a related simian immunodeficiency virus (SIV) from its native host to humans (17, 18). It is known that HIV-2 originated from at least eight independent transmission events of SIV_{sm}, which is a naturally occurring lentivirus in sooty mangabeys (17, 26–28). This gave rise to the eight lineages of HIV-2, labeled A-H, though only A and B have spread to an appreciable degree (17). HIV-2 is far less pathogenic than HIV-1, and typically displays lower viral loads and poor transmission (17). Many individuals who become infected do not progress to AIDS, though those who do have symptoms that are indistinguishable from HIV-1-related AIDS (17).

HIV-1 transmission occurred from at least four independent transmission events of a related lentivirus, SIVcpz from chimpanzees and gorillas that gave rise to subgroups M, N, O, and P (17, 18, 29). Of these four groups, N and P have remained confined to a handful of cases in Cameroon, O has spread to a limited degree in West Africa and accounts for approximately 1% of the global incidence and group M is the highly-diversified virus that is the cause of the global pandemic (17, 18). Group M has been further divided into 9 subtypes, A-D, F-H, J, and K, with additional recombinant forms between them that number greater than 40 (17). Global migration of these subtypes can be easily mapped, as many of them are now the predominant virus in different areas of the world (18). Subtype C, for example, has migrated to southern Africa, where it is now by far the dominant species of HIV-1. From there it has spread to India and Southeast Asia (17). Alternatively, subtype B initially was brought to Haiti, from where it spread to become the predominant virus in North America and Europe (17).

Each group and subgroup within HIV-1 contains an immense amount of diversity. Due to both error-prone replication strategies and a short reproduction time, HIV evolves a million times faster than human DNA does, allowing it to quickly outpace our natural defenses to infection (30, 31). It has been reported that within subgroup variation is typically between 8-17% at the amino acid level, though as high as 30% variation has been observed (18). Additionally, intra-subgroup variation ranges from 17-35%, with as high as 42% for some subgroups (18). Group O virus has also been divided into subgroups I-V, which show similar between group variation as group M, though less intra-group variation has been observed due to generally more restricted spread of the

virus (18). Together, this complicates efforts to develop diagnostics, antiretroviral treatments and vaccine candidates that will work on a diverse array of viruses.

Viral evolution and lineages- Simian Immunodeficiency Virus (SIV)

HIV is a member of the lentivirinae family of viruses whose life cycle is defined by reverse transcription of the viral plus stranded RNA genome into double stranded DNA that is subsequently integrated into the host cell genome (32). Lentiviruses are a distinct subclass of the larger, retrovirus family which all reverse transcribe and integrate the host genome (32). Lentiviruses are unique amongst retroviruses in that they encode mechanisms to import the reverse transcribed viral dsDNA into the host nucleus independent of cellular division and are thus able to infect non-dividing cells (32). Both HIV-1 and HIV-2 are each derived from one of more than 40 described SIV strains circulating in African primates (17). Interestingly, SIVs have only been detected in African old world monkeys (OWMs), indicating that they infected African OWMs after speciation from Asian NHPs and new world monkeys in the Americas, which occurred six to ten million years ago (17).

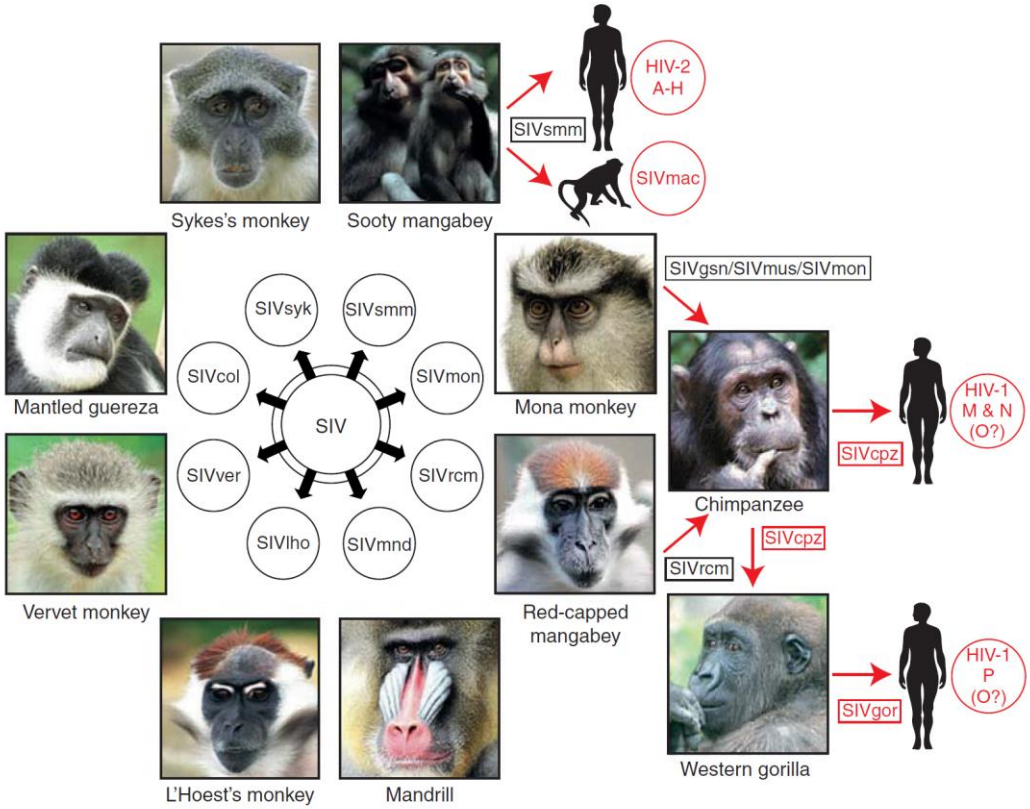


Figure 1. Origins of human AIDS viruses.

Old World monkeys are naturally infected with more than 40 different lentiviruses, termed simian immunodeficiency viruses (SIVs) with a suffix to denote their primate species of origin (e.g., SIV_{smm} from sooty mangabeys). Several of these SIVs have crossed the species barrier to great apes and humans, generating new pathogens (see text for details). Known examples of cross-species transmissions, as well as the resulting viruses, are highlighted in red. (17) Figure and legend from Sharp, *et al.* Cold Spring Harbor Perspectives in Medicine, 2011.

While there have been reported cases of SIV infections in Asian macaques, these occurred mostly in captivity (33). SIV_{sm} was transmitted to Asian macaques via experimental injections and co-housing with sooty mangabeys (Fig. 1) (17, 33). This created the new strain, SIV_{mac}, which is pathogenic and results in AIDS-like disease in Asian macaques (33). This species jump is of particular interest because SIV_{sm} is non-pathogenic in its natural host, sooty mangabeys, but gains pathogenicity following zoonotic transmission, suggesting host factors influence the course of disease (Fig. 1). Similarly, SIV_{sm} has jumped into human populations on several instances, which has resulted in pathogenic HIV-2 epidemic in West Africa (26–28). Sooty mangabeys are frequently hunted as agricultural pests, and it is thought that exposure to humans occurred during instances of hunting where individuals were exposed to contaminated blood or tissue (17, 34). How sooty mangabeys tolerate SIV infection without noticeable symptoms remains unclear and is of research interest. Understanding these mechanisms might result in development of a functional cure for HIV infection.

Unlike HIV-2, HIV-1 was derived from a cross-species jump of SIV_{cpz}, likely through the capture or consumption of bushmeat (Fig. 1) (29, 34). SIV_{cpz} is, itself a mosaic virus derived from recombination between SIV red-capped mangebey (SIV_{rcm}) and an SIV from the *Cercopithecus* species including the greater spot-nosed, mustached, and mona monekys (SIV_{gsn}/SIV_{mus}/SIV_{mon}) (35). *Env*, as well as some of the accessory genes, including *vpu*, *tat*, and *rev* are derived from SIV_{gsn}/SIV_{mus}/SIV_{mon}, while the viral LTRs, the 5’ half of the genome and *nef* all more closely resemble SIV_{rcm} (35). Exposure of chimpanzees to SIVs from other monkeys is thought to be due primarily to predatory

behavior (17). Field studies using non-invasive sampling techniques have defined the spread of SIV_{cpz} amongst the 5 species or subspecies in western and central Africa (36–38). Only 2 of these, the central and eastern chimpanzees, have detectable SIV infection (36–38). Infection rates among colonies can range from as high as 50% to non-existent. Contrary to what was noted with SIV_{sm}, increasing amounts of data show that SIV_{cpz} can cause AIDS-like symptoms and increased morbidity and mortality in chimpanzees (36–40). The recent nature of the jump from red-capped mangabeys and monkeys to great apes likely has limited the amount viral tolerance that has developed in the new host (17). Interestingly, in addition to jumping to humans, SIV_{cpz} has also spread to gorillas, though mode of transmission remains unclear, since gorillas are herbivores that do not pray hunt or eat other mammals (17, 41, 42). The transmission of SIV_{cpz} that gave rise to HIV-1 group P and potentially group O have been mapped to gorilla-derived lineages (17, 18, 37, 43). It is less clear if group O is gorilla-derived, since it lies phylogenetically somewhere between known chimpanzee and gorilla lineages and may have arisen through contact with a chimpanzee lineage that also spread to gorillas (17, 18). Due to the limited amount of field studies of gorilla colonies in Africa, the extent of SIV spread and whether it results in pathological disease remain unclear. Though there is still a good amount that is unknown about disease pathogenesis during infection of diverse primates, pathogenesis of HIV-1 and HIV-2 in humans has been well characterized.

Pathogenesis

Due to the limited tropism of HIV, infection requires direct contact with immune cells normally resident in blood, immune organs and at mucosal sites. This restricts the

routes of HIV transmission to sexual contact, both intra-vaginal and intra-rectal, direct injection into the blood stream, either through transfusion of contaminated blood products or needle sharing during injection drug use, transplantation of organs from infected individuals, or mother-to-child transmission during child birth or breast feeding (44). The risk of transmission varies for each of these events, ranging anywhere from 1 in 200 to 1 in 3000 for heterosexual transmission, 1 in 20 to 1 in 300 for men who have sex with men (MSM) transmission, 1 in 5 to 1 in 20 for mother-child transmission and 95 in 100 to 1 in 150 for transmission via the bloodstream (44). A genetic "bottleneck" is frequently observed during transmission where one or very few viral clones establish infection in a new individual (18, 44). These single-founder events occur with high frequency in all infection routes, with approximately 80% of heterosexual transmission, 60% of MSM transmission, and 40% of intravenous transmission occurring from one or few founder viruses (18, 45). These founder viruses are typically CCR5-tropic (one of two co-receptors used by HIV, as discussed further below), and dissimilar from the diverse quasispecies of virus in the transmitting host (44). This is generally thought to be due to the relatively low efficiency of viral transmission, resulting in an extreme bottleneck where only the most fit of transmitted viruses are able to establish an infection in a new host (44).

Acute disease

After establishment of a new infection, acute disease is typically characterized by flu-like symptoms, consisting of a fever, rash, sore throat, and swollen/tender lymph nodes, which is often severe enough for the individual to seek medical attention (46, 47).

Due to the non-specific nature of these symptoms, patients are typically diagnosed with a non-specific viral infection and testing for HIV is rarely done (46). After exposure to virus, a successful infection begins with an eclipse phase where the virus replicates in the local environment, typically a mucosal site, before being trafficked to local lymph nodes by dendritic cells (DCs) or infected $CD4^+$ T cells (45). The eclipse phase typically occurs about 10 days before viral RNA is detectable in the blood. After the eclipse phase, virus trafficked to the lymph nodes establishes a robust infection and begins to spread systemically, infecting all immune sites in the body, including the gut-associated lymphoid tissues (GALT) (45). At 21-28 days post infection, peak viremia is attained with viral RNA reaching 10^6 copies/mL in the plasma (45). Peak viremia is accompanied by a drastic loss in circulating and lymph-associated $CD4^+$ T cells, the main target of infection. HIV mainly infects activated, $CCR5^+$ (memory) $CD4^+$ T cells, which are present in the blood and lymph tissues and highly enriched in the GALT. In the first 3 weeks of infection, approximately 80% depletion of GALT-associated $CD4^+$ T cells can occur due to direct cytopathic effects of infection or indirect effects of systemic immune activation associated with acute infection (45). Acute infection is characterized by a cytokine storm, driven by detection of viral infection from innate immune cells including conventional and plasmacytoid DCs (cDCs or pDCs), macrophages and natural killer (NK) cells (45). The antiviral cytokines interleukin (IL) 15, type I interferons (IFNs) and INFγ inducible protein 10 (IP-10) increase rapidly but transiently, while the proinflammatory cytokines IL-18, tumor necrosis factor α (TNFα), IFNγ, and IL-22 increase rapidly and are maintained in the serum (45, 46). During this time, the levels of

circulating DCs also drop, either from activation-induced cell death or increased migration to the lymph nodes, where levels notably increase (45). Concurrently, the adaptive immune response begins to mount a response to viral infection. B cell specific responses can begin to be detected at 8 days post detectable plasma viremia and production of the first viral envelope-specific antibodies occurs between 13 and 27 days post plasma viremia (45). T cell-specific responses occur more rapidly and drive viral diversification as the virus tries to escape detection. Early T cell responses are typically specific to the viral Env or Nef proteins, while later responses develop against $p24^{gag}$ and Pol, which are thought to help keep viral levels in check (45). In the 12-20 weeks post infection, viral loads decrease and reach a "set-point," and maintained at a fairly consistent level by the adaptive immune system (45, 46). During this time, plasma levels of $CD4^+$ T cells rebound but GALT-associated $CD4^+$ T cells do not (45). After these initial, early events in infection, viral load and $CD4^+$ T cell count reach an equilibrium and disease progresses into its chronic stage.

Chronic disease

Before the onset of anti-retroviral therapies (ART), chronic infection was characterized by persistent levels of immune activation, production of proinflammatory cytokines and a slow decline in immune function and $CD4^+$ T cell levels (46). Consistently high levels of IL-6, TNFα, and coagulation-associated protein d-dimer drive chronic immune activation (48, 49). Production of antiviral IFNs are typically difficult to detect, but a consistent signature of interferon stimulated genes (ISGs) is noted in transcriptional analysis of cells from infected patients (50, 51). T cells, B cells and

antigen presenting cells (APCs), including DCs and macrophages which coordinate initiation of an adaptive response all show phenotypic and functional evidence of persistent activation (4, 52). T cells display increased expression of activation markers CD38 and HLA-DR, as well as increased expression of senescence and exhaustion markers CD57 and programmed cell death protein 1 (PD-1) (53–56). These markers are associated with decreased ability of the T cells to respond to T cell receptor stimulation and decreased functionality (53–56). There is also increased levels of cell-turnover and proliferation, indicated by Ki-67^{+} staining, which may be the result of homeostatic mechanisms of the immune system trying to fill the void of viral-induced cell death (46). During this time, the structure of primary and secondary lymphoid tissue begins to deteriorate (57). The thymus, which is the source of new, naïve T cells, undergoes severe morphological damage, which is thought to be responsible for some of the decline in circulating $CD4^{+}$ T cells (46, 57). The intestine, which contains 40% of all lymphocytes in the body, also undergoes morphological changes, including increased epithelial cell apoptosis and crypt hyperplasia (46, 58, 59). The immediate and drastic loss of $CD4^{+}$ T cells is thought to be the driving force for these changes (3–5, 46, 60). Th17 $CD4^{+}$ cells are responsible for maintenance of the mucosal barrier and are reported to be amongst the first infected cells in pathological models of SIV infection (61). These cells are almost entirely depleted in chronic infection, which may be the reason for intestinal barrier breakdown and increased translocation of microbial products from the gut (46, 60, 62). These microbial products, including bacterial lipopolysaccharide (LPS), are thought to be

some of the driving force behind the high levels of serum proinflammatory cytokines (46).

During end-stage HIV infection, or onset of AIDS, $CD4^+$ T cell levels drop below 200 cells/mL and the host immune system essentially collapses (3–5, 46). At this point, the individual becomes highly susceptible to secondary infections including mycobacteria, cytomegalovirus, or infection by *Pneumocystis jirovecii*, *Toxoplasma gondii*, *Streptococcus pneumoniae*, or *Cryptococcus* (46). They also have increased rates of very specific malignancies including non-Hodgkin's lymphoma and Kaposi's sarcoma (46). This can occur anywhere from several months to 10 years after initial infection, though in rare cases of so-called "elite controllers," they may not progress to disease in 20+ years (46, 63, 64).

Since the advent of highly-active antiretroviral therapy (HAART), a combination of drugs that target multiple steps in the viral life cycle (described later in this section), course of disease during chronic HIV infection has been dramatically altered (65). In most cases, after HAART initiation, serum HIV drops to undetectable levels, $CD4^+$ T cell levels rebound, and systemic inflammation decreases, though does not disappear entirely (46). Studies of individuals who initiate HAART early after infection have revealed that the thymus is able to regenerate/repair itself, if treatment is started early enough (57). In some cases, reconstitution of the GALT has also been observed, though other studies have reported that GALT-associated $CD4^+$ T cell count remains low even post treatment (66–71). Early HAART treatment is associated with reduced disease progression and better restoration of $CD4^+$ cell levels (46).

In some individuals, typically those that initiate HAART later during their course of disease, $CD4^+$ T cell levels are not able to rebound, despite undetectable viral load in the plasma (46). In these individuals, lymphoid tissue fibrosis, especially in the gut, is irreversible, and microbial product translocation as well as serum proinflammatory cytokines are maintained (46). These individuals also maintain higher levels of activation and proliferation markers on their circulating T cells, as well as decreased ability of theirs cells to respond to stimulation (46). These residual disease effects are all collectively associated with worse disease outcomes and increased morbidity and mortality.

With the advent of HAART, HIV has become a chronic disease. Individuals now are more likely to suffer from HIV-associated non-AIDS conditions (HANA) that are thought to be driven by the underlying inflammatory signature that even HAART cannot alleviate (72). These HANAs include cardiovascular disease, increased incidence of cervical and lung cancers, liver disease specifically in hepatitis co-infected individuals, and a variety of non-AIDS related malignancies (73–75). All of these are more common in HIV-infected individuals than the general population and correlated with $CD4^+$ T cell count (46, 73). It is thought that the low level of viral replication that occurs, specifically in isolated tissue reservoirs where drug penetrance is low, is the driving force behind these inflammation-linked disorders. There is hope that better treatment to reduce residual replication or a functional cure for infection could prevent HANA entirely. In order to effectively design better treatments, in depth knowledge of the viral life cycle is necessary.

Molecular mechanism of disease: viral life cycle

HIV and SIV replication, like with all viruses, initiates with attachment and entry into the target cell (Fig. 2). For all lentiviruses, binding is mediated by a virally encoded env protein (76). The HIV-1 Env is a trimer, each composed of the transmembrane anchor, gp41 and the receptor binding motif, gp120 which are proteolytically cleaved from one polyprotein encoded in the genome (76–78). For HIV-1, fusion requires the presence of the primary virus receptor, CD4, as well as a co-receptor, either CCR5 or CXCR4 (79–86). Receptor and co-receptor requirement limits HIV-1 cell tropism to $CD4^+$ T cells, macrophages and dendritic cells (87, 88). The HIV-1 fusion protein is usually specific for usage of either CCR5 or CXCR4, though dual tropic viruses, although rare, have been isolated as well (89). This has given rise to the nomenclature CCR5- or CXCR4-tropic viruses. HIV-2 also utilizes CD4 as its primary receptor but is known to have a broader range of co-receptor usage including CCR1, CCR3, CXCR6, and GHOST(3) (90–92). SIVs, similar to HIV-2, are also thought to have a broader range of co-receptor usage and may be able to enter cells independent of CD4 expression with certain co-receptors (93–95). In HIV-1, binding to CD4 by the env protein allows for rearrangement of the V1, V2, and V3 loops of the viral env so that the co-receptor can be engaged (76). Co-receptor engagement is thought to be the trigger for insertion of the fusion peptide of gp41 into the host plasma membrane (76). Then, a six-helix bundle composed of two viral Env trimers with fusion peptides inserted into the host membrane undergo a conformational change that pulls the two membranes together (76, 96, 97). Once the virus fuses with the cell membrane, entry and uncoating occur (32).

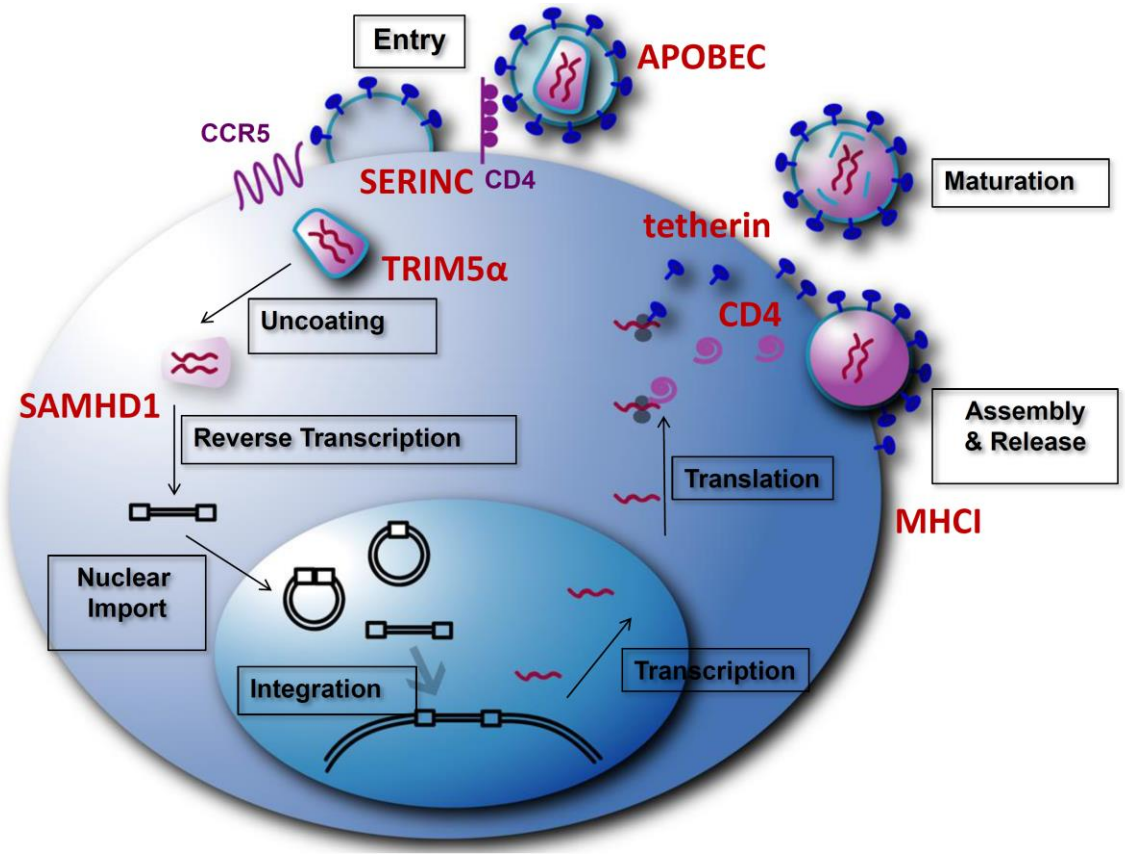


Figure 2. HIV life cycle and viral restriction factors.

Steps of the HIV life cycle are denoted with black, boxed labels and host restriction factors that are counteracted by virally encoded proteins are shown in red.

The viral core, or capsid must enter into the host cytoplasm and begin to come apart, or uncoat, releasing the viral genetic material to allow for infection (Fig. 2) (98). Timing is important in this process, as evident by the effects of the restriction factor TRIM5α (99–102). TRIM5α is a host protein shown to restrict SIV/HIV infection of cells derived from its non-native host during a cross-species transmission event (99–101). TRIM5α binds to capsid and mediates premature uncoating, resulting in viral restriction (Fig. 2) (102). It remains unclear whether this process occurs in the host cytoplasm or after the core reaches the host cell nucleus (98). During uncoating, reverse transcription is initiated by the viral reverse transcriptase (Fig. 2) (98).

Reverse transcription utilizes a virally encoded polymerase to convert the single-stranded RNA genome to double stranded DNA (103, 104). Reverse transcription initiates using a tRNA primer that binds to the primer binding site on the viral RNA, just downstream of the long terminal repeat or LTR (105, 106). The LTR contains sequences (R or repeat region) that flank both ends of the viral genome allowing for successful reverse transcription and U3 for initiating transcription from integrated viral DNA (105). HIV preferentially utilizes a lysine tRNA for reverse transcription initiation (103, 104, 107, 108). Then the viral reverse transcriptase begins to add dNTPs to the tRNA primer in the direction of the 5’ LTR using the viral RNA as a template (Fig. 3) (32, 105). The viral reverse transcriptase, in addition to polymerase activities, also has RNAse H activity and degrades the template RNA as it copies it into DNA (32, 105). Once the polymerase extends through the 5’ end, it reaches the minus-strand strong stop, where it pauses until

the whole tRNA-reverse transcriptase-DNA complex will jump to the other end of the viral RNA (Fig. 2) (32, 105). Base pairing between the newly reverse transcribed R and 3’ R (RNA) allows for continued duplication of the rest of the genome into DNA (Fig. 3) (32, 105). During this second extension, RNAse H is unable to degrade a short track of RNA called the polypurine tract, which acts as a primer for creation of the second strand of DNA (Fig. 2) (32, 105). The viral reverse transcriptase will then copy the first strand of DNA in the 3’ direction until it reaches the initial tRNA primer at the positive strand strong stop (Fig. 3) (32, 105). Again, the reverse transcriptase will pause until the newly made second strand DNA base pairs with the first DNA strand at the primer binding site, forming a loop like structure (32, 105). DNA replication can continue around the loop to form a complete, double stranded DNA genome (32, 105).

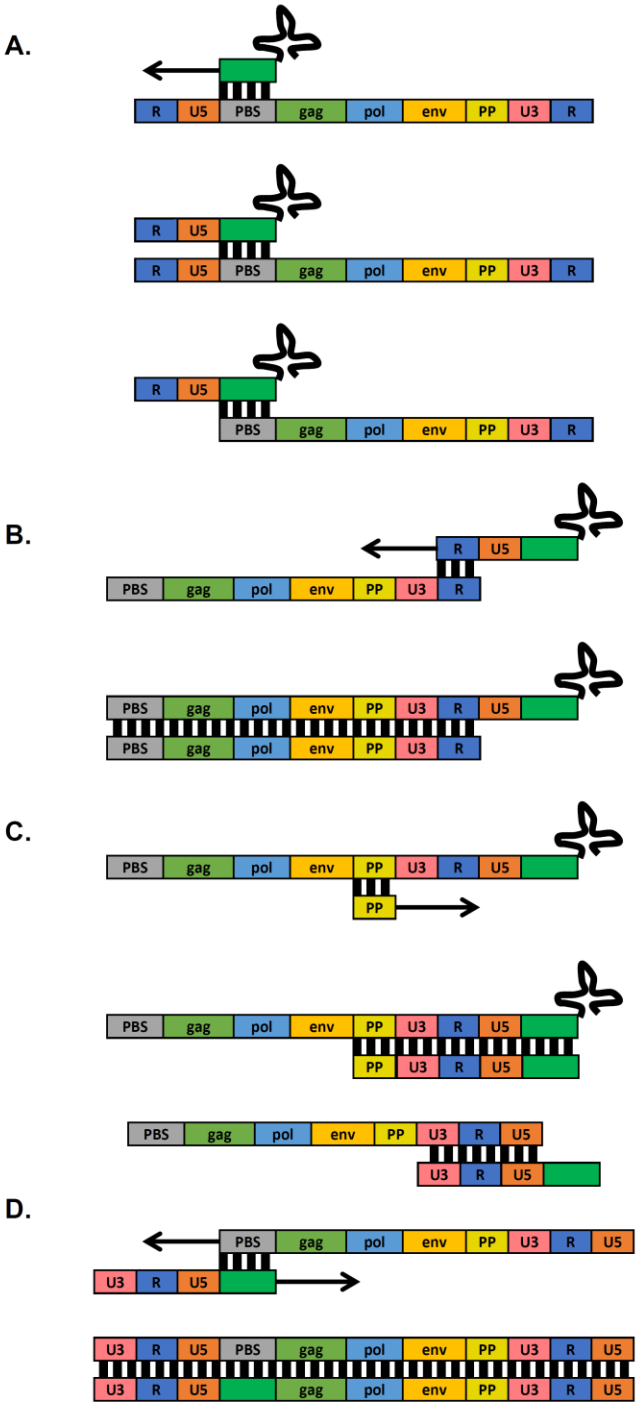

A.
R U5 PBS gag pol env PP U3 R
R U5
R U5 PBS gag pol env PP U3 R
R U5
PBS gag pol env PP U3 R
B.
R U5
PBS gag pol env PP U3 R
PBS gag pol env PP U3 R U5
PBS gag pol env PP U3 R
C.
PBS gag pol env PP U3 R U5
PP
PBS gag pol env PP U3 R U5
PP U3 R U5
PBS gag pol env PP U3 R U5
U3 R U5
D.
PBS gag pol env PP U3 R U5
U3 R U5
U3 R U5 PBS gag pol env PP U3 R U5
U3 R U5 gag pol env PP U3 R U5

Figure 3. Viral Reverse Transcription.

(A) Viral reverse transcription initiates via a tRNA primer binding to the primer binding site (PBS). The viral polymerase, reverse transcriptase, will then proceed to copy the U5 and R sequence while the RNAse H portion of the viral polymerase degrades the template strand. When the polymerase comes to the end of the R sequence, base pairing can occur the homologous R sequence at the other end of the genome, resulting in the whole DNA-polymerase complex to jump in a process referred to as strand transfer (B). The viral reverse transcriptase will again begin to copy the viral RNA into DNA, degrading the template RNA as it goes. The polypurine track (PP) is resistant to RNAse H degradation, and will remain base paired to the newly synthesized DNA. (C) The PP then acts as a primer, and the viral reverse transcriptase will use the newly synthesized DNA to as a template. Once U3, R and U5 have been synthesized, the second strand transfer will occur (D), and base pairing at the PBS will allow for extension in both directions to make a complete DNA copy of the viral genome.

After the double stranded DNA reaches the host cell nucleus, another virally encoded protein, integrase, incorporates the viral DNA into the host cell's chromatin creating a provirus, or a permanently incorporated viral genome encoded by the host (Fig. 2) (109). Integration is directed by the viral integrase protein along with a number of host cell proteins that are recruited to the viral DNA (109). These include the cellular protein Barrier to autointegration (BAF) that prevents the viral DNA from integrating into itself (110, 111). A pre-integration complex (PIC) is generated, which includes integrase and matrix and capsid proteins, all of which contribute to nuclear import (109, 110). This complex of proteins associate with nuclear pore proteins transportin 3 and Nup358 and facilitate transfer of the PIC across the nuclear membrane (109). Once in the nucleus, the PIC associates with the cellular protein LEDGF/p75, which is thought to help tether the PIC to the host DNA and play some role in integration site selection, though this process remains poorly understood (112–115). Though the mechanism of site selection remains poorly defined, integration site mapping has revealed that HIV preferentially integrates in euchromatic regions where active gene transcription is occurring (109). During the process of integration, the viral integrase removes two nucleotides from each 3' end of the linear viral DNA (Fig. 4) (109, 110, 116). These 3' ends, facilitated by the catalytic domain of integrase, attack the target host DNA at a phosphodiester bond at a major grove in the DNA (Fig. 4) (109, 117). This joins the 3' ends of the viral DNA to the host DNA with a five-nucleotide, single strand gap in the host DNA between joining sites and a two-nucleotide, 5' flap of viral DNA (Fig. 4) (109). Next, host cell machinery must remove the two-nucleotide 5' overhang and fill in single stranded gaps (118). If this

process does not occur, host DNA replication for cell division will stall at the joining sites (119). These single strand gaps are also hot spots for accumulation of additional DNA damage like double strand breaks, which trigger cellular apoptosis if not repaired (119). Once this process is complete, the integrated viral genome can be transcribed to produce viral mRNA or remain dormant, not undergoing any transcription through a process called latency (32). Since the viral genetic material is incorporated into the host genetic material, the virus becomes very difficult to purge from an individual once infected, particularly when it is in a latent state (120). This remains one of the largest barriers to a cure.

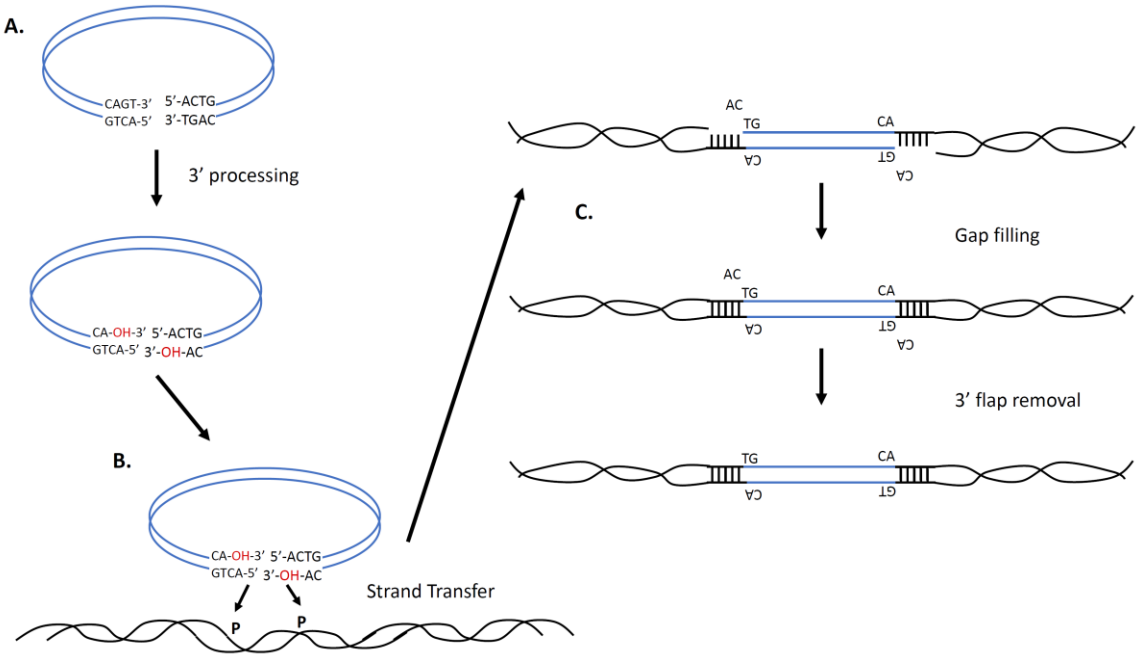


Figure 4. Retroviral Integration.

Integration begins with 3' processing (A) catalyzed by the viral integrase. This process removes two nucleotides to create 3' -OH groups that are attached to host DNA at phosphate groups (B) during strand transfer. This joining results in a five base pair gap and two nucleotide flap that must be repaired by host cell machinery (C). Cellular DNA repair proteins will fill in the gap, ligate the newly synthesized DNA to the host DNA, and remove the two nucleotide flap to create a transcriptionally competent provirus.

After integration, the virus utilizes host cell machinery to undergo transcription and translation in order to make new viral proteins (Fig. 2) (32). These assemble in the cytoplasm and bud from the host cell membrane (32). This budding process incorporates host cell lipids and plasma membrane proteins into the viral membrane, which is thought to be advantageous to viral spread and immune evasion (121, 122). As a final step in the replication process, a virally encoded protease must cleave Gag and Pol polyproteins that make up the viral core (32). This makes the newly budded virion fully infectious and able to initiate a new infection in a neighboring cell (32). This entire process of viral replication is mediated by only a handful of virally encoded proteins which co-opt key cellular processes to assure propagation of virus.

Virally encoded proteins and their functions

All primate lentiviruses encode three main structural and enzymatic proteins that are essential to replication as well as a number of accessory proteins that facilitate replication *in vivo* in cells that have high barriers to infection (32). The three main proteins are conserved across primate lentiviral evolution and include the polyproteins Gag and Pol as well as Env (Fig. 5) (32). Gag encodes the three main structural proteins, matrix, capsid, and nucleocapsid that provide the structure of the virion as well as form that viral core that protects the viral mRNA during infection and shields it from sensing by host machinery (32). Pol encodes the three enzymatic proteins, reverse transcriptase, integrase and protease (32). Reverse transcriptase converts the viral RNA to double stranded DNA through a process known as reverse transcription (103, 104). Integrase then incorporates this double stranded DNA into the host cell DNA (109, 110). Viral

protease is responsible for maturation (proteolytic cleavage) of both Gag and Pol polyproteins after viral budding, creating a mature, infectious virion (32). Without protease, the newly budding virions remain in an immature form and are non-infectious (123). Env, the viral envelope protein, mediates binding and fusion of the virion to the host cell. Env is extensively glycosylated during endoplasmic reticulum (ER) processing and is cleaved into its two components, gp120 and gp41 by the cellular protease, furin during protein processing in the ER (124, 125).

In addition to these structural and enzymatic proteins, HIV encodes six other proteins important for infection (Fig. 5). The proteins Tat and Rev are conserved across primate lentiviruses, and are both critical to viral replication (126). Tat, also known as trans-activator of transcription, is a highly potent HIV/SIV transcriptional enhancer that is critical for mediating high levels of transcriptional output from the integrated provirus (127). Tat binds a RNA-stem loop structure called the transactivation-responsive element (TAR), which recruits proteins that prevent premature RNA polymerase II pausing on nascent viral transcripts (128, 129). In the absence of Tat, only low levels of viral transcription can occur, often with premature termination at terminator sequences within the viral genome (127). The viral Rev protein is important for splicing and export of viral RNA (130). The viral genome encodes four different splice donor sites and eight splice acceptor sites, allowing for more than 40 different viral transcripts to be made, likely more if cryptic splice sites were included (131). It is critical to the viral life cycle that some viral RNAs remain unspliced in order to be packaged as new viral genomes or only partially spliced for certain viral proteins to be expressed (131). Rev binds to a short

RNA sequence within the *env* portion of the viral genome known as the rev responsive element (RRE) (132, 133). Binding of rev induces a conformational change in the viral RNA, allowing for multimerization of Rev which is necessary for viral RNA export (131). Rev then facilitates singly-spliced or unspliced RNA export from the nucleus through associations with Crm1 for translation or virion incorporation, respectively (131, 134–136). After nuclear export, Rev is released from the viral RNA and returns to the nucleus via associations with importin-β (136–139). Without Rev, mRNA encoding the viral enzymatic and structural genes *gag*, *pol*, and *env* would not be translated and new, progeny virions could not be made (131).

Finally encoded by primate lentiviruses are a number of accessory proteins. Accessory proteins are not necessary for replication *in vitro* but are absolutely essential for replication *in vivo* to counteract host restriction factors that normally would inhibit infection (140). Among the accessory proteins, Nef, Vif, and Vpr are encoded by all primate lentiviruses, while Vpu is unique to the HIV-1/SIV_{cpz} lineage and Vpx is unique to SIV_{sm}, SIV_{mac}, and HIV-2 lineage (Fig. 5) (140). Most of these proteins have multiple functions during infection, many of which have been thoroughly studied and are well defined in the literature.

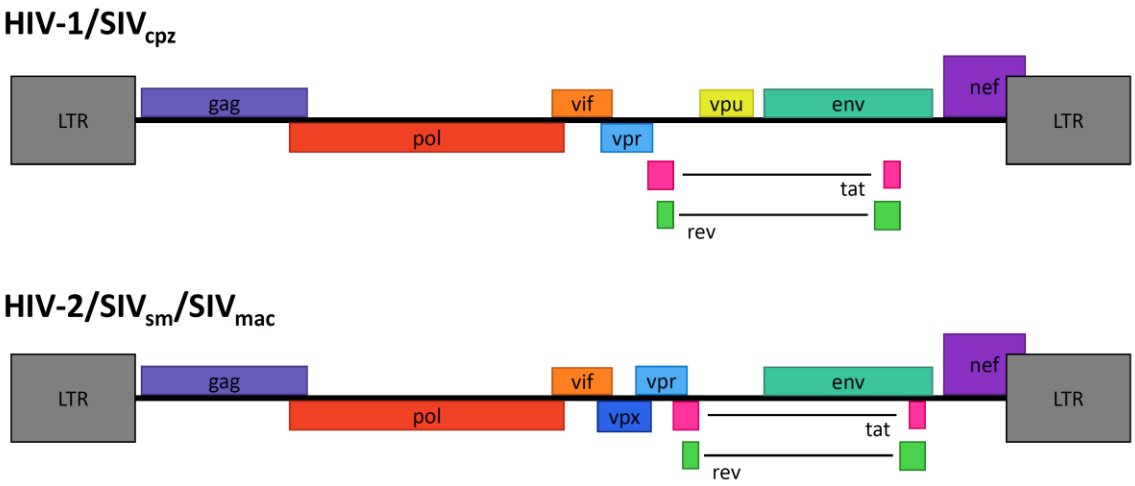


Figure 5. HIV-1/SIV$_{cpz}$ and HIV-2/SIV$_{sm}$/SIV$_{mac}$ viral genomes.

The viral genomes (not to scale) of HIV-1/SIV$_{cpz}$ lineage viruses and HIV-2/SIV$_{sm}$/SIV$_{mac}$ lineage viruses. The most significant difference is the presence or absence of the viral accessory protein Vpu or Vpx.

Viral accessory proteins and restriction factors

In the arms race between pathogen and host, accessory proteins are the virus's best defense against host restriction (Fig. 2). These proteins are dispensable in some *in vitro* systems but are absolutely essential *in vivo* to counteract host immune defense proteins that restrict viral infection (140). During the zoonotic transmission events that resulted in HIV-1 and HIV-2, the simian virus had to adapt to its new host in order to replicate (17, 18). We diverged from our most recent common ancestor with apes and old world monkeys (OWM) approximately 25 million years ago, so divergence of host restriction factors that block infection is a major obstacle to cross species transmission (17, 140). Every time a transmission event occurs, the viral accessory proteins must evolve to counteract their cognate restriction factor in order to successfully infect its new host (140, 141). This process also places pressure on the host species to accumulate polymorphism in viral restriction factors to enhance survival, resulting in the positive selection that is observed amongst lentivirus restriction factors in the primate lineage (140, 141). The accessory proteins Vpr and Vpx will be discussed later, as they are the main focus of this dissertation. Of the remaining accessory proteins, the function of viral infectivity factor (Vif) is the most clearly defined. It, like most of the viral accessory proteins, uses a conserved pathway to target a host restriction factor for degradation (140). This conserved pathway utilizes a cullin scaffolding protein to assemble a Rbx/Rox RING finger protein and an E2 conjugating enzyme to form a ubiquitin ligase complex (140, 142). In the case of Vif, this complex targets apolipoproteinB mRNA-editing enzyme catalytic polypeptide-like 3G and 3F (APOBEC3G, APOBEC3F) and to

a lesser extent, some of the other members of the APOBEC family for proteasomal degradation (140, 143, 144). In the absence of Vif, APOBEC proteins will be packaged into the budding virion and will convert cytosine residues to uracil during early reverse transcription (145–147). When this strand of cDNA is used during late reverse transcription as a template, these changes will become fixed in the viral genome as guanosine to adenosine mutations in a process called G to A hypermutation (140, 146). Approximately 10% of the Gs can be mutated through this process, resulting in error catastrophe which prevents further viral spread (140). In the presence of Vif, APOBEC is poly-ubiquitinated and proteasomally degraded, preventing viral restriction (140).

Similar to Vif, viral protein U (Vpu) also interacts with a ubiquitin ligase complex, but Vpu uses this complex to target multiple, highly divergent proteins (140). Vpu utilizes the cullin1-Skp1 complex to target both CD4 and BST-2 for proteasomal degradation (148, 149). CD4 is the main viral receptor for both HIV and SIV, as discussed earlier. Downregulation of this receptor allows for efficient viral egress and prevents super-infection of the host cell (140). Vpu utilizes the ubiquitin ligase complex to target env-bound CD4 in the ER during processing, preventing it from co-trafficking to the cell surface with viral Env (149). Vpu poly-ubiquitinates the cytoplasmic tail of CD4, targeting it for degradation via the proteasome (150–152). In addition to downregulation of CD4, Vpu is also able to target the viral restriction factor BST-2 or tetherin (153, 154). It was noted that in the absence of Vpu, infectious virus becomes stuck or tethered to the surface of infected cells, reducing viral spread (155, 156). Tetherin is an interferon inducible plasma membrane protein able to bind and retain budding virus to the surface

of the cell (153, 154, 157). Its cytoplasmic tail contains signaling motifs able to initiate the NF-κB signaling cascade and induce pro-inflammatory cytokine secretion. Vpu binds the cytoplasmic tail of tetherin at the plasma membrane and targets it for ubiquitination and proteasomal degradation (158). Vpu is unique to SIV lineages that gave rise to HIV-1, SIV_{cpz}, and SIV_{gor} (159, 160). In other lineages, the Env protein and/or the Nef proteins have shown the ability to downregulate tetherin and CD4 (140).

Interestingly, most SIV and HIV Nef proteins retain the ability to downregulate CD4 and MHCI independent of expression of Vpu via an ubiquitin-independent mechanism (161–163). Nef associates with the cytoplasmic tail of CD4 at the plasma membrane and recruits endocytosis machinery including AP-2 and clathrin to endocytose CD4 and traffic it to the lysosome for degradation (164). Recently, this has been suggested to help shield infected cells from antibody-dependent cell-mediated cytotoxicity by preventing pre-triggering of the viral glycoprotein by surface CD4 (165). In addition to CD4, Nef has been shown to induce endocytosis of the plasma membrane protein major histocompatibility complex I (MHC I) (166). MHC I is an antigen-presenting molecule on the surface of all cells that displays both self and foreign antigens. When a cell becomes infected, antigen from the infection can be displayed on MHCI and sensed by nearby immune cells including NK cells and cytotoxic T cells (167, 168). These immune cells will respond by killing the infected cell, limiting its ability to transmit the infection. It is highly advantageous to HIV and SIV to downregulate these presentation molecules to limit immunological detection (167, 168).

In addition to plasma membrane protein targets, it is well established that Nef has effects on virion infectivity and that this is a highly conserved function across primate lentiviruses (169, 170). Virions made from cells infected with a nef-deficient virus have been shown to be less infectious on a per particle basis than nef-expressing virus, but this phenotype is evident in only certain types of cells (169). For instance, viruses derived from cell lines such as HEK293T (human embryonic epithelial kidney cell line) or HeLa (human cervical epithelial cell line) cells show no difference in infectivity in the presence or absence of Nef, while virions derived from primary $CD4^+$ T cells or Jurkat T cell lines are significantly less infectious in the absence of nef (171–173). Recently, two labs have discovered the reason for this and also a third family of proteins targeted by nef. Work done by both the Pizzato and the Göttlinger laboratories show that nef targets a family of proteins called SERINCs, in particular SERINC3 and SERINC5 (174, 175). Normally, these proteins are incorporated from the host plasma membrane into the budding virion. In the presence of nef, the SERINCs are endocytosed and recycled from the plasma membrane (174, 175), preventing incorporation. While it remains unclear what direct effect the SERINCs are having on infectivity, it is thought that they may be limiting membrane fluidity of the virion, making it too rigid to fuse fully with a target cell and preventing entrance of the capsid into the cytoplasm.

The remaining two accessory proteins, Vpr and Vpx are closely related and are the main focus of this work (176–178). Vpx antagonizes the viral restriction factor SAMHD1, a dNTPase that prevents reverse transcription in monocytes, macrophages, DCs, and resting $CD4^+$ T cells (179–183). Vpr has also been shown to enhance infection

in DCs, macrophages, and resting $CD4^+$ T cells, though the exact function of the protein remains poorly defined (184–186).

Viral protein R (Vpr): size, structure, encapsidation

Vpr is a 96-amino acid, 14 kDa accessory protein encoded by HIV-1 (187). It is expressed from a singly-spliced Rev-dependent mRNA (188). Though an individual crystal structure has not been solved, NMR has revealed that the protein is composed of three alpha helices with flexible N- and C-terminal domains (189). Alpha helices span amino acids 17-33, 38-50 and 56-77 (189). These three alpha helices are folded around a hydrophobic core consisting of leucine, isoleucine and valine residues (189). The N-terminus of Vpr is responsible for associations with the p6 region of gag, which allows for incorporation into virions (190–195).

Vpr evolution: duplication, rise, function of Vpx

Vpr can trace its origins back through primate lentiviral evolution (177, 178, 196). All described primate lentiviral isolates contain a functional Vpr gene, suggesting that it plays a critical role during infection (177, 178, 196). In many old-world monkey (OWM) lentiviruses, like SIV_{agm}, Vpr has two ascribed functions, initiation of G_2 cell cycle arrest, which is conserved amongst all Vpr alleles in their host cells, and degradation of the restriction factor SAMHD1 (178). SAMHD1, a dNTPase, is highly expressed in myeloid cells, including monocytes, macrophages and dendritic cells, as well as resting $CD4^+$ T cells (179, 180, 183), that lowers the resting dNTP pool in cells, thus affecting the kinetics and magnitude of reverse transcription (181, 182, 197). The ability of Vpr to degrade SAMHD1 has been lost, or more likely never existed in some SIV lineages,

including that which gave rise to SIV_{cpz}/HIV-1 (178). In others, Vpr underwent a duplication event after which the two functions of the protein diverged, giving rise to Vpx that encoded the SAMHD1 antagonism (176, 178). Vpx has been found only in two lentivirus lineages, the SIV_{sm}/HIV-2 lineage and a lineage that includes SIV red capped mangabeys (SIV_{rcm}) (178). Though, surprisingly, pathogenesis studies suggests Vpx may be more important than Vpr in primate models of infection (198, 199).

Transactivation

One of the original prescribed functions for Vpr is its ability to transactivate the viral LTR. It has been suggested that some of the differences in viral replication seen in the presence or absence of Vpr may be due to the ability of Vpr to transactivate, or stimulate transcription from the viral LTR (200). It has been shown by a number of groups that Vpr acts in primary human $CD4^+$ T cells and T cell lines to enhance output from the viral LTR (200–202). Work from Gummuluru, *et al* shows that this process does not occur in primary human macrophages, suggesting that it may be limited to $CD4^+$ T cells or cycling cells where Vpr expression results in cell cycle arrest (201, 203). Transactivation is a conserved function of all primate lentiviral Vprs, suggesting it plays an important role in the viral life cycle (204). Vpr from SIVagm, a distant relative to strains that gave rise to HIV-1 and HIV-2 maintains the ability to transactivate in human cells, even though it loses the ability to induce cell cycle arrest and apoptosis, suggesting that Vpr functions independently of host-cell machinery in order to increase viral transcription (205). It is also possible that transactivation is just an outcome of the viral

LTR being more active in G_2 phase, which cycling cells are arrested in in the presence of Vpr (203).

Vpr function and interactions: G2 arrest/apoptosis

The most well described and studied function of Vpr is its ability to induce G_2 cell cycle arrest during infection of cycling cells (206, 207). G_2 arrest is conserved across all characterized primate lentiviral Vprs studied within their own host cells, though function is sometimes lost during infection of cells from other species (207, 208). It has been suggested that Vpr-induced G_2 arrest increases viral progeny production, since the viral LTR has been shown to be most active in G_2 phase (203). It is thought that arrest at G_2 prevents further cellular resources from going into cell division and DNA replication, allowing for their use in manufacturing new, progeny virions (203). In addition to the enhancement of viral transcription, expression of Vpr results in the induction of apoptosis (209–211). It remains somewhat of a debate if apoptosis is a result of G_2 arrest or occurs independently, being driven by other functional regions of Vpr or through associations of Vpr with the mitochondrial cell death pathway (212–215). Regardless of the mechanism, induction of apoptosis is a driving force for loss of Vpr expression upon serial passage of HIV-1 in cells *in vitro*; cell death selects for Vpr-null mutations (216). In contrast, inactivating mutations in Vpr are selected against in vivo in both experimental SIV_{mac} infections of Asian macaques (217). Furthermore, long-term non-progressor (LTNP) populations have been described with inactivating mutations in Vpr (218–220), suggesting that maintenance of Vpr function is required for pathogenesis in vivo. The differences that determine selection for maintenance or deletion in cell lines versus *in*

vivo remain unclear. However, it is well understood that G_2 arrest is mediated by interactions with the Cul4A/DCAF/DDB1 ubiquitin ligase complex ($DCAF^{CRL4}$ complex) (221–224).

DCAF complex and DNA-damage proteins

It has been well characterized that induction of G_2 arrest is reliant on Vpr associating with the $DCAF^{CRL4}$ complex (221–224). It is generally thought that Vpr associates with the $DCAF^{CRL4}$ complex to target an unidentified host restriction factor for ubiquitin-mediated proteasomal degradation. The complex is similar to that used by other HIV-1 accessory proteins and the same as that used by HIV-2/SIV_{sm}/SIV_{mac} Vpx to target SAMHD1 for degradation (140, 225–227). Multiple groups have performed proteomics studies to find potential binding partners for the Vpr-$DCAF^{CRL4}$ complex, which have resulted in the identification of a number of targets. The first identified target of Vpr-$DCAF^{CRL4}$ was uracil DNA glycosylate 2 (UNG2), which excises uracil that has been misincorporated into DNA (228). Vpr expression mediates proteasomal degradation of UNG2, though the effect of UNG2 on the viral life cycle remains unclear (229–231). Alternatively, it has been proposed that Vpr interacts with UNG2 to recruit it to the viral DNA for removal of misincorporated uracils, but the clear reduction in UNG2 levels in the presence of HIV-1 Vpr provide contrary evidence to this suggestion (232–234). Additionally, interactions with UNG2 do not correlate with induction of G_2 arrest, suggesting that UNG2 may not be the primary target of the Vpr-$DCAF^{CRL4}$ complex (235).

Association with DNA damage response (DDR) proteins is thematic for Vpr-DCAF^{CRL4}. This complex also associated with the structure specific endonuclease 4 complex (SLX4com), which is a complex of proteins involved in Holliday junction repair (236). It has been suggested that Vpr recruits this complex to induce a DDR, which results in the observed G_2 arrest (236). Not all primate lentiviral Vprs interact with SLX4com, and it has been shown that interaction does not necessarily mediate cell cycle arrest (235–237). Similar to SLX4com, helicase like transcription factor (HLTF) associates with HIV-1 Vpr in complex with DCAF^{CRL4}, but interaction does not mediate G_2 arrest, nor is the interaction conserved amongst primate lentiviral Vprs (238, 239). HLTF is a DNA translocase involved in repair of damaged replication forks (238, 239). Though, it remains unclear what role these interactions have in the viral life cycle, association of Vpr with cellular proteins involved in the induction of DDR is a conserved function for all lentiviral Vpr alleles.

The Vpr-DCAF^{CRL4} complex interacts and degrades a handful of other proteins that are less clearly associated with induction of G_2 arrest. Vpr has been shown to degrade the miRNA processing protein DICER, which was shown to enhance infection of macrophages (240). Vpr also degrades certain histone deacetylases (HDACs) which remove acetyl groups from histones, condensing DNA (241, 242). It has been suggested this this interaction enhances transcriptional output by reducing quiescent or latent viral integration (241, 242). Again, the importance of these interactions to the viral life cycle remains unclear, but many of them have been linked to a Vpr-mediated regulation of IFN.

Interferon (IFN) regulation

Since induction of type I IFN responses are rarely observed during HIV-1 infection in vitro, a hypothesis that the virus encodes a protein that specifically blocks this induction has been pervasive in the literature. IFN-I is highly restrictive to most viral infection, including HIV (243–245). To counteract this, many viruses, including paramyxoviruses, arenaviruses, influenza and filoviruses encode viral proteins that shut down IFN signaling in order to allow for infection (246–251). The role of Vpr in regulation of IFN-I, if any, remains relatively unclear. There are publications arguing divergent hypotheses, suggesting both downregulation and upregulation of IFN-I responses by Vpr (236, 252–259). There have been a number of reports that Vpr specifically down-modulates IFN-I signaling during infection at IRF3, either through direct degradation or sequestration of IRF3 in the cytoplasm to prevent signaling (256, 257, 260). Other, contradictory reports have either attributed this function to Vpu and others have shown no difference in IRF3 levels and signaling during infection of both primary cells and cell lines with any of the viral accessory proteins (255, 261–264). Additionally, other groups have looked at IFN induction downstream of IRF3. Mashiba, *et al* noted in primary macrophages, infection with a Vpr-null virus resulted in a ten-fold increase in *IFNA1* mRNA in primary human macrophages (252). Work from Laguette, *et al* in HeLa cells also shows induction of IFN-α and IFN-β mRNA in response to infection with a Vpr-null virus in an SLX4com-dependent manner (236). Alternatively, multiple groups have shown a Vpr-specific activation of IFN-response during infection, though the benefit of such a response to viral fitness remains unclear (255, 258, 265). Vermeire,

et al recently reported that Vpr amplifies cGAS-dependent sensing of viral transcripts but Vpu acts to counteract IFN production (255). It is possible that differences in cell types and viral isolates used, as well as divergent Vpr-expression systems, as opposed to productive infections may account for these differences, though more work is necessary to clarify what role Vpr plays in IFN-I immune signaling during infection.

Regulation of viral env production in macrophages and dendritic cells (DCs)

In addition to potentially modulating type I IFN signaling, some recent studies have suggested that Vpr may regulate HIV-1 Env stability and processing. Data from Mashiba, *et al* shows that during infection of macrophages, Env is degraded via the lysosome in the absence of Vpr (252), resulting in a defect in viral production and viral spread (252). This work stands in contrast to that published by others in the field who show little to no replication defect in macrophages in the absence of Vpr (238). In a follow up paper from the same lab, they extend their work to show that this defect results in a defect in spread to $CD4^+$ T cells. In the absence of Vpr, they see significantly reduced viral infection of $CD4^+$ T cells co-cultured with infected macrophages in the absence of Vpr (266). Vpr-mediated enhancement of Env production has also been shown to occur in moDCs and certain cell lines by the Zheng lab by mediating proper folding of HIV-1 Env in the ER (267). In the absence of Vpr, enhanced ER stress due to accumulation of misfolded proteins induced an unfolded protein response that shuttles Env to the lysosome for degradation (267). Together, their work suggests that Vpr may play a role in promoting production of HIV-1 Env during infection of myeloid-derived cells. Since Env expression in productively infected cells is a late event in the viral life

cycle, modulation of Env production is unlikely to be determined by incoming virion-associated Vpr, but rather dependent on de novo expressed Vpr. Whether Vpr has roles at both early and late steps in the viral life cycle remains to be validated.

Replication defect in macrophages/DCs

The effects of Vpr on cell-type specific viral replication have been well studied, but remain somewhat controversial. It is well established that Vpr is not necessary for efficient replication in most cell lines, but effects in primary cells remain unclear (186). It was first reported by Balliet, *et al* in 1994 that Vpr is important for infection of macrophages, but dispensable for infection of PBMCs, (268). Additional evidence for this hypothesis was reported the following year when Connor *et al* published similar data, again showing that Vpr is dispensable for infection of resting or activated PBMCs but was required for infection of monocytes and macrophages (186). Since then, there have been a number of contradictory reports. Eckstein, *et al* also show a requirement for Vpr in tissue resident cells (269). Alternatively, Gummuluru, *et al* show contradictory work with single cycle viruses, indicating that Vpr enhances transcription from primary $CD4^+$ T cells, but may not have an effect on single cycle viral production from macrophages (201). Höhne, *et al* recently has shown the necessity of Vpr for infection of resting $CD4^+$ T cells (185), which may diverge from previously reported findings because they are the first to use purified, resting $CD4^+$ T cells rather than resting PBMCs. Additionally, work from the Kathleen Collins' lab has shown that Vpr-mediated regulation of Env production is essential for viral replication in macrophages and that this process enhances spread from macrophages to $CD4^+$ T cells (266), though this is contradicted by work

from Lahouassa, *et al* again showing that presence of Vpr has little effect on viral replication in macrophages (238, 252, 266). Work done in our lab has shown significant donor-to-donor variability in replication of Vpr-deficient viruses in macrophages, with some donors displaying a replication defect for Vpr-deficient virus while macrophages derived from other donors showing no differences in replication between WT and Vpr-deficient viruses (Akiyama, unpublished data). While these reports are confusing at best, what has been clearly defined in the literature is the effect of Vpr on replication in DCs. Our work, along with work from de Silva *et al* and Zhang *et al* are all in agreement that Vpr-deficient viruses replicate poorly in DCs, though the identified cause of this defect differs amongst the studies (184, 267). Our work identifies virion incorporated Vpr as being necessary for enhancing viral LTR-driven transcription in single round and spreading infection, possibly due to Vpr-mediated regulation of integration, whereas de Silva, *et al* identified *de novo* synthesized Vpr as being important for enhancing reverse transcription and viral LTR-driven transcription (184). Alternatively, work by Zhang, *et al* indicates that Vpr is important for proper Env production in DCs, implying that the replication defect only occurs over multiple rounds of replication (267). Published and unpublished work from our group suggests that this is not the case; I do not observe any differences in Env production in infected DCs and I cannot rescue replication with the ERAD inhibitors utilized by Zhang, *et al* in their studies (267).

Together, there are still many unanswered questions about the role for Vpr during infection. My work focusing on Vpr function during infection of DCs, discussed in this document, attempts to clarify some of the controversies regarding Vpr and extend the

understanding of its function. It is my hope that with better understanding of the molecular mechanisms of infection, better therapeutics will be developed to counteract infections.

Current therapeutic approaches

Current pharmacological approaches targeting HIV-1 infection can be roughly divided into two main strategies, either targeting the virus early, during acute infection with high doses of HAART and latency limiting agents or targeting the virus during chronic infection using the "shock and kill" strategy (270). The "shock and kill" strategy utilizes latency reversing agents (LRAs) which target and reactivate latent virus in the host cell (270). After reactivation, it is thought that a combination of the host immune system and high doses of HAART could act to purge the virus from the infected individual, though to date no studies have achieved robust enough reactivation (270). Though both approaches have met with limited success in patients, they have provided important insights into HIV-1 pathogenesis that will help shape future therapeutic approaches (270).

Treating with high doses of HAART and other pharmacological agents early, during acute infection, is becoming a popular area of study after a number of case reports of undetectable viral load in HIV-infected individuals (271, 272). HAART treatment can either be initiated at extremely high doses early post infection, like with the case of the recent "Mississippi baby" or coupled with an agent that limits seeding of a latent reservoir by killing or limiting the expansion of memory $CD4^+$ T cells, such as hydroxyurea (273, 274). In the case of the "Mississippi baby," an HIV-positive infant

was treated with high doses of HAART 30 hours after birth/viral detection and remained on the treatment for 18 months (274). At this time, the child was removed from therapy because of lack of parental consent, after which she remained HIV-negative (within limits of detection) for 27 months before her virus rebounded (274). While the "Mississippi baby" was not cured like many scientists had hoped, she did show long lasting control of virus replication and provided insight into disease pathogenesis that may lead to better treatment regimens in the future (274).

An alternative approach to the treat-early strategy is to couple HAART with another drug regime that limits seeding of a latent reservoir. One such agent, hydroxyurea, a cytostatic drug that halts the cell cycle in the G_1 phase, has had mixed results (270, 273). There are several reports that early treatment with combination HAART/hydroxyurea can decrease viral load, in one case to undetectable levels (272). These studies are limited to few individuals, and while experiments in a SIV_{mac} model of infection has reaffirmed these findings, use of hydroxyurea is not recommended due to possible liver and pancreatitic toxicity (275–277). Continued research is underway to determine if a treatment regimen coupled with hydroxyurea may be a viable therapeutic approach, as well as to identify other possible drugs with similar reservoir-limiting effect (270). Unfortunately, the treat-early approach will never work for many patients, since most HIV-positive individuals are not identified until they are in the chronic stage of infection (270).

For chronic infection, the current therapeutic approach primarily under investigation is called the "shock and kill" strategy (270). During the "shock" phase, a

latency reversing agent (LRA) is used to reactivate HIV in latently infected cells so that they begin to make new virus (270). During the "kill" phase, either viral cytopathic effect or the host immune system will target and eliminate the infected, newly-transcribing cells (270). These therapies are employed while the patient remains on HAART to prevent reinfection and reseeding of the reservoir (270). Pharmacological agents under investigation include chromatin modifying compounds like histone deacetylate inhibitors or bromodomain inhibitors, cytokines like IL-7, and T cell activating compounds like phorbol 12-myristate 13-acetate (PMA) or anti-CD3/CD28, though use of pan-T cell activating agents is typically restricted to *in vitro* work (270). So far, clinical data from these studies has yielded mediocre results, likely due to incomplete viral reactivation by these compounds *in vivo* (270). Currently, there remains hope that development of better LRAs paired with multiple rounds of "shock and kill" could eventually purge viral reservoirs from infected individuals (270). Until now though, the only therapeutic strategy that has resulted in a functional cure was the result of a bone marrow transplant.

Bone marrow transplants

The only successful treatment to date has come from the treatment of high risk HIV+ patients with secondary malignancies (270, 278). In this singular case, the "Berlin patient," later identified as Timothy Brown, underwent an aggressive combination treatment of ablative chemotherapy and radiotherapy to treat acute myeloid leukemia (270, 278). After which, he was placed on immune suppression drugs and received a allogeneic stem cell transplant from a donor who was homozygous for the Δ32 deletion of the *CCR5* gene, shown to confer protection against HIV-1 transmission (270, 278). At

the time of therapy, HAART treatment was ceased, and his viral load since that point has remained undetectable in both his blood and tissues, even in the absence of HAART (270, 278). While there is some evidence that there may be some residual HIV infection in Mr. Brown, he remains the only successful case of a functional cure, where viral load is successfully controlled to an undetectable level by the host immune system.

In order to understand the contribution of the Δ32*CCR5* deletion, several other studies employing bone marrow transplantation have been conducted. One, conducted on two "Boston patients" utilized allogeneic stem cell transplantation from donors with wild type *CCR5* alleles (279, 280). It was the hope that graft-versus-host response would rapidly eliminate the remaining virally infected cells within the infected individuals before the virus is able to spread to the engrafted immune system (279, 280). Post transplantation, both patients were kept on HAART to minimize the ability of the virus to spread to the transplanted cells (279, 280). Both had no detectable HIV DNA in the periphery while on antiretroviral therapy. Furthermore upon treatment interruption, patients remained HIV(-) for a period of 12 to 32 months (or weeks) post HAART interruption before viral rebound (279, 280). Together, this suggestion that stem cell transplant alone is inadequate to purge the latent host reservoir in the absence of a protective mechanism like the Δ32*CCR5* deletion. Since the frequency of the Δ32*CCR5* deletion is low, ~1% of the Caucasian population is homozygous for it, alternative strategies, including gene therapy approaches, are being investigated for inducing protection from reinfection after stem cell transplants (270, 278).

Despite these rapid advances in stem cell therapy approaches, treatment is unlikely to provide a viable cure for most of the world's HIV-positive population. High cost of care is highly restrictive, limiting treatment availability primarily to developed countries (270, 278). Additionally, the procedure is incredibly risky for patients and has a high mortality rate due to treatment complications and uncontrollable graft-versus-host disease (270, 278). Altogether, while advance in bone marrow transplant techniques may be able to provide a functional cure for some, it is likely that these procedures will remain limited, since patients are subject to high-risk secondary complications including bone marrow disorders and leukemias. It is the hope of many researchers that development of pre-exposure therapeutics, like an effective vaccine, may help to stem infection in the absence of an accessible cure.

Vaccine development

Development of a broadly effective HIV-1 vaccine poses many challenges. Typically, vaccine development is modeled around mimicking a successful, sterilizing immune response in an infected host, but a sterilizing response to infection with HIV-1 has never been reported (281, 282). With no information to determine what a protective immune response against HIV might look like, scientist and vaccine developers are at a severe disadvantage in the fight against HIV. The first challenge posed by the virus is its sheer diversity; there are nine clades that may vary as much as 45% at the amino acid level, which makes it difficult to design a vaccine that provides broad protection against many or all clades (18). Additionally, HIV-1 is able to rapidly mutate its surface exposed proteins in order to avoid detection by host antibodies, allowing the virus to escape

detection and neutralization by a primed host immune system (283–285). To date, there have been a handful vaccine trials in humans, only one of which has had any efficacy in protecting against infection and provided only mild protection, at best (282). Current strategies in vaccine design are now frequently focusing on designing vaccines to illicit broadly neutralizing antibodies (bnAbs).

Broadly neutralizing antibodies

The discovery of bnAbs has given new hope that long lasting protection against HIV is possible. BnAbs target and neutralize a broad range of gp120 trimers across all clades of HIV (282). Examples include PG9 and PG16 that neutralize approximately 80% of HIV strains, VRC-01 which neutralizes 90% and 10E8 which neutralizes 98% of tested HIV-1 viruses (282). Studies conducted to identify similarities between bnAbs have shown that they all target one of four conserved areas on gp120, either the membrane-proximal region of gp41 which anchors the protein to the viral membrane, the first or second variable region on gp120 which are highly mutable to prevent antibody recognition, the V3 region on gp120 which determines co-receptor usage, or the CD4 binding site (286, 287). Additionally, these antibodies are also unique in containing extensive hypermutation and/or an unusually long complementary-determining region (282). These antibodies are exciting due to their potential therapeutic value. Studies using passive immunization against SHIV (chimeric simian-human immunodeficiency virus that encodes HIV-1 Env) challenges in non-human primates (NHPs) have shown robust protection against infection (282). Unfortunately, passive immunization with bnAbs is costly and time consuming for at-risk individuals, and thus is not practical for use as a

wide-spread therapeutic (282). An ideal therapy would be a vaccine that induces production of these broadly effective antibodies in the host (282). Unfortunately, their extensive hypermutation and long complementary-determining regions suggests that these antibodies come from B cells that have undergone significant affinity maturation in the presence of continuous antigen stimulation during chronic disease (282). Even amongst chronically infected individuals, they are quite rare, occurring in only 10-30% of individuals (282). Together, this suggests that stimulating production of bnAbs *in vivo* will be quite challenging. Despite challenges in therapeutic design, there have been some successes in development of strategies to prevent transmission. Pre-exposure prophylaxis (PrEP) has had enormous success in the prevention of transmission.

Pre-exposure prophylaxis

PrEP is a relatively new strategy to counteract HIV-1 infection. PrEP is blanket term for one of several possible HAART regimens high risk individuals can take to reduce the potential of transmission of HIV-1 in the event of an exposure (288). Development of PrEP is based on work done using SIV_{mac} models of transmission that characterized what dosing of antiretrovirals is required to provide protection from transmission, both pre- and post-exposure (289–292). Post-exposure studies indicated that 3-4 weeks of continuous HAART, initiated within hours of an exposure event is required to significantly reduce the likelihood of infection, which has come to be defined as the standard of care for accidental laboratory or hospital exposures (288, 293–295). Post-exposure studies in macaques have revealed two different antiretroviral regimens that are efficacious in human trials. The first requires a daily dose of either tenofovir

disoproxil fumarate (TDF) in combination with emtricitabine (FTC), also called Truvada, or TDF alone (296). Effectiveness of this approach has been shown in men who have sex with men (MSM), heterosexual men and women, serodiscordant couples, and injection drug users (297–300). In the iPrEx human trial in populations of MSM, daily dosing shows a 44% risk reduction overall (300). If the trial group is broken down further, this increases to 73% when limited to high self-reported adherence and 92% when drug was detectable in the plasma (300). While this trial and others like it have shown high efficacy under high adherence, daily PrEP is not without its faults. Tenofovir-based treatment is known to have potential side-effects including reduced bone density as well as renal toxicity in uninfected individuals (301–304). Sub-optimal drug adherence may also increase the prevalence of tenofovir-resistant HIV circulating in infected populations, ultimately limiting the long-term efficacy of PrEP (305) and the high cost of daily medication, especially during periods of low risk, remains a major hurdle for many at-risk groups. Despite these downfalls, the WHO is now recommending daily PrEP for high-risk populations to combat the spread of HIV (306).

The second antiretroviral regimen developed is an event-based strategy that has individuals take antiretrovirals based on high-risk behavior or potential exposures. With event-based PrEP, two doses of Truvada are taken before intercourse and one dose per day for two days after (307, 308). Two studies, both PROUD and IPERGAY have investigated this efficacy in MSM populations and both reported an 86% reduction in HIV acquisition (307, 308). This approach has the advantage of using far less doses of Truvada than daily dosing and to-date has not produced any tenofovir-resistant mutations

(296). Additionally, there is lower risk of drug toxicity than daily dosing, reducing any long-term side effects of treatment (288). Event-based dosing is not without its pitfalls as well. There are concerns that the complexity of the regimen will lead to lower adherence and it is thought to be a less effective therapy in women due to low vaginal drug levels (288, 309). Currently this strategy is only recommended for MSM populations in certain areas of the world (296).

Though research on viral protein functions and studies on viral life cycle in vitro have provided numerous approaches to therapeutic development, and a number of these therapies have provided clear benefit for HIV-infected individuals, a cure remains out of reach, primarily because of the ability of the virus to establish to a latently infected tissue reservoir that has proven difficult to purge.

Dendritic cells: role in immune response

HIV-1 infection of DCs is the primary focus of this work. DCs are critical sentinel cells that lie at the interface of innate and adaptive immunity (310). They are antigen presenting cells able to initiate T cell immunity and help develop B cell immunity (310). There are a number of different DC subsets, all with crucial roles in innate and adaptive immune response. Plasmacytoid DCs (pDCs) are a small subset of DCs that mostly localize to the blood and lymphoid organs (311, 312). They have a tightly controlled range of pattern recognition receptors that are specialized to enhance pathogen detection that results in production of ant-viral type I interferons (type I IFNs) (311, 312). Plasmacytoid DCs, more than any other cell, are able to make enormous quantities of type I IFNs in response to viral pathogen detection (311, 312). Conventional DCs (cDCs)

make up the other main class of DCs that localize to tissue compartments and are critical for T cell immunity (311, 312). Upon pathogen detection, these cells will enter the lymphatics and travel to the paracortical T cell zone of the regional lymph nodes, engage naïve T cells and initiate a specific T cell response to the detected pathogen (313). Conventional DCs also localize to the marginal zone of the spleen where they interact with blood to acquire circulating blood and tissue antigens for T cell presentation (311, 312). The final subset are monocyte derived DCs (moDCs), also known as inflammatory DCs (311, 312). Unlike other subsets of DCs that differentiate from hematopoietic precursors, moDCs differentiate from circulating blood monocytes under inflammatory conditions or during infection (311, 312). These cells are the most abundantly studied human DC subset, due to the ease of isolation and creation *ex vivo* (311, 312, 314). Monocytes are relatively abundant in the circulating blood and can be isolated by $CD14^+$ selection. They can then be differentiated in the presence of interleukin 4 (IL-4) and granulocyte-monocyte colony-stimulating factor (GM-CSF) to differentiate into $MHCII^+$, $CD11c^+$, $CD25^+$ and DC-$SIGN^+$ immature moDCs (311, 312). These cells can subsequently be matured with a variety of stimuli including LPS, IFNγ, TNFα and CD40 ligand (311, 312). While they share many characteristics of cDCs, they remain an imperfect model and studies should be carefully conducted to reaffirm results in cDCs.

Initiation of an immune response

Initiation of a CD4- or CD8- immune response requires more than just MHC-antigen recognition. A secondary signal is required in order to limit immune over-reaction to an innocuous antigen (315). These secondary signals are typically provided by

the antigen presenting cell, either through co-stimulatory molecules on the cell surface or cytokine secretion (315, 316). Both of these responses are initiated in the antigen presenting cell by triggering one of a number of pathogen recognition receptors (PRRs) by a pathogen association molecular pattern (PAMP) (317, 318). DCs are highly enriched in these pathogen detection molecules that allow them to sense and initiate an immune response in reaction to infection (317, 318). There are a number of different initiation proteins, present in the cytoplasm and endosomes as well as cell-surface exposed sensors on the plasma membrane (317, 318). These PRRs are able to detect conserved patterns on or within common pathogens including bacteria, viruses, fungi and parasites (317–322). Commonly detected PAMPs include surface structural molecules unique to bacteria or prokaryotic cells or viral nucleic acids (319–322). There are a number of different PRRs, including toll-like receptors (TLRs) which detect bacterial and viral proteins, glycans or lipids, and nucleic acid sensors such as, RIG-I-like receptors (RLRs) and cyclic GMP-AMP synthase (cGAS) which detect abnormal nucleic acid structures in the cytoplasm (319–322). All of these molecules result in initiation of signaling cascades that culminates in upregulation of co-stimulatory molecules and secretion of cytokines, though the exact response varies slightly based on which PRR is triggered (317–322). The ability of DCs to initiate an immune response to a foreign pathogen complicates their interactions with HIV.

Role in HIV-1 infection

It is a limitation of HIV research that most studies, ours included, have focused on interactions of the virus solely with moDCs, due mostly to ease of attainability (314).

Despite this, much has been discovered using *ex vivo* IL-4 and GM-CSF stimulated, moDCs as a model system for HIV infection (314). In general, CCR5-topic HIV-1 infects immature moDCs at a low but measurable level (Fig. 6) (184). This restriction is due primarily to the presence of the dNTPase SAMHD1, but it has also been reported that a low molecular weight form of APOBEC3G may play a role in viral restriction as well (179, 180, 323). SAMHD1 is present in DCs regardless of activation status and acts to lower dNTP pools, preventing successful reverse transcription (179–182). Alternatively, APOBEC3A is upregulated with maturation, and may be partially responsible for complete viral restriction that is observed in mature DCs (323, 324). In mature moDCs, additional restriction from the downregulation of CCR5 limits viral entry (323). Viruses that do manage to enter and complete reverse transcription suffer from additional post-integration restriction of viral transcription (323). *In vivo* studies of patient cohorts have been able to detect low levels of infected tissue DCs and studies using the SIV_{mac} model of infection have shown that tissue resident DCs are amongst the first cells to become infected after exposure, indicating that they are relevant target cell for infection (61, 325). In addition to cis-infection, DCs mediate HIV trans-infection.

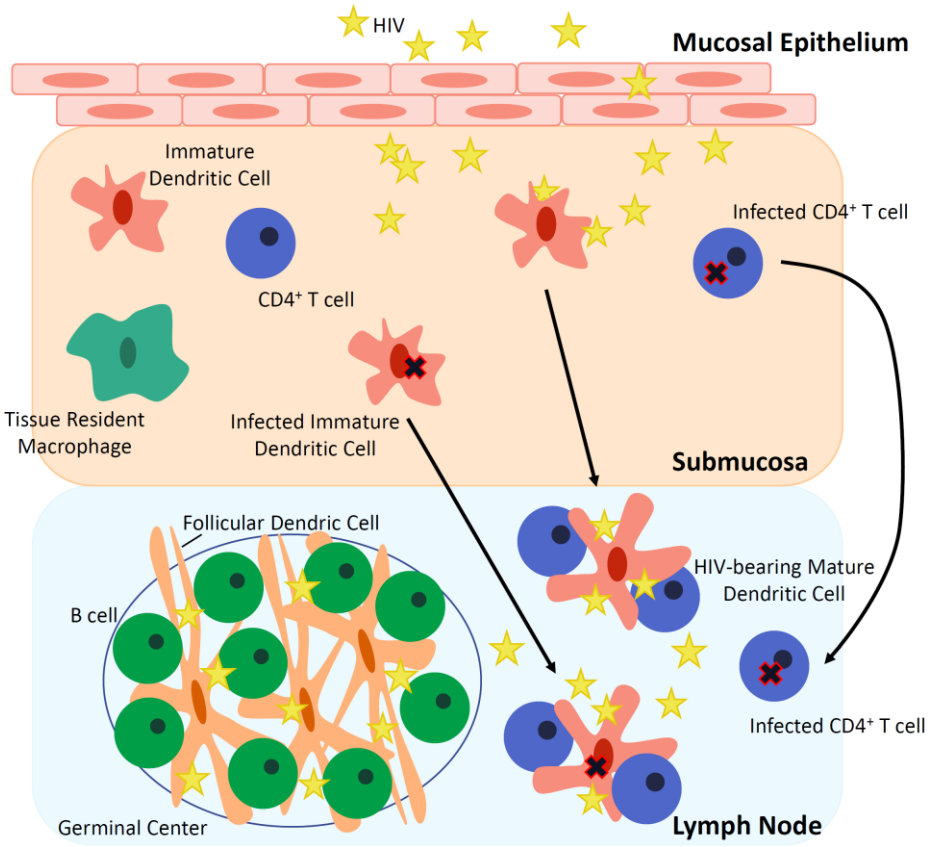


Figure 6. Summary of HIV-1 interactions with dendritic cells.

Typical exposure to HIV-1 first occurs at mucosal surfaces. HIV can cross mucosal barriers, either through active transport or via cell associated transport. Once the virus is in the submucosal layer, it can interact with a number of tissue-resident immune cells that are directly susceptible to infection, including macrophages, $CD4^+$ T cells, and immature DCs. Mature DCs, present because of either HIV- or non-HIV-related inflammatory signals can capture the virus with high efficiency. Both DCs and $CD4^+$ T cells are migratory and will travel to nearby lymph nodes where there is a high concentration of target $CD4^+$ T cells. Follicular dendritic cells in germinal centers will also capture and retain virus, increasing spread.

HIV trans-infection is the process by which DCs capture virus through surface receptor binding to a viral component and transfer it with high efficiency to target cells (Fig. 6) (326). This process is far more efficient than cell-free infection and is mediated almost exclusively by mature DCs (327, 328). It was originally thought that the surface receptor responsible for this interaction was dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), which is capable of binding to lectins on the surface of the virion (329). DC-SIGN is expressed highly on immature DCs, which are poor conductors of trans-infection, and is downregulated with maturation (327, 328). Mature DCs instead utilize CD169, a type I IFN-induced cell surface protein that binds sialic acid residues on the lipid membrane of the virion (121, 122, 330, 331). Our lab and others have characterized these interactions and have shown that CD169 binding to virions results in receptor clustering and formation of a surface-connected compartment that protects the virus until transmission to $CD4^+$ T cells (332). Dissemination of virus via mature DC-mediated trans infection pathway may be a critical early step in establishment of infection in the peripheral mucosal tissues (Fig. 6).

In this work, I have tried to address some of the unanswered questions regarding the role of Vpr in the HIV infection cycle. Based on what was reported by de Silva, *et al* (184) I hypothesized that infection of MDDCs might be a robust system to address some of the controversies and unanswered questions about the role of Vpr during infection. I found that infection of MDDCs with Vpr-deficient virus is attenuated as compared to wild type (WT) infection. MDDCs are a unique system in which to study Vpr function since they are the only cell type to consistently show differences in infection in the

absence of Vpr. I sought to determine what step of the viral life cycle was affected in the absence of Vpr, as well as clarify some of the controversial roles prescribed to Vpr during infection, including regulation of Env production and IFN induction.

I also sought to determine whether the functions I characterized for HIV-1 Vpr in MDDCs were maintained in diverse primate lentiviral Vprs. As discussed above, Vpr is present in all primate lentiviruses, suggesting a critical function during *in vivo* infection (177, 196). Decreased pathogenicity has been seen in both SIV_{mac} models of infection and cohorts of long term non-progressors that have mutations in Vpr (198, 218–220). I hypothesized that a critical function of Vpr would be conserved across lineages, similar to functions ascribed to Vif and Nef (140). In the process, I discovered a novel role for Vpr in regulating sensing of integration. In the absence of Vpr, I observe increases in the antiviral cytokine IFN, which would restrict replication *in vivo* (333–335). I propose that this function is critical for *in vivo* pathogenesis and the reason for maintenance of Vpr expression.

CHAPTER ONE

Introduction

HIV-1 encodes a number of proteins that allow for entry and replication in human cells. In addition to the structural or enzymatic proteins that have well defined functions in the replication cycle, there are also a number of small, accessory proteins. Accessory proteins encoded by HIV-1 are not always necessary for replication *in vitro*, but are absolutely essential for replication *in vivo* (140). These proteins serve to counteract host restriction factors that would normally limit HIV-1 infection (140, 336). Of the accessory proteins encoded by HIV-1, Vpr is the only one whose function remains relatively unclear.

Vpr is a small, 96 amino acid, 14 kDa protein that is packaged into the budding virion through associations with the p6 region of Gag (187, 191–194, 337–339). This association allows Vpr to be present in the cell at a relatively high quantity (~200-300 molecules/virion) upon initial infection (340). Previous studies have extensively characterized the outcome of Vpr expression in various cell types. In cycling cells, Vpr expression results in G2/M cell cycle arrest which culminates in induction of apoptosis (206, 341, 342). It is well established that Vpr-mediated G2/M cell cycle arrest is mediated though its association with the Cul4A/DCAF/DDB1 E3 ($CRL4^{DCAF1}$) ubiquitin ligase complex (224, 343, 344). In addition, HIV-1 Vpr recruits and degrades a number of DNA-damage response (DDR) proteins, including the SLX4-SLX1/MUS81-EME1 structure-specific endonuclease complex (SLX4com), Uracil DNA glycosylase 2 (UNG2), and helicase-like transcription factor (HLTF) (228, 236, 238, 239) via the

$CRL4^{DCAF1}$ complex resulting in G2/M cell cycle arrest though it still remains unclear what role this process plays during HIV-1 infection.

Though a number of previous studies have examined the requirement of Vpr on HIV-1 replication in various cell types, including primary $CD4^+$ T cells and monocyte-derived macrophages (MDMs), differences in virus replication have not been consistently observed (185, 236, 238, 266, 268, 269, 345). Vpr expression is dispensable for infection in activated $CD4^+$ T cells *in vitro* (266, 268, 269, 345, 346), presumably due to the well characterized cytostatic and cytopathic functions of Vpr in cycling cells (341). In contrast, recent studies in MDMs suggest that Vpr is necessary for HIV-1 envelope (Env) expression, and the purported consequence of infection of MDMs with Vpr-deficient viruses was reported to be decreased viral production and reduced cell-to-cell spread to $CD4^+$ T cells (252, 266). Notably, there has been considerable heterogeneity in replication differences between wild type and Vpr-deficient viruses and host responses to virus infection in MDMs, presumably due to donor and experimental variability between studies (186, 341, 347). Additionally, it has also been reported that Vpr expression in macrophages can both inhibit or induce type I interferon (IFN) responses (236, 252, 256, 258, 265, 348).

Dendritic cells (DCs) are sentinel cells that bridge innate and adaptive immunity (310). They actively patrol peripheral tissues, including mucosal sites of HIV-1 transmission, in search of foreign pathogens. Because of this, MDDCs are among the first cells to interact with HIV-1 upon sexual transmission of the virus (61, 349–352). While MDDCs are less susceptible to infection than activated $CD4^+$ T cells and macrophages,

they are still able to be infected *ex vivo* at a low but consistent level (184, 267, 353, 354). In contrast to work with MDMs and $CD4^+$ T cells, there have been isolated descriptions of effects of Vpr on HIV-1 replicative capacity in MDDCs (184, 267), with no consensus on the mechanisms accounting for Vpr-mediated enhancement of virus replication. In this study, I use MDDCs as a model system to investigate the role of Vpr during infection. I found a robust replication defect of Vpr-deficient HIV-1 in MDDCs and, contrary to previous studies (267), the replication defect was not due to decreased Env expression in Vpr-deficient HIV-1 infected cells. Rather, the block to ΔVpr virus infection was at the step of viral transcription and could be rescued by addition of Vpr *in trans* into the virion in a single round infection analysis. I found that mutations, Vpr-Q65R and Vpr-H71R, which ablate association of Vpr with the $CRL4^{DCAF1}$, or Vpr-R90K which does not induce G2 cell cycle arrest (201, 221, 222, 224, 355–357), displayed similar decreases in replication and viral transcription in single round of infection analysis. Together these data show a novel post integration block to HIV-1 replication in MDDCs at the point of viral transcription that is alleviated by virion-associated Vpr.

Results

Vpr-deficient viruses display a replication defect in DCs.

HIV-1 replication in MDDCs is restricted at the reverse transcription step by SAMHD1 that controls the size of the cytosolic dNTP pools (179, 180). Despite the presence of SAMHD1, MDDCs remain susceptible to HIV-1 infection *in vitro* at a low but measurable level (267, 358–360). I infected MDDCs with replication competent wild

type (WT) or Vpr-deficient (ΔVpr) CCR5-tropic Lai-YU2 and harvested cells for intracellular $p24^{Gag}$ expression by flow cytometry analysis 3 days post infection. Input for these infections was normalized based on infectious titer of the viruses on TZM-bl cells. As expected, $CD11c^{+}$ DC-$SIGN^{+}$ MDDCs were susceptible to viral infection, albeit to low levels (Fig 7A and B). Interestingly, Lai-YU2/ΔVpr failed to establish a robust infection in MDDCs (Fig. 7A and B), and there was a reproducible 3- to 5-fold decrease in percentage of $p24^{Gag+}$ cells in ΔVpr virus infections as compared to WT virus infections (Fig. 7B). To determine the functional consequences of Vpr-deficiency on virus spread, DCs and PHA/IL-2-activated $CD4^{+}$ T cells were infected with infectious viruses (MOI = 1) and cell-free culture supernatants were harvested every 3 days and analyzed for $p24^{Gag}$ content by an ELISA. While there was some donor variability, Lai-YU2/ΔVpr infection of MDDCs derived from 3 independent donors consistently resulted in significantly lower levels of replication than wild type Lai-YU2 infection (Fig. 7C). In contrast to the substantial attenuation of virus spread in Lai-YU2/ΔVpr infected DCs, both viruses replicated to a similar extent in activated $CD4^{+}$ T cells (Fig. 7E), in agreement with previously published studies (266, 268, 269, 345, 346). These results suggest that Vpr plays an important role in facilitating HIV-1 infection of DCs.

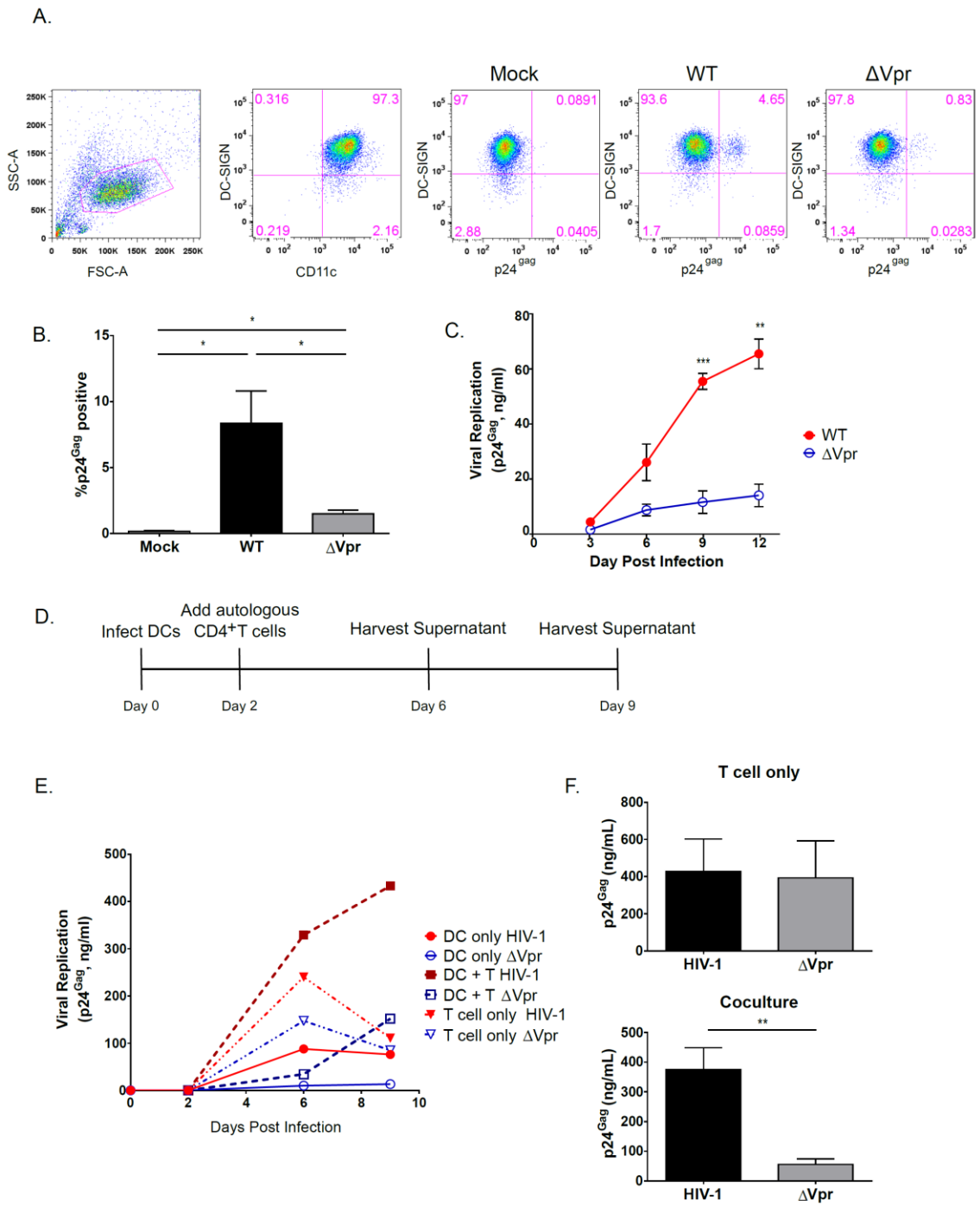

A.
Mock
WT
ΔVpr
SSC-A
FSC-A
DC-SIGN
CD11c
p24 gag
0.316
97.3
0.219
2.16
97
0.0891
2.88
0.0405
93.6
4.65
1.7
0.0859
97.8
0.83
1.34
0.0283
B.
%p24Gag positive
Mock
WT
ΔVpr
C.
Viral Replication (p24Gag, ng/ml)
Day Post Infection
WT
ΔVpr
D.
Infect DCs
Add autologous CD4+T cells
Harvest Supernatant
Harvest Supernatant
Day 0
Day 2
Day 6
Day 9
E.
Viral Replication (p24Gag, ng/ml)
Days Post Infection
DC only HIV-1
DC only ΔVpr
DC + T HIV-1
DC + T ΔVpr
T cell only HIV-1
T cell only ΔVpr
F.
T cell only
p24Gag (ng/mL)
HIV-1
ΔVpr
Coculture
p24Gag (ng/mL)
HIV-1
ΔVpr

Figure 7. Infection with Vpr-deficient HIV-1 results in attenuated virus replication in MDDCs and MDDC-T co-cultures.

(A) FACS profiles of mock infected MDDCs or MDDCs infected with Lai-YU2 or Lai-YU2/ΔVpr (MOI =1) at day 3 post infection. Cells were stained for CD11c, DC-SIGN and $p24^{Gag}$. From left to right, plots shown depict the gating strategy for the flow cytometry analysis and include plots of forward scatter/side scatter to exclude cellular debris, anti-CD11c/anti-DCSIGN staining to identify MDDC population, and DC-SIGN/$p24^{Gag}$ staining to identify productively infected MDDCs in mock infected, or WT (Lai-YU2) and ΔVpr infected DCs. (B) The mean (± SEM) percentage of DC-$SIGN^{+}$ intracellular $p24^{Gag}$ positive MDDCs determined from infections of cells derived from three donors infected as in (A). (C) Replication kinetics of Lai-YU2 and Lai-YU2/ΔVpr in MDDCs infected at MOI =1. MDDC supernatants were harvested every three days and analyzed for $p24^{Gag}$ content by an ELISA. Data shown are the mean (± SEM) for three independent experiments with MDDCs derived from three independent donors. (D) Schematic of DC-T cell co-culture set up. MDDCs were infected with Lai-YU2 or Lai-YU2/ΔVpr (MOI = 1). At two days post infection, autologous $CD4^{+}$ T cells (PHA/IL2 treated) were added at a 2:1 ratio to MDDCs or infected with cell-free virus in parallel (MOI = 1). Supernatants were harvested on day 6 and day 9 post infection (day 3 or 6 for cell-free $CD4^{+}$ T cell infection), and the $p24^{Gag}$ content in the culture supernatants determined by an ELISA. (E) The data shown is the kinetics of $p24^{Gag}$ production in cell culture supernatants from a representative infection of MDDCs only, $CD4^{+}$ T cell only or MDDC - $CD4^{+}$ T cell co-cultures. (F) The mean (±SEM) $p24^{Gag}$ present in the

supernatant from five independent donor infections of $CD4^+$ T cells only or DC-$CD4^+$ T cell co-cultures at day 6 post infection (day 3 post infection for cell free $CD4^+$ T cell infections). Significance calculated using paired student's T tests where *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Numerous studies have demonstrated robust HIV-1 replication in DC-T cell co-cultures at levels greater than that observed in infections of either cell type alone, and is dependent on rapid highly efficient transmission of DC-derived progeny virions to $CD4^+$ T cells across infectious synapses (326, 353, 354, 359–364). I sought to determine the effect, if any, of Vpr-deficiency on DC-mediated virus spread to $CD4^+$ T cells. MDDCs were first infected with wild type Lai-YU2 or Lai-YU2/ΔVpr and cultured for two days, prior to initiation of co-culture with autologous activated $CD4^+$ T cells (Fig. 7D). There was a substantial enhancement of virus replication in co-cultures infected with WT virus, compared to ΔVpr virus infections (Fig. 7E and F; ~7-fold increase). Interestingly the difference between WT and ΔVpr virus replication in DC-T cell co-cultures was greater than that observed in infections of MDDCs or $CD4^+$ T cells alone (Fig. 7E and F). Together, these results suggest that the replication defect observed in MDDCs infected with HIV-1/ΔVpr translates to $CD4^+$ T cells during cell-to-cell contact and transmission.

Defects in Vpr infection are independent of viral glycoprotein expression.

Previous studies have suggested a requirement for Vpr in maintaining robust HIV-1 gp120 expression in MDMs and MDDCs by counteracting a myeloid cell-intrinsic mechanism of Env degradation (252, 266, 267). To begin to understand the underlying mechanism accounting for the replication defect of HIV-1/ΔVpr in DCs, I examined viral protein expression in MDDCs infected with wild type Lai-YU2 or Lai-YU2/ΔVpr (MOI = 3). Infected cells were lysed 6 days post infection for quantitative western blot analysis. I did not observe any steady-state differences in gp120 expression when normalized to

Gag (p55 and p24) levels in MDDCs infected with WT or ΔVpr viruses (Fig. 8A). Quantification of immunoblots from infected MDDC lysates derived from four independent donors showed no significant differences in gp120 expression (Fig. 8B). I next sought to determine if Vpr-deficiency might result in decreased gp120 incorporation in virus particles derived from productively infected DCs. MDDC culture supernatants were harvested on multiple days post infection and pooled supernatants were concentrated over a sucrose cushion prior to western blot analysis. I again failed to observe any significant differences in levels of gp120 incorporation between virus particles derived from WT or ΔVpr infected MDDCs (Fig. 8C and D). The consistency of the replication defect of HIV-1/ΔVpr virus in MDDCs in the absence of any significant differences in gp120 expression suggests that previously hypothesized Vpr-dependent enhancement of gp120 production is unlikely to account for the observed replication defect in the present study (252, 267).

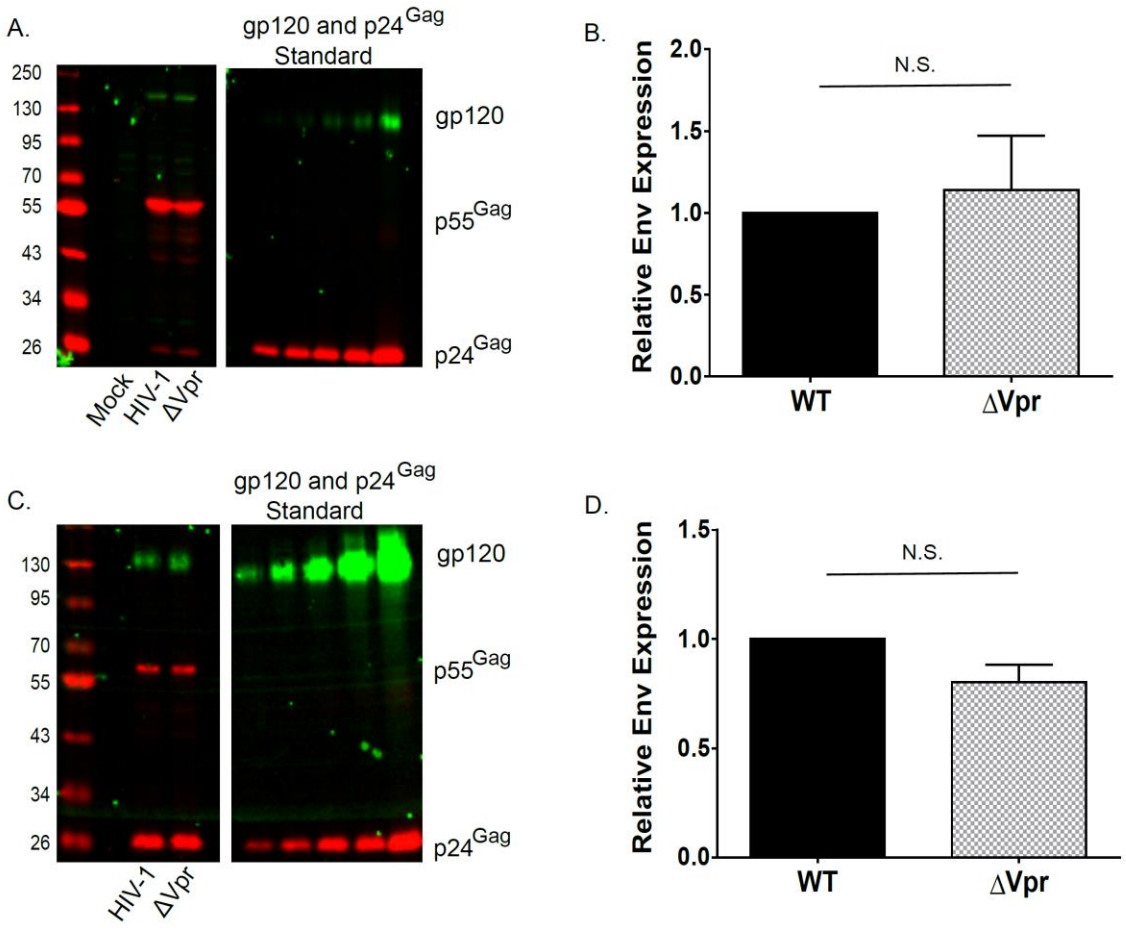


Figure 8. Vpr does not regulate Env expression in infected MDDCs or incorporation of Env into MDDC-derived virions.

(A) Western blot analysis of mock infected, Lai-YU2 (WT) or Lai-YU2ΔVpr infected MDDCs (MOI = 3) for $p55^{Gag}$ and gp120 expression at day 6 post infection. (B) Quantification of western blots for $p55^{Gag}$ and gp120 in infected MDDCs as in (A) from four independent experiments. The gp120 band intensity was quantified and normalized to $p55^{Gag}$ from experiments with infected MDDCs derived from 4 donors. Data shown are mean (± SEM). (C) Western blot analysis of $p24^{Gag}$ and gp120 expression in mock infected, Lai-YU2 (WT) or Lai-YU2ΔVpr infected MDDCs (MOI = 5). MDDC culture

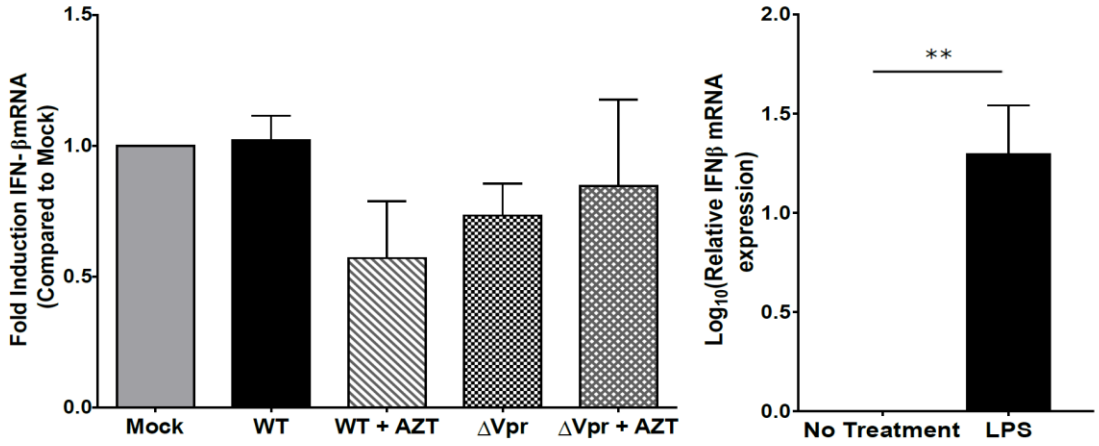
supernatants were harvested at days 3, 6, and 9 post infection, pooled and concentrated over a 20% sucrose cushion and virus pellets lysed for western blot analysis. (D) Quantification of western blot analysis from MDDC-derived virions from three independent donors. The band intensity for gp120 was quantified and normalized to $p24^{Gag}$ band intensity. Data shown are mean (± SEM). Significance calculated using a one sample T test where N.S>0.05.

Infection with Vpr-deficient HIV-1 does not induce type 1 IFN.

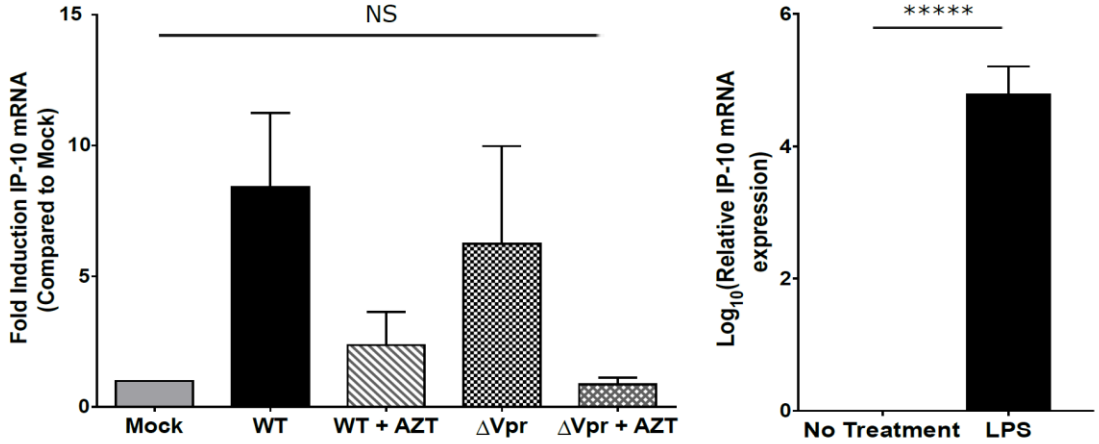
Exposure of target cells to type I IFN potently restricts HIV-1 replication *in vitro* (243, 245, 333–335, 365–368). In addition, recent studies have suggested that infection with ΔVpr virus induces type I IFN (236, 252, 256, 258, 265, 348). Hence, I sought to determine if induction of an early type I IFN response in HIV-1/ΔVpr infections of MDDCs accounts for the restricted virus replication and spread. MDDCs infected with wild type Lai-YU2 or Lai-YU2/ΔVpr virus were harvested 48 h post infection, and the mRNA expression levels of IFNβ and the type I IFN-inducible protein, interferon-γ-inducible protein 10 (IP-10) were quantified by qRT-PCR. At 48 h post-virus exposure, I did not detect significant increases in IFN-β mRNA levels in wild type or ΔVpr infected cells compared to mock infected cells (Fig. 9A). While expression of the ISG, IP-10, was robustly induced by establishment of productive HIV-1 infection of DCs, differences in IP-10 mRNA levels between WT and ΔVpr virus infections were not statistically significant (Fig. 9B). Note that pre-treatment of cells with azidothymidine (AZT) reduced induction of IP-10 mRNA levels to that observed in mock infected cells, suggesting that induction of IP-10 expression in virus-exposed cells was dependent on *de novo* reverse transcription. In contrast, LPS treatment of MDDCs for 4 hours resulted in robust increases of both IFN-β and IP-10 mRNAs (Fig. 9A, B). Inability to detect differences in mRNA expression levels of IFNβ in MDDCs infected with WT and ΔVpr viruses was also mirrored with the absence of differences in protein levels in infected MDDC culture supernatants (data not shown). I used a sensitive bioassay to measure type I IFN production in infected MDDC supernatants, and failed to detect any type I IFN

production in HIV-1 infected MDDCs over mock infected controls (data not shown) (369). In contrast, IP-10 was robustly secreted in both Lai-YU2 (WT) and Lai-YU2ΔVpr infected MDDC culture supernatants at day 3 post-infection, though the magnitude of IP-10 induction was donor-dependent (Fig. 9C). Furthermore, I observed a significant increase in IP-10 production upon WT virus infection of MDDCs as compared to mock-infected cells (Fig. 9C). Again, AZT pre-treatment reduced secretion of IP-10 indicating that IP-10 production is dependent on completion of reverse transcription (Fig. 9C). Taken together, these results suggest that Vpr deficiency does not result in the induction of type I IFNs during establishment of productive HIV-1 infection of MDDCs and is unlikely to play a role in the restriction of HIV-1/ΔVpr in DCs.

A.


Fold Induction IFN-βmRNA (Compared to Mock)
1.5
1.0
0.5
0.0
Mock
WT
WT + AZT
ΔVpr
ΔVpr + AZT
Log10(Relative IFNβ mRNA expression)
2.0
1.5
1.0
0.5
0.0
**
No Treatment
LPS

B.


Fold Induction IP-10 mRNA (Compared to Mock)
15
10
5
0
NS
Mock
WT
WT + AZT
ΔVpr
ΔVpr + AZT
Log10(Relative IP-10 mRNA expression)
6
4
2
0
*****
No Treatment
LPS

C.

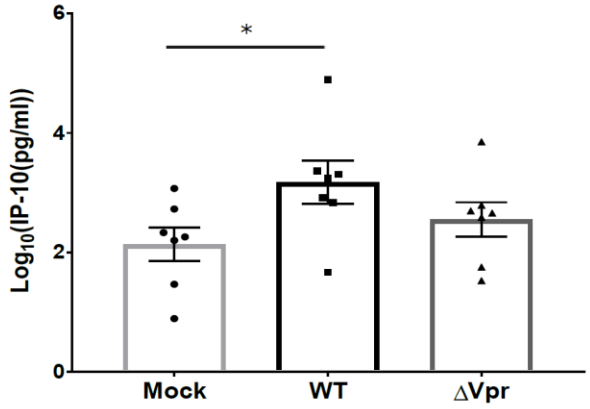

Log10(IP-10(pg/ml))
6
4
2
0
*
Mock
WT
ΔVpr

Figure 9. Vpr-deficiency does not result in enhanced type I IFN production in productively infected MDDCs.

Quantitative RT-PCR for IFNβ (A) and IP-10 (B) transcripts in infected MDDCs at 48 hours post infection. MDDCs were mock-infected or infected with Lai-YU2 or Lai-YU2/ΔVpr (MOI = 2) in the presence or absence of AZT (10 μM). The amount of IFNβ or IP-10 transcripts in infected MDDCs was normalized to the number of cells using a GAPDH control, and reported as relative to that of mock infected MDDCs (set as 1) for four independent donors. LPS treatment for 4 hours was used as a positive control for IFNβ and IP-10 production. Data is the log-transformed mean (± SEM) of seven donors. (C) Secreted IP-10 in MDDC culture supernatants infected with Lai-YU2 or Lai-YU2/ΔVpr (MOI = 1) at day 3 post infection was measured by an ELISA. The data shown are the log-transformed mean (± SEM) of independent experiments with MDDCs derived from four donors for (A) and (B) and six donors for (C). Significance calculated using a paired student's T test or a one value T test (when comparing normalized data) where N.S>0.05, *p<0.5, **p<0.1, ***p<0.01, ****p<0.001, *****p<0.0001.

Infection with ΔVpr viruses results in decreased infection in a single round of replication and is rescued by virion-associated Vpr.

To identify the step of the virus replication cycle in MDDCs that is affected by Vpr, I next performed single cycle of infection analysis. MDDCs were infected with HIV-1 reporter viruses pseudotyped with VSV-G and expressing luciferase upon establishment of infection that do (Lai-luc Δenv/G or WT) or do not express Vpr (Lai-luc Δenv/G ΔVpr or ΔVpr). Infection with ΔVpr virus resulted in a 3- to 5- fold decrease in luciferase expression compared to infection with WT virus (Fig. 10A), suggesting that Vpr acts early in the HIV-1 replication cycle in MDDCs at steps preceding virion assembly and maturation. Since Vpr is a virion-associated protein, I next sought to determine whether incoming virion-associated Vpr was sufficient or if *de novo* synthesized Vpr was required for enhancement of virus replication in DCs. I produced Lai-luc Δenv/G ΔVpr complemented with HA-epitope tagged Vpr *in trans* (Lai-luc Δenv/G Vpr-*trans*) via co-transfection of HEK293T cells with a functional HA-Vpr expression plasmid and the Lai-lucΔenv/G ΔVpr proviral plasmid. HA-Vpr was efficiently incorporated in ΔVpr virus particles to levels similar to that observed in WT virus particles (Fig. 10B). I then infected MDDCs with Lai-luc Δenv/G-WT, ΔVpr, or Vpr-*trans* viruses and lysed the cells on day 3 post-infection. Incorporation of Vpr *in trans* within incoming virus particles rescued ΔVpr virus infection in a single-round assay (Fig. 10C), suggesting that virion incorporated Vpr is sufficient for overcoming cell-intrinsic blocks to early steps in HIV-1 replication in DCs.

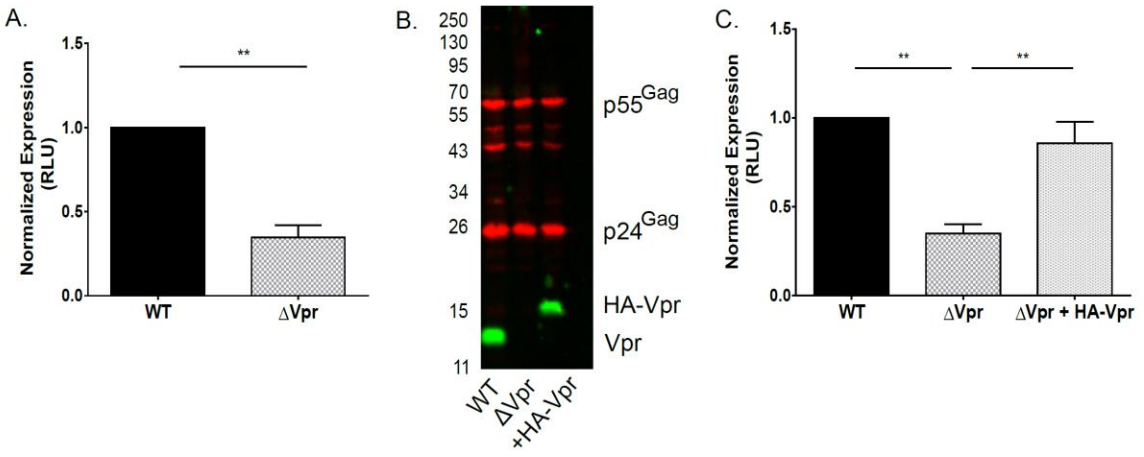


Figure 10. Infection of MDDCs with Vpr-deficient viruses results in block to HIV-1 replication in single round infection analysis.

(A) MDDCs infected with 40 ng $p24^{Gag}$ equivalent of VSV-G pseudotyped Lai-luc Δenv (WT or ΔVpr) were lysed 3 days post infection, and viral replication was quantified by measuring luciferase activity in cell lysates. The luciferase activity in ΔVpr infected cell lysates was normalized to that of WT virus-infected MDDC lysates and reported as mean (± SEM) of four independent experiments with MDDCs derived from four independent donors. (B) Western blot analysis of Vpr incorporation in virus particles (Lai-luc Δenv/G, Lai-luc Δenv/G ΔVpr, or Lai-luc Δenv/G Vpr-*trans*) derived from transient transfection of HEK293T cells. (C) MDDCs were infected with 40 ng $p24^{Gag}$ equivalents of viruses (Lai-lucΔenv, Lai-lucΔenvΔVpr, or Lai-lucΔenvΔVpr + HA-Vpr), and lysed 3 days post infection. Cell lysates were analyzed for luciferase activity and the data reported is normalized to that observed with WT-virus infection and is mean (± SEM) from 4 independent experiments. Significance calculated using a paired student's T test or a one value T test (when comparing normalized data) where *$p<0.05$, **$p<0.01$.

Proviral LTR-mediated transcriptional activity is attenuated in Vpr-deficient virus infection in DCs.

Since the block to HIV-1/ΔVpr infection in MDDCs is evident within a single round of replication, and is independent of the mode of virus entry (VSV-G pseudotyped virus infection was also restricted, Fig. 10A), I assessed the effect of Vpr-deficiency on HIV-1 reverse transcription (RT) and integration efficiency in DCs. I used qPCR to measure RT-products and the number of proviruses at day 3 post-infection using R-U5 and *Alu*-Gag primer pairs, respectively (370, 371). Infections were also performed in the presence of AZT to control for contaminating input plasmid DNA. In contrast to previously published findings (184), I saw no decrease in the number of RT products (Fig. 11A) or integrants (Fig. 11B, C) upon infection with ΔVpr virus compared to WT virus infections (Fig. 11A, B and C). Previous studies have suggested that Vpr can modulate HIV-1 LTR transcriptional activity (186, 200, 201, 204, 372). I therefore asked if the block to HIV-1/ΔVpr infection occurs at the stage of viral transcript production. To determine the effect of Vpr on LTR-mediated transcription from proviruses, I used qRT-PCR to measure multiply-spliced *tat/rev/nef* transcripts at 48 h post infection (Fig. 11D). Similar to my findings with luciferase reporter expression in infected DCs, I observed a 4-fold decrease in the number of multiply-spliced HIV-1 transcripts in HIV-1/ΔVpr-infected cells suggesting that Vpr-deficiency results in inhibition of proviral LTR-mediated transcription in DCs.

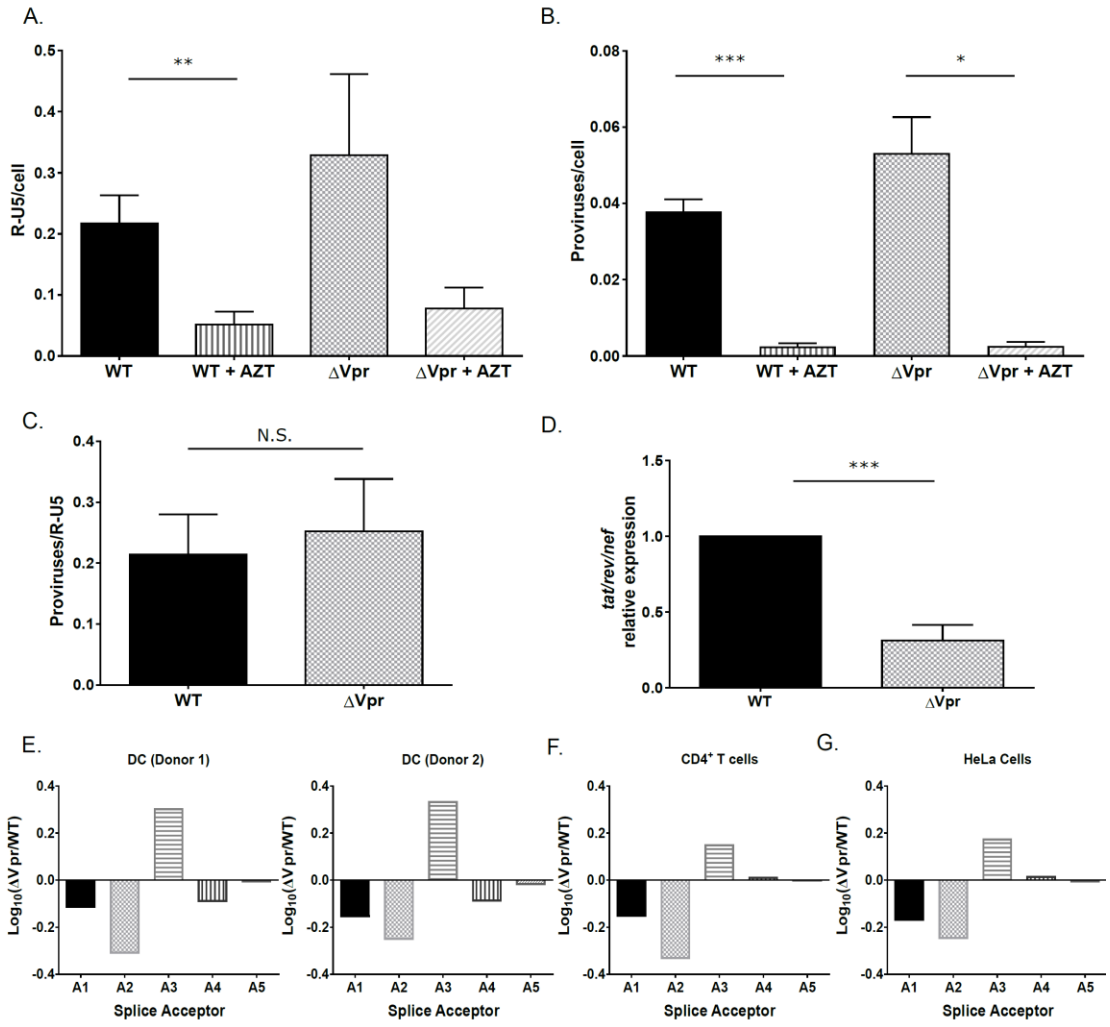


Figure 11. Viral transcription is attenuated in ΔVpr virus infected MDDCs.

(A-C) MDDCs infected with WT or ΔVpr viruses (MOI = 2) in the presence or absence of AZT (10 μM) were lysed 72 h post infection, and processed for DNA isolation. Note that infected cells were cultured in the presence of indinavir (1μM) to prevent viral spread. QPCR was used to detect early RT products (A) and integrated proviruses (B) by R-U5 and *Alu*-PCR primer sets and the number of integrated proviruses normalized to early RT products for each infection is shown in (C). The data reported is the mean (± SEM) of three independent experiments. (D) The numbers of multiply-spliced viral

transcripts (*tat-rev-nef*) in MDDCs infected with Lai-YU2 or Lai-YU2/ΔVpr (MOI = 1) was determined at 48 hours post infection by qRT-PCR. Viral transcripts were measured using primers specific to *tat/rev/nef* multiply-spliced transcripts. Data shown are mean (± SEM) of four independent experiments with MDDCs derived from four donors. (E) Quantification of 4 kb class of splice variants for MDDCs infected with Lai-YU2 or Lai-YU2/ΔVpr (MOI = 2) for 72 hours. The data was normalized, log10 transformed, and then graphed according to slice acceptor usage. Histograms show fold changes in splicing from D1 to each of the 5 viral splice acceptor sites A1 through A5 relative to a WT control. Splicing was quantified using a PrimerID-splicing assay for MDDCs from two independent infections (e), productively infected $CD4^+$ T cells (F) and HeLa cells (G) and is data from a single deep sequencing experiment. Significance calculated used unpaired student's T test where *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

I next sought to determine if the decrease in multiply-spliced viral mRNA levels in HIV-1/ΔVpr-virus infected MDDCs were driven by changes in the pattern of viral mRNA splicing. I, with the help of collaborators used a novel PrimerID-tagged deep sequencing assay (373, 374) to determine the relative abundance of different splice variants in WT and ΔVpr infected DCs, and compared viral splice site usage to that observed in WT or ΔVpr-infected $CD4^+$ T cells and HeLa cells (Fig. 11E-G). Data depicts the relative quantity of 4 kb singly-spliced mRNA for each splice acceptor and is reflective of the changes observed in the 1.8 kb multiply-spliced mRNA (data not shown). We detected minor differences in splice acceptor usage between WT and ΔVpr infections in MDDCs. We observed small decreases in the use of the Vif [A1] and Vpr [A2] splice acceptors and a small increase in the use of the Tat [A3] splice acceptor, but these differences were well within the normal range of splicing variation seen in productive viral infections (374). These small differences in splice site usage were consistently observed in infections of $CD4^+$ T cells and HeLa cells. Since the differences in splicing are both relatively small and observed in two cell types (primary activated $CD4^+$ T cells and HeLa cells) that do not restrict ΔVpr virus replication, it is unlikely that efficiency of viral mRNA splicing or choice of mRNA splice acceptor sites is a contributing factor to the restricted replication of ΔVpr virus in MDDCs.

Mutations in the C-terminal end of Vpr or those that disrupt binding to CRL4^{DCAF1} ubiquitin ligase attenuate viral replication in DCs.

A range of functions have been attributed to Vpr, including G2/M cell cycle arrest, enhancing fidelity of reverse transcription, nuclear import and/or nuclear tethering of the pre-integration complex, and induction of apoptosis (184, 201, 206, 341, 342, 347, 375). To clarify which of the known functions of Vpr are important for enhancing HIV-1 replication in DCs, a panel of mutations were introduced in Vpr ORF with previously characterized effects on Vpr functions. HEK293Tderived virus particles were analyzed by quantitative western blotting to assess incorporation of mutant Vpr proteins into viral particles (Fig. 12A). While all viral mutants expressed and incorporated Vpr in virus particles, the mutants Vpr-F34I and Vpr-H71R had slightly decreased incorporation levels of Vpr compared to wild type viruses (Fig. 12A), though both wild type and Vpr-mutant viruses were equally infectious on TZM-bl cells on a per particle basis (Fig. 12B). MDDCs were infected with replication competent HIV-1 (WT or Vpr-mutants) at equal MOIs and the extent of viral replication was measured by periodic quantification of p24^{Gag} in cell-free culture supernatant by an ELISA (Fig. 12C). Since there was donor-to-donor variability in the kinetics and extent of virus replication in DCs, I calculated the area under the curve of replication kinetics obtained from four independent infections (Fig. 12D). As depicted in Fig. 12C and D, infection with both Vpr-Q65R and Vpr-H71R mutant viruses resulted in significantly attenuated virus replication and spread, similar to what was observed with ΔVpr virus replication in MDDCs (Fig. 12C and D). In contrast, replication of both Vpr-F34I and Vpr-W54R mutants was not significantly different from

that observed with wild type virus infections (Fig. 12C and D). Cumulative analysis revealed that replication of Vpr-Q65R and Vpr-H71R mutants, which lack the ability to associate with the $CRL4^{DCAF1}$ complex (201, 222, 224, 235, 236, 344, 347, 355, 376), was significantly reduced ($p<0.01$), similar to that observed with ΔVpr virus infection (Fig. 12D). Interestingly, replication of Vpr-F34I mutant which incorporates reduced levels of Vpr in virions (Fig. 12A), and displays reduced association with the nuclear envelope, (347, 355, 357) was slightly enhanced over that observed with wild type virus replication (Fig. 12D; $p<0.01$), suggesting a threshold amount of functional Vpr that is still present in the incoming virus particle is sufficient for establishment of productive infections in DCs. The mutation Vpr-W54R, which ablates binding of Vpr to UNG2 (228, 230, 232, 235, 355) had a negligible effect on viral replication in DCs.

I next sought to determine which of these Vpr mutants could recapitulate the single cycle of replication defect observed with HIV-1/ΔVpr infection in MDDCs (Fig. 10A). I infected MDDCs with either replication competent viruses (Lai-YU2, WT or Vpr mutants, MOI = 1) in the presence of a protease inhibitor (indinavir) or with equal amounts of $p24^{Gag}$ equivalents of Lai-luc Δenv/G encoding the various Vpr mutations. Similar to the results observed with replication competent viruses, both the number of $p24^{Gag}$-postitive cells (Fig. 12E) and luciferase production (Fig. 12F) from infections with Vpr-Q65R and Vpr-H71R mutants were significantly attenuated in a single round of infection compared to isogenic WT viruses. While the host protein targeted by HIV-1 Vpr to induce G2 cell cycle arrest has not been identified, the C-terminal tail of the protein has been proposed to bind the unknown host factor, and mutations in the C-

terminal tail of Vpr abrogate the ability of Vpr to induce G2 cell cycle arrest (355). To determine the role of Vpr-mediated G2 cell cycle arrest on virus infection enhancement in DCs, an additional mutation, Vpr-R90K was introduced in GFP-expressing single-cycle virus (Lai-GFP Δenv/G). The Vpr-R90K mutant can bind $CRL4^{DCAF1}$ complex but fails to induce G2 arrest in cycling cells (235, 355, 357). Despite equivalent incorporation into the virion as WT Vpr (Fig. 12G), infection of MDDCs with Vpr-R90K mutant resulted in significant infection defect in single round analysis (Fig. 12H), similar to what was observed in infections with ΔVpr or Vpr-Q65R viruses, suggesting that interaction with a putative host factor whose degradation is critical for the induction of G2 cell cycle arrest is required to enhance HIV-1 infection of DCs. Together, my data suggests that there is a novel block to HIV-1 infection in MDDCs in the absence of Vpr that is present in a single round of infection and manifests at the stage of viral transcription. Further studies are underway to determine the exact mechanism by which Vpr alleviates the DC-intrinsic block to HIV-1 replication.

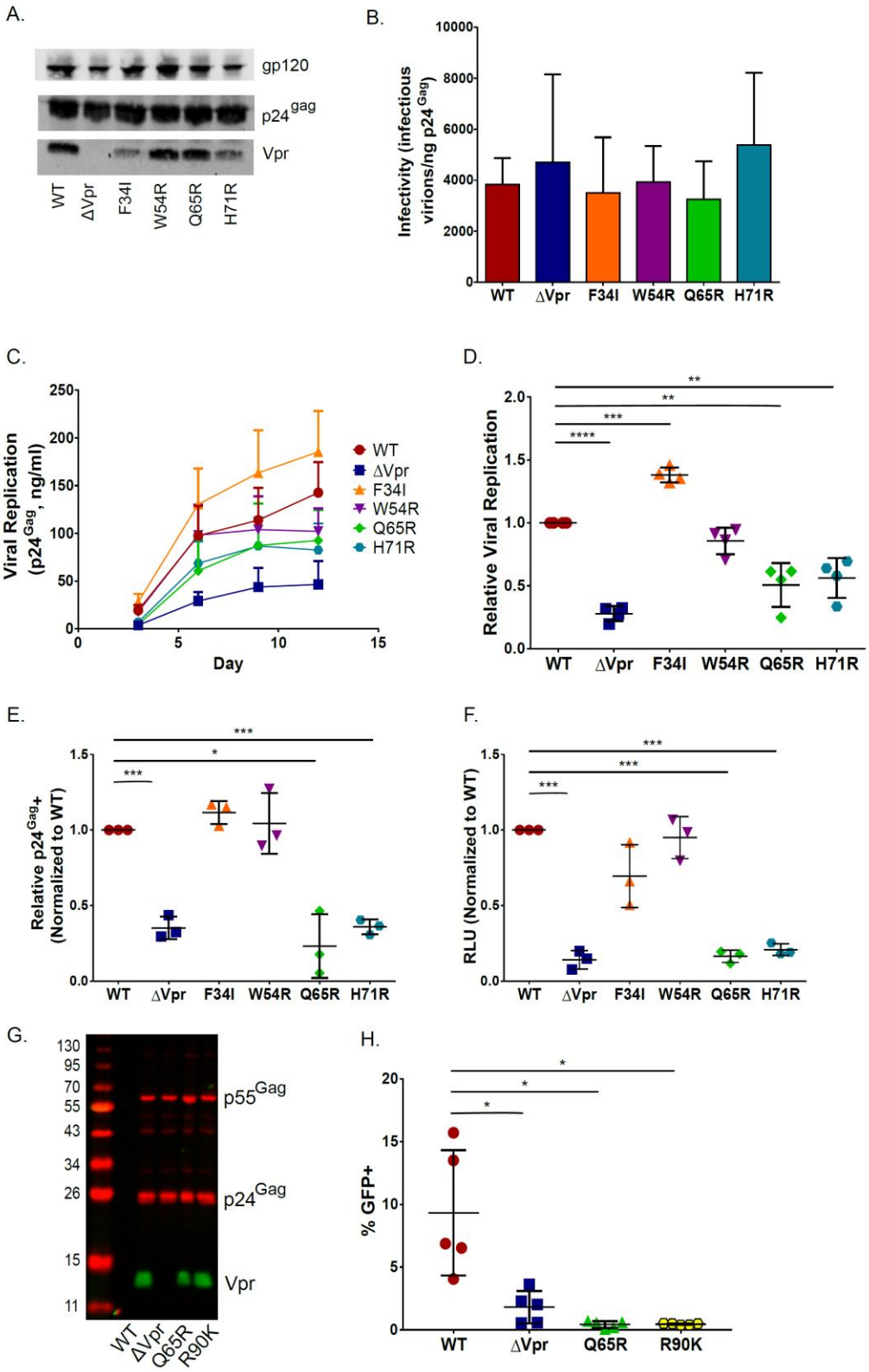

A.
gp120
p24gag
Vpr
WT
ΔVpr
F34I
W54R
Q65R
H71R
B.
Infectivity (infectious virions/ng p24Gag)
10000
8000
6000
4000
2000
0
WT
ΔVpr
F34I
W54R
Q65R
H71R
C.
Viral Replication (p24Gag, ng/ml)
250
200
150
100
50
0
0
5
10
15
Day
WT
ΔVpr
F34I
W54R
Q65R
H71R
D.
Relative Viral Replication
2.0
1.5
1.0
0.5
0.0
**
**
***
****
WT
ΔVpr
F34I
W54R
Q65R
H71R
E.
Relative p24Gag+ (Normalized to WT)
1.5
1.0
0.5
0.0
***
*
***
WT
ΔVpr
F34I
W54R
Q65R
H71R
F.
RLU (Normalized to WT)
1.5
1.0
0.5
0.0
***
***
***
WT
ΔVpr
F34I
W54R
Q65R
H71R
G.
130
95
70
55
43
34
26
15
11
p55Gag
p24Gag
Vpr
WT
ΔVpr
Q65R
R90K
H.
% GFP+
20
15
10
5
0
*
*
*
WT
ΔVpr
Q65R
R90K

Figure 12. Vpr mutants deficient for interaction with DCAF1/DDB1/E3 ubiquitin ligase and inducing G2 cell cycle arrest are attenuated in a single cycle of replication analysis in MDDCs.

(A) Representative western blot analysis of HEK293T- derived Lai-YU2 (WT) and indicated Vpr mutant viruses used for MDDC infections. Blots were probed with anti-$p24^{Gag}$, anti-Vpr and anti-gp120 antibodies. (B) Infectivity of Lai-YU2 and corresponding Vpr mutants in TZM-bl cells is reported as the number of infectious units (blue cells) per ng of $p24^{Gag}$ equivalent and are the mean (± SEM) of three independent viral preparations. (C) Viral growth curves of four independent infections of MDDCs with Lai-YU2 and indicated Vpr mutants in DCs. Viral growth was determined by analyzing $p24^{Gag}$ release into cell culture supernatants at days 3, 6, 9 and 12 post infection and determined by ELISA. (D) Area under the curve compiled for four independent MDDC infections represented in (C) normalized to WT virus infection, set as 1 (mean ± SEM). (E) The percentage of $p24^{Gag}$ positive MDDCs at day 3 post infection as measured by intracellular $p24^{Gag}$ staining and FACS analysis. Cells were treated with indinavir (1 μM) post virus exposure to prevent viral spread. The data was normalized to WT virus infection, set as 1, and depicts the mean (± SEM) of three independent infections of MDDCs from three donors. (F) MDDCs infected with 40 ng $p24^{Gag}$ equivalents of Lai-luc Δenv/G (WT or Vpr mutants) were lysed 3 days post infection, and viral replication was quantified by measuring luciferase activity in cell lysates. The luciferase activity in Vpr-mutant infections was normalized to that of WT virus infections, set as 1, and the data shown are the mean (± SEM) for three independent experiments. (G) Western blot

analysis of HEK293T-derived Lai-GFP Δenv/G (WT) or indicated Vpr mutant virus particles. (H) MDDCs infected with Lai-GFP Δenv/G (WT) or indicated Vpr-mutants (MOI = 3) were harvested at day 3 post infection and processed for FACS analysis. The data shown is the mean percentage of GFP^+ cells (± SEM) of five independent experiments with cells derived from five independent donors. Significance calculated using a paired student's T test or a one value T test (when comparing normalized data) where *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Discussion

In the work presented here, I examined the role of Vpr in establishing productive HIV-1 infection of DCs. Previous work in the field suggests that Vpr likely regulates a complex network of host interactions that may vary depending on the cell type infected. I find that, unlike what has been previously observed in activated $CD4^+$ T cells and MDMs (186, 238, 266, 268, 269, 345), infection of MDDCs with ΔVpr viruses was significantly attenuated when compared to WT HIV-1 infections (Fig. 7), similar to the findings reported by de Silva et al (184). Interestingly, Vpr-mediated enhancement was observed within both a single round viral infection as well as in spreading infections, contrary to what has been reported previously (184, 252). Furthermore, the single round replication defect could be rescued by complimenting back Vpr *in trans* in the incoming virion (Fig. 10) indicating that incoming virion-associated Vpr is necessary for the establishment of efficient HIV-1 infection of DCs. Initiating infections with the Vpr mutants, Vpr-Q65R, Vpr-H71R and Vpr-R90K that either lack the ability to engage the $CRL4^{DACF1}$ complex or bind the yet-to-identified host factor(s) necessary for inducing G2 cell cycle arrest, displayed similar replication deficits to that observed with ΔVpr virus in both spreading infections and single round infection analysis (Fig. 12).

Surprisingly, the block to ΔVpr virus replication in MDDCs was evident at a post-integration step and resulted in reduced numbers of viral mRNAs, suggesting that Vpr is acting either directly or indirectly to enhance transcription from the viral LTR (Fig. 11). It has been reported previously that Vpr can transactivate the viral LTR in a number of cell types and that this function correlates with the ability of Vpr to induce G2 cell cycle

arrest (185, 186, 200, 201, 372). Previous studies have also shown that both SIV_{mac} and SIV_{agm} Vpr can also transactivate their respective LTRs (204, 205, 377), suggesting that this is a conserved function among non-human primate lentiviral Vpr proteins. While it is possible that Vpr-mediated transactivation could be more robust in DCs than in $CD4^+$ T cells (Fig. 7E), another hypothesis is that Vpr is indirectly activating transcription to promote infection in cells that have a higher barrier to infection.

Unlike most of the other lentiviral accessory proteins, Vpr is actively packaged into the budding virion through associations with the p6 region of Gag (187, 191, 194, 337, 339). Our work in MDDCs suggests that there may be a novel role for virion-associated Vpr to enhance viral transcription and increase infection of DCs. These findings are at odds with recently published studies on the role of Vpr in modulating *de novo* HIV-1 Env production in productively infected macrophages and MDDCs (252, 267). While I do occasionally see a decrease in viral Env production during infection with ΔVpr virus in MDDCs (one out of four donors tested), infection of MDDCs from most of the donors revealed no differences in Env expression or virion incorporation (Fig. 8). It is possible that the use of different viral clones, primary cell variation derived from multiple donors, or different infection conditions might play a role in the differences between my results and those described previously. Since I observed infection differences in a single-round infection assay, putative effects of Vpr on Env expression are unlikely to play a role in establishment and spread of virus infection in MDDCs and DC-T cell co-cultures.

HIV-1 is not unique among primate lentiviruses in expressing a protein that functionally allows for infection of DCs. HIV-2 and certain SIV lineages express Vpx, another small accessory protein that targets host restriction factor SAMHD1 for proteasomal degradation by recruiting it to the $CRL4^{DACF1}$ complex, and facilitates infection of MDDCs (179, 180, 378). Interestingly, Vpr-mediated replication enhancement in MDDCs was substantially attenuated upon infection with Vpr mutants (Q65R or H71R; Fig. 12F) that lack ability to interact with $CRL4^{DCAF1}$ complex, or upon infection with Vpr-R90K mutant (Fig. 12H), that fails to interact with the host factor(s) hypothesized to be recruited to the $CRL4^{DCAF1}$ complex for proteasomal degradation. Since Vpr is introduced into target cells along with the incoming virion because of its association with the viral capsid, I hypothesize that early interactions of Vpr with a host factor and recruitment of that protein to the $CRL4^{DCAF1}$ complex for proteasomal degradation is essential for promoting HIV-1 replication in DCs, similar to the ability of Vpx from SIV_{mac}/SIV_{smm}/HIV-2 lineages to promote infection of DCs.

Across primate lentiviral Vpr evolution, induction of DDR and G2 cell cycle arrest are conserved functions, and Vpr proteins from diverse primate lentiviruses have been shown to associate with and degrade many DDR regulatory proteins including the SLX4com, HLTF, and UNG2 (228, 229, 232, 234–239, 379). While DDR activation may represent a cell-intrinsic antiviral response, it has been suggested that both RNA and DNA viruses induce DDR signaling to promote cellular conditions that are favorable for viral replication (205, 207, 208, 380, 381). For instance, induction of DDR signaling activates ataxia-telangiectasia mutated (ATM) kinase which results in nuclear factor

kappa-light-chain-enhancer of activated B cells (NF-κB) activation (382, 383). Additionally, the DDR pathway also directly activates pro-inflammatory responses through the induction of interferon regulatory factors (IRFs) or through the recruitment of co-activators and chromatin modifying complexes, such as ten-eleven translocation methylcytosine (TET) dioxygenases, which I hypothesize might also activate viral transcription (384). Since the barrier to successful establishment of infection in non-cycling, metabolically quiescent cells like MDDCs is higher than that in activated $CD4^+$ T cells or MDM, Vpr-mediated activation of NF-κB and co-activator recruitment to the viral LTR might be a viral strategy for overcoming the restrictive cellular environment and for optimal production of progeny virions. In line with this hypothesis, numerous studies have documented that Vpr is able to modulate NF-κB activity in different cell lines and primary cells, though these studies rarely agree on the mechanism of regulation or direction of modulation (185, 253, 254, 372, 385–388). Recent work from Höhne, et al has shown similar effects of Vpr on viral replication in non-activated primary $CD4^+$ T cells, which have similar barriers to infection as MDDCs including increased expression of SAMHD1 and low baseline NF-κB activity (183, 185, 389). Some studies have shown virion-associated Vpr-dependent activation of NF-κB occurs via a transforming growth factor-β-activated kinase 1 (TAK1) signaling cascade, while other studies have shown that secreted or synthetic Vpr stimulates NF-κB signaling through a TLR4-dependent mechanism (253, 254, 372, 387). My data also demonstrates upregulation of IP-10 upon HIV-1 (WT) infection (Fig. 9C) which is also dependent on NF-κB activation (390–392).These results suggest a link between Vpr-mediated NF-κB activation in MDDCs

and enhanced viral gene expression and pro-inflammatory cytokine secretion, which may act *in vivo* to enhance recruitment, activation and infection of $CD4^+$ T cells, resulting in increased viral dissemination (Fig. 7E) (346, 372, 387).

Studies with peripheral blood myeloid MDDCs and monocyte-derived MDDCs from HIV-1 elite controllers have shown that these cells may be critical for viral control, acting to capture virus and enhance T cell-specific immunity to HIV-1, while being less susceptible to HIV-1 infection compared to MDDCs from healthy controls (393, 394). Understanding the mechanisms that control HIV-1 replication in MDDCs which are overcome by Vpr, might lead to new insights on viral dissemination and persistence *in vivo*, and development of novel anti-HIV-1 therapeutics.

CHAPTER TWO

Introduction

During the course of the HIV-1 replication cycle, viral genomic RNA is reverse transcribed to dsDNA, which is incorporated into the host cell genome via a virally encoded integrase. Due to poorly defined restrictions to virus integration or because of actions of host DNA repair machinery, fully reverse transcribed viral DNA can be maintained as linear DNA or as recombination circles (395). These circles are the result of two different types of recombination events. Non-homologous end joining (NHEJ) results in the joining of the ends of linear viral DNA, forming 2-LTR circles and homologous recombination at the viral LTRs results in the looping out of one of the LTRs, forming 1-LTR circles (395). While linear viral DNA is degraded with time via the action of nuclear exonucleases, 1-LTR and 2-LTR circles are relatively stable and only decrease with cell death or division (396, 397). All three forms of unintegrated DNA can be transcribed to make new viral proteins and virions which can spread to neighboring cells (398). Mathematical models predicting the relative contribution to infection of unintegrated DNA could be as high as 20% *in vivo* (399). Clinical studies have shown that the majority of viral DNA in patient cells is episomal and that accumulation of unintegrated DNA in neuronal tissue is associated with development of AIDS-related dementia, suggesting that these forms of extrachromosomal DNA are relevant to *in vivo* pathogenesis (400–403). Furthermore, with the use of integration inhibitors as part of HAART regimens, the accumulation of unintegrated HIV DNA and its role in HIV pathogenesis needs to be carefully explored.(395).

Expression from unintegrated HIV-1 DNA has been described and a number of groups have correlated expression from unintegrated DNA with the presence of Vpr (404, 405). Whether Vpr regulates expression of unintegrated DNA in primary cells and if the function of Vpr is conserved in other primate lentiviruses is not known (406, 407). Here, I characterize the ability of HIV-1 Vpr to enhance expression from unintegrated DNA in diverse cells, including primary human MDDCs, human MDMs, murine BMDCs and murine BMDMs. In the presence of Vpr, I find that there is an increase in formation of 2-LTR circles in human MDDCs. Additionally, I show that residues important for Vpr-mediated induction of DDR, are also important for mediating viral gene expression from unintegrated DNA. Finally, I show that Vpr from diverse primate lentiviruses is able to maintain expression of unintegrated DNA in human MDDCs and similar DDR mutations in SIV_{mac} Vpr also map to unintegrated DNA maintenance.

Results

HIV-1 Vpr promotes expression of unintegrated DNA in a variety of cell types

It has been reported by other groups that Vpr is able to enhance expression of unintegrated DNA in multiple cell lines and CD4+ T cells, but little work has been done to characterize this function in primary myeloid cells. I have developed several tools to explore Vpr function, including a panel of diverse single cycle of replication competent primate lentiviral clones lacking Vpr as well as proviral clones encoding point mutations in Vpr that abrogate DDR induction. I first wanted to ask if Vpr enhances expression of unintegrated DNA in primary human MDDCs and MDMs. MDDCs were infected with

40 ng $p24^{gag}$ content of Lai-luc Δenv/G or Lai-luc Δenv/G ΔVpr in the presence of the integration inhibitor raltegravir. Cells were lysed and luciferase expression was assessed at three days post infection. Similar to what other groups have observed in cell lines and $CD4^+$ T cells, a low level of luciferase expression was detected in WT-virus infections in the presence of raltegravir that was ablated in the absence of Vpr (Fig. 13A). I observed similar effects in MDMs, in PMA-differentiated THP-1 cells (human monocytoid cell line) and in HeLa cells, suggesting that Vpr-mediated enhancement of gene expression from unintegrated viral DNA is observed in a variety of human cell types (Fig. 13B-D). Furthermore, the ability of Vpr to enhance expression from unintegrated viral DNA was independent of its effects on enhancing expression from integrated viral LTR in these cells (Fig. 13C and D). While contribution of unintegrated DNA to infection is modest compared to untreated controls, it may still significantly impact *in vivo* spread in the presence of an integration inhibitor.

Previous studies have suggested that there is exquisite species-specificity to Vpr functions (208). Hence, I wanted to assess whether Vpr-mediated enhancement of viral gene expression from unintegrated DNA was conserved across diverse species. I infected mouse (C57/Bl6) bone marrow derived macrophages (BMDMs) with Lai-luc Δenv/G or Lai-luc Δenv/G ΔVpr in the presence of raltegravir and measured luciferase expression in cell lysates at day 3 post infection. Interestingly, I observed no difference in luciferase expression in the presence or absence of Vpr from unintegrated viral DNA (Fig. 13E). These results suggest that host factor(s) involved in Vpr-mediated expression from unintegrated DNA have diverged between mice and humans to be unusable by Vpr.

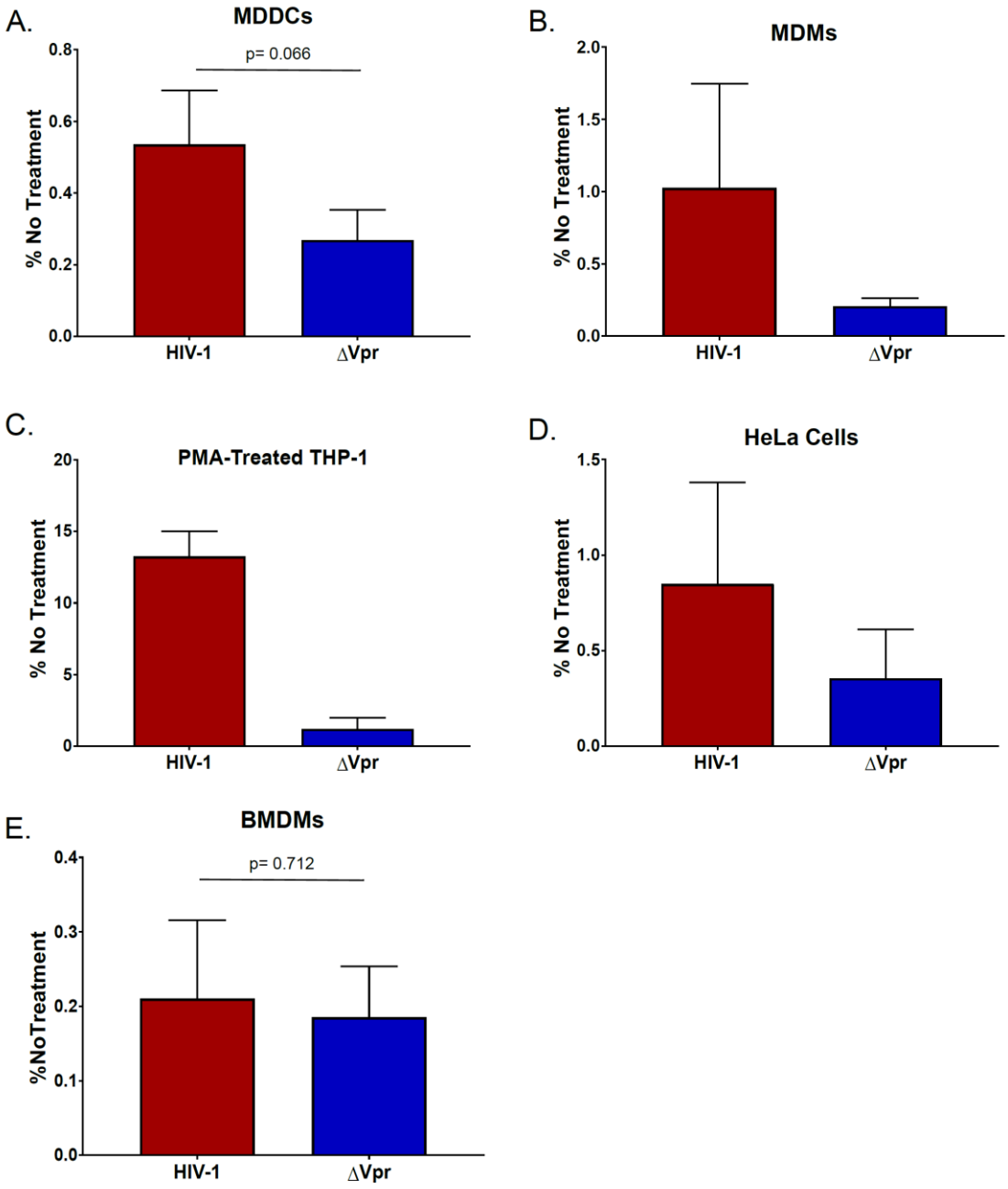

A.
MDDCs
p= 0.066
% No Treatment
0.8
0.6
0.4
0.2
0.0
HIV-1
ΔVpr
B.
MDMs
% No Treatment
2.0
1.5
1.0
0.5
0.0
HIV-1
ΔVpr
C.
PMA-Treated THP-1
% No Treatment
20
15
10
5
0
HIV-1
ΔVpr
D.
HeLa Cells
% No Treatment
1.5
1.0
0.5
0.0
HIV-1
ΔVpr
E.
BMDMs
p= 0.712
%NoTreatment
0.4
0.3
0.2
0.1
0.0
HIV-1
ΔVpr

Figure 13. Vpr enhances expression of unintegrated DNA.

Luciferase expression from (A) MDDCs (n=3), (B) MDMs (n=2), (C) PMA-differentiated THP-1s (n=2), or (D) HeLa cells (n = 2) infected with Lai-luc Δenv/G -WT or -ΔVpr in the presence of 30 μM raltegravir to block integration. (A). MDDCs were infected with 40 ng $p24^{gag}$ per $1x10^5$ cells and harvested at day 3 post infection. (B). MDMs were infected at MOI = 2 and lysed for luciferase production at day 2 post infection. (C) THP-1s were stimulated with PMA (0.1 μM) for two days and then seeded at $5x10^4$. Cells were infected with 50 ng $p24^{gag}$ and lysed at 3 days post infection. (D) $1x10^4$ HeLa cells were infected with 3 ng $p24^{gag}$ and harvested at 2 days post infection. Data shown had background subtracted and is depicted as percent of luciferase expression from untreated infections with the respective virus. (E). $5x10^5$ Black-6 (B6) BMDMs were infected at MOI = 2 with Lai-luc Δenv/G -WT or -ΔVpr in the presence of 30 μM raltegravir to block integration. Data is the mean +/- SEM of four independent experiments. Significance was calculated using a paired student's T test.

To confirm that Vpr mediated expression from unintegrated viral DNA is not due to off-target effects of raltegravir treatment, I constructed a catalytically inactive mutant of integrase (D116N) that fails to catalyze the strand transfer reaction and invasion of the viral DNA into the host genome (408). HeLa cells and PMA differentiated-THP1s were infected with luciferase-expressing viruses (Lai-luc Δenv/G) encoding the integrase catalytic site mutant IntD116N that did (WT) or did not (ΔVpr) express Vpr. I observed a similar enhancement in unintegrated DNA expression from WT but not ΔVpr-infected cells (Fig. 14A and B).

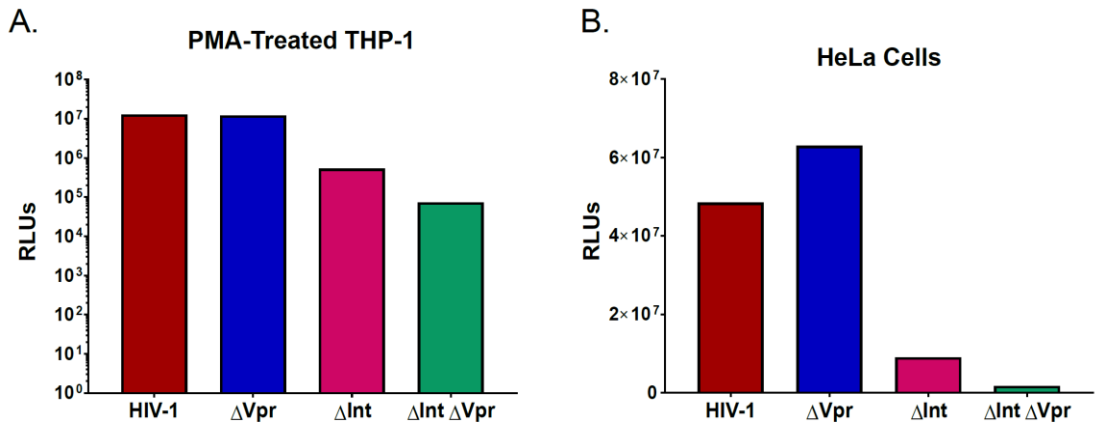


Figure 14. Vpr enhances expression of HIV-1 containing the catalytic mutation IntD116N.

(A-B) $5x10^4$ PMA-THP-1s (A) or $1x10^4$ HeLa cells (B) were infected as in (Fig. 13C) with Lai-luc Δenv/G -WT, -IntD116N, -ΔVpr, or -IntD116N/ΔVpr. Data represents single replicates.

Vpr expression of unintegrated DNA correlates to its ability to regulate DDR responses

I hypothesized, due to the role of Vpr in coordinating DDR responses during infection, Vpr may be acting to promote viral DNA repair such as NHEJ to form 2-LTR circles. 2-LTR circles are considered dead-end products of viral infections, though they can be maintained episomally in the nucleus and used as a template for viral transcription (396–398). To test this, I first wanted to determine if episomal expression from unintegrated DNA was fleeting or if it could be maintained for an extended period of time. I infected human MDMs with Lai-luc Δenv/G -WT or -ΔVpr. Cells were harvested for luciferase expression at 3, 6, and 9 days post infection. Expression from unintegrated DNA was maintained over this time in the presence of Vpr, suggesting that Vpr acts to maintain expression from unintegrated DNA in the nucleus (Fig. 15A). Interestingly, this enhancement was not observed at any of the times post virus infection of mouse BMDMs or BMDCs, nondividing cells that would turn over unintegrated DNA relatively slowly (Fig. 15B, C).

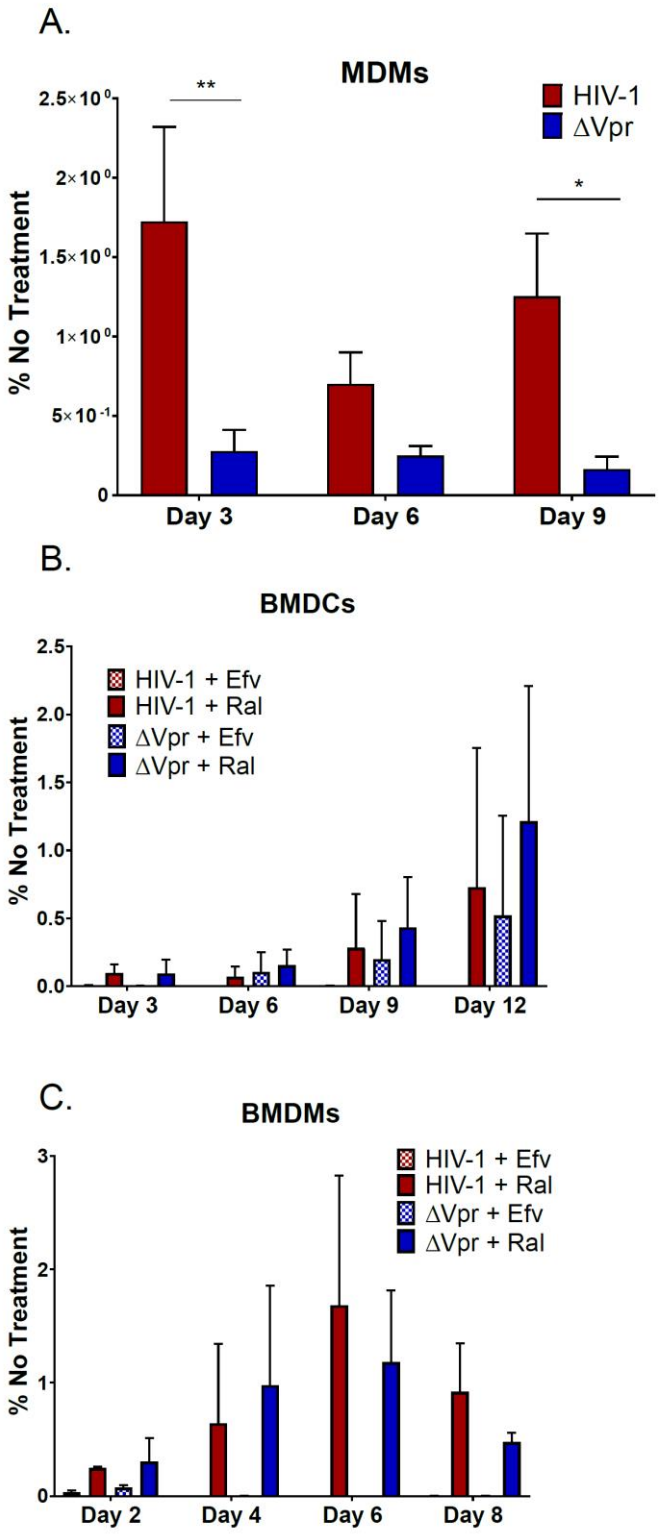

A.
MDMs
HIV-1
ΔVpr
**
*
% No Treatment
2.5×10 0
2×10 0
1.5×10 0
1×10 0
5×10 -1
0
Day 3
Day 6
Day 9
B.
BMDCs
HIV-1 + Efv
HIV-1 + Ral
ΔVpr + Efv
ΔVpr + Ral
% No Treatment
2.5
2.0
1.5
1.0
0.5
0.0
Day 3
Day 6
Day 9
Day 12
C.
BMDMs
HIV-1 + Efv
HIV-1 + Ral
ΔVpr + Efv
ΔVpr + Ral
% No Treatment
3
2
1
0
Day 2
Day 4
Day 6
Day 8

Figure 15. Expression from unintegrated DNA is maintained with time in the presence of Vpr.

(A). Human MDMs were infected with 40 ng $p24^{Gag}$ content per $2x10^5$ cells. Cells were lysed on day 3, 6, or 9 post infection for luciferase content. Data is the mean +/- SEM of infections with three independent donors. (B-C) $1x10^4$ (B6) BMDCs or $5x10^4$ B6 BMDM (C) were infected with 40 ng $p24^{Gag}$ in the presence of 30 μM raltegravir and lysed every 2 (C) or 3 days (B). Data shown from two independent experiments. (C) MDMs were infected with 3 ng $p24^{Gag}$ content Lai-luc Δenv/G -WT or -ΔVpr in the presence of 30 μM raltegravir for 3, 6, or 9 days before lysis for luciferase expression. Data represents three independent experiments with three donors. Data shown had background subtracted and is depicted as percent of luciferase expression from untreated infections with the respective virus. Data shown represents the mean +/- the SEM, where applicable. Significance was calculated using a paired student's T test where *$p<0.05$, **$p<0.01$.

Linear DNA is subject to degradation at higher rates, due to the presence of cellular endonucleases. I hypothesized that Vpr stabilizes unintegrated DNA by promoting end joining and production of 2-LTR circles. I measured 2-LTR circles in the presence and absence of Vpr in MDDCs. I chose to focus on MDDCs as an infection model because of its *in vivo* relevance and my previous findings showing that Vpr expression impacts infection of these cells (Chapter 1). MDDCs were infected with Lai-YU2 or Lai-YU2 ΔVpr at MOI = 3 for 12, 24 or 48 hours and 2-LTR circles were measured by qPCR. The RT-inhibitor efavirenz was used as a negative control. I observed similar levels of 2-LTR circles at 12 hours, suggesting similar input of virus was achieved, but by 24 and 48 hours, there was an increase in 2-LTR circles in WT-infected MDDCs as comparted to ΔVpr (Fig. 16), suggesting that Vpr promotes formation of 2-LTR circle form of unintegrated viral DNA.

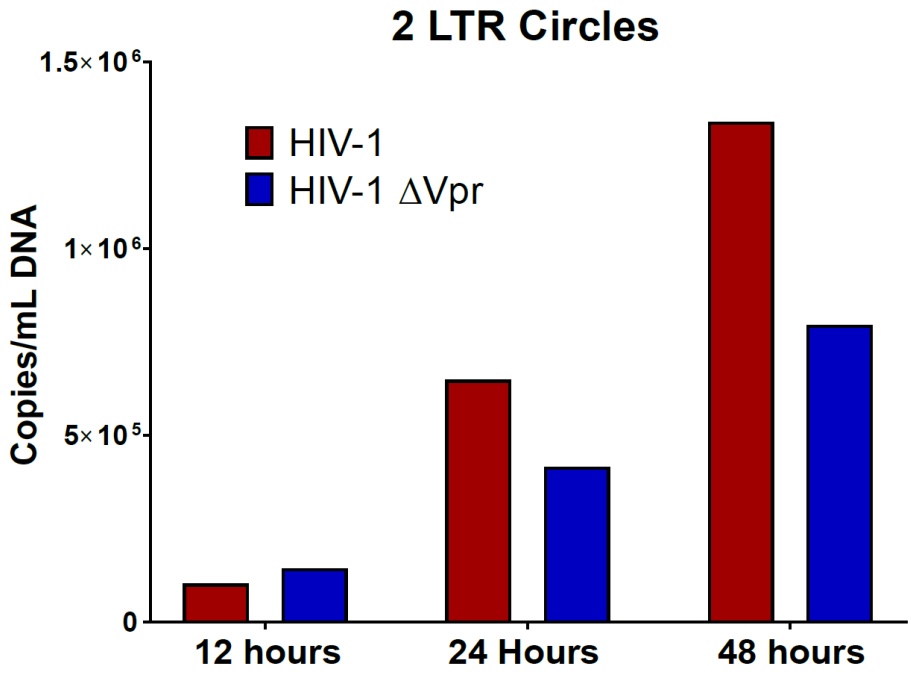


Figure 16. Vpr increases 2-LTR circles during HIV-1 infection of MDDCs. MDDCs were infected at MOI = 3 with Lai-YU2 or Lai-YU2 ΔVpr for 12, 24, or 48 hours before lysis for DNA. Efavirenz (1 μM) was used as a control for plasmid DNA input. QPCR was used to analyze 2-LTR circle content as compared to a standard curve. Data represents a single experiment.

I next wanted to determine what functional domain of Vpr is important for preservation of unintegrated DNA. I infected Hela cells or MDMs with HIV-1 Vpr mutants, Vpr-W54R which lacks association with UNG2, Vpr-Q65R, which lacks association with the SLX4com and $DCAF^{CRL4}$, and Vpr-H71R, which lacks association with $DCAF^{CRL4}$ in the presence or absence of raltegravir. I observed a similar increase in expression of unintegrated DNA from Vpr-W54R, but not -Q65R or -H71R, both of which lack the ability to associate with $DCAF^{CRL4}$ complex (Fig. 17A, B). The association of Vpr with the $DCAF^{CRL4}$ complex results in G_2 arrest, which is thought to be the outcome of Vpr-mediated induction of a DDR (195, 207, 237, 355, 375). This suggests that the ability of Vpr to induce DDR is important for enhancing expression from unintegrated viral DNA.

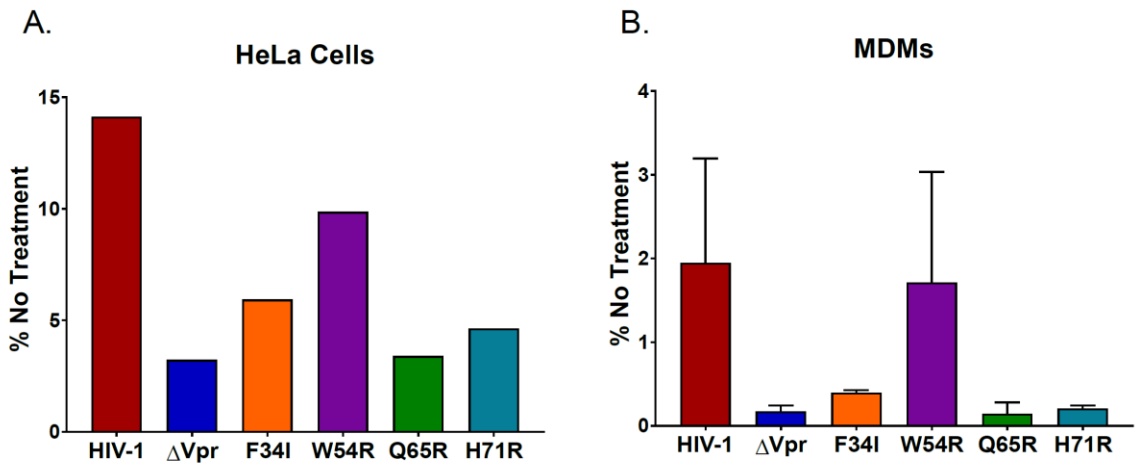


Figure 17. Vpr maintenance of stable forms of episomal DNA is reliant on its ability to associate with the DCAF^{CRL4} complex.

(A). HeLa cells were infected with 40 ng Lai-luc Δenv/G -WT or indicated Vpr mutant in the presence of 30 μM raltegravir. Data represents a single experiment. (B). MDMs were infected with 40 ng p24^{gag} per 5x10^4 cells with Lai-luc Δenv/G -WT or indicated Vpr mutant for 3 days before lysis. Data represents two independent experiments. Data shown represents the mean +/- the SEM, where applicable.

Vpr-mediated preservation of unintegrated DNA is conserved among primate lentiviruses and correlates with the ability of SIV_{mac} Vpr to regulate DDR responses

I next asked if Vpr-dependent enhancement of viral gene expression from unintegrated viral DNA was a conserved function amongst Vpr alleles from different primate lentiviruses. I utilized GFP-expressing single cycle of replication competent viruses from SIV_{sm}, SIV_{mac} or HIV-2 (viral clones SIV_{sm}-GFP Δenv/G, SIV_{mac}-GFP Δenv/G and HIV-2 Rod9-GFP Δenv/G, respectively) that encoded WT or Vpr null-mutations. I infected MDDCs at MOI = 3 in the presence or absence of raltegravir. GFP expression in infected cells was determined by FACS analysis. I found Vpr from all three lentiviruses maintained the function of enhanced gene expression from unintegrated viral DNA in the presence of raltegravir (Fig. 18A, B, C). This expression from unintegrated viral DNA was not observed in cells infected with the corresponding ΔVpr viral clones in the presence of raltegravir (Fig. 18D, E, F). It should be noted that SIV_{sm}/SIV_{mac}/HIV-2 ΔVpr viruses were much more infectious in MDDCs than HIV-1 ΔVpr due to their expression of Vpx.

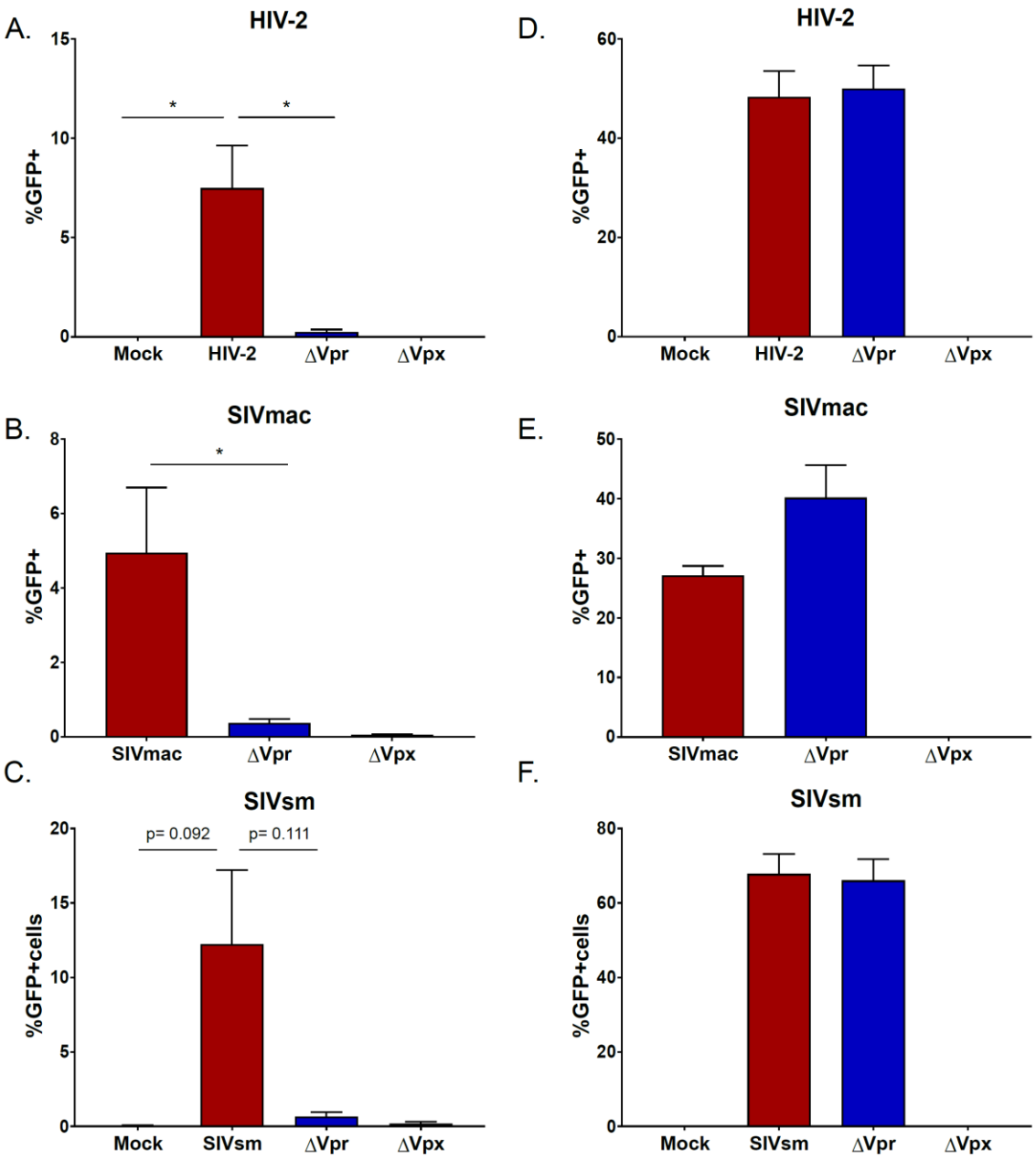

A.
HIV-2
%GFP+
15
10
5
0
*
*
Mock
HIV-2
ΔVpr
ΔVpx
D.
HIV-2
%GFP+
60
40
20
0
Mock
HIV-2
ΔVpr
ΔVpx
B.
SIVmac
%GFP+
8
6
4
2
0
*
SIVmac
ΔVpr
ΔVpx
E.
SIVmac
%GFP+
50
40
30
20
10
0
SIVmac
ΔVpr
ΔVpx
C.
SIVsm
%GFP+cells
20
15
10
5
0
p= 0.092
p= 0.111
Mock
SIVsm
ΔVpr
ΔVpx
F.
SIVsm
%GFP+cells
80
60
40
20
0
Mock
SIVsm
ΔVpr
ΔVpx

Figure 18. Diverse primate lentiviral Vprs promote expression from unintegrated DNA.

(A-F). MDDCs were infected at MOI = 3 with HIV-2/Rod9-GFP Δenv/G -WT or -ΔVpr (A, D), SIVmac-GFP Δenv/G -WT or -ΔVpr (B, E), or SIVsm-GFP Δenv/G -WT or -ΔVpr (C, F) in the presence of 30 μM raltegravir. GFP expression was analyzed by FACS analysis 3 days post infection. Raltegravir treated infections are depicted in (A-C) and corresponding untreated controls in (D-F). Data represents seven (A, D), six (B, E), or four (C, F) independent experiments. Data shown represents the mean +/- the SEM. Significance was calculated using a paired student's T test.

I next asked what domains of SIV_{mac}Vpr are important mediating expression of unintegrated DNA. I introduced two mutations in the open reading frame of SIV_{mac} Vpr, VprV21A and VprS81A. I characterized their expression in cells and ability to be incorporated into virions (Fig. 19A). Both mutants have been previously characterized to lack G_2 arrest capacity (409). Work is currently underway to characterize this further, as well as determine other functions of Vpr these mutations impact. I next infected MDDCs with SIV_{mac}-GFP Δenv/G encoding WT, ΔVpr, or the Vpr mutants in the presence and absence of raltegravir and assessed expression of unintegrated DNA by FACS analysis. I found one of the mutants that lacked the ability to induce a DDR response, Vpr-S81A, also lacked the ability to preserve unintegrated DNA expression (Fig. 19B). Together, these results suggest that formation of and expression from unintegrated viral DNA is a conserved function of primate lentiviral Vpr alleles and requires Vpr-association with DDR.

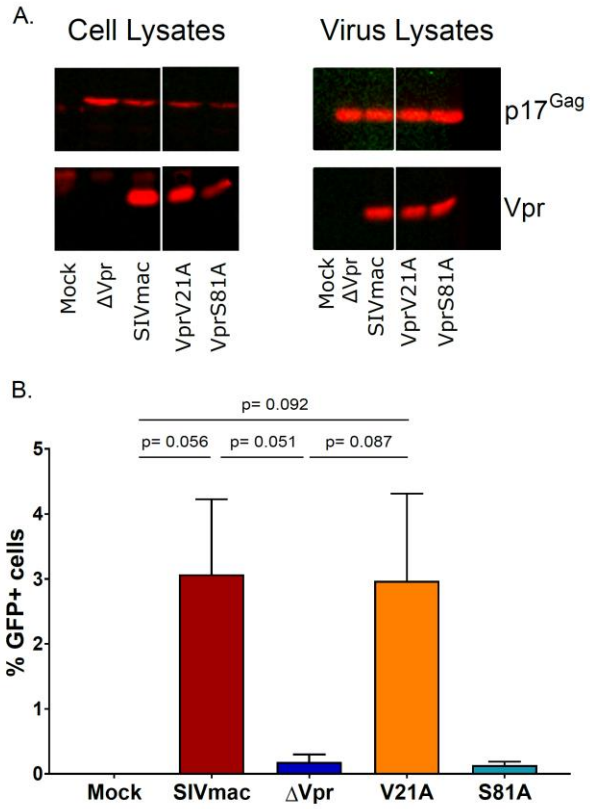


Figure 19. The ability of SIV_{mac} Vpr to promote expression of unintegrated DNA correlates with induction of cell cycle arrest.

(A). Flag-tagged expression constructs of SIV_{mac} Vpr -WT and mutants -V21A and -S81A were co-expressed with SIV_{mac} Δenv/G ΔVpr in 293T cells via transient transfection. Cell lysates (left) and concentrated virion lysates (right) were analyzed for $p27^{Gag}$ and Flag-Vpr content via western blot analysis. (B). MDDCs were infected at MOI = 3 with SIVmac-GFP Δenv/G -WT or indicated Vpr mutant for 3 days before GFP analysis by FACS. Data represents five independent experiments with five different donors. Data shown represents the mean +/- the SEM. Significance was calculated using a paired student's T test.

Future Work

Experiments to further characterize Vpr-mediated maintenance of unintegrated DNA expression are currently ongoing. In my future work, I plan on using the DDR response inhibitor Caffeine and PARP-1 inhibitors during HIV-1 infection in the presence of raltegravir. I hypothesize that I will observe reduced maintenance of and expression from unintegrated viral DNA in both HeLa cells and MDDCs from WT infection in the presence of these inhibitors. Work is also underway to characterize the SIV_{mac} Vpr mutants further, as well as to determine if I observe similar results with integrase-null SIV_{mac} as those observed with HIV-1.

Discussion

Together, these data suggest a conserved role for Vpr in enhancing expression from unintegrated DNA. This effect is seen in human cells with all primate lentiviruses tested, but not observed during HIV-1 infection of murine cells. As was discussed previously in this manuscript, it has been shown that a number of DDR response proteins have undergone positive selection in the primate lineage, though the reason for this selection remains unclear (410, 411) Mutations in DNA-repair proteins often result in genomic instability, making the host more likely to develop malignancies, like in the case of BRCA1 and BRCA2 mutations (410). The overlap I observe between Vpr-mediated expression of unintegrated DNA and the ability of Vpr to block IFN secretion during infection of MDDCs, described in Chapter 1, suggests that the two effects of Vpr may be linked. It is possible that the integration sensor proposed in Chapter 2 may be part of the

DDR pathway that regulated degradation of extrachromosomal DNA. In the presence of Vpr, this pathway is inhibited, allowing for other DNA-repair machinery to convert viral DNA into stable 2-LTR circles.

Despite a measurable enhancement in 2-LTR circles in WT HIV-1 infections as compared to ΔVpr, this difference I observed is only about 2-fold. I see a much bigger difference in transcription from unintegrated DNA in the presence of Vpr (10- to 20-fold enhancement). This result suggests that 2-LTR circle accumulation may not be the sole determinant for unintegrated DNA expression. In Chapter 1 of this dissertation, I describe the ability of Vpr to enhance transcriptional output from the proviral (integrated) LTR during infection of MDDCs, resulting in increased expression. It is possible that my observations on unintegrated DNA expression may also be regulated by a Vpr-mediated enhancement of transcription, rather than a DNA-repair mechanism. Vpr has been reported to transactivate the viral LTR, though this work has mostly focused on $CD4^+$ T cells or T cell lines (200–202). It has also been noted that immediately after entry of the viral pre-integration complex (PIC) into the nucleus, histones are loaded on linear viral DNA (412). Histones on the viral DNA can, in theory, be modified to promote or inhibit transcription of the viral DNA, either pre- or -post integration. Vpr has been shown, at least indirectly, to modify histone markers through its ability to regulate levels of histone deacetylases (HDACs), which modify histones to transcriptionally repress areas of DNA (241, 242). Vpr may be changing the overall transcriptional state of the cell by reducing HDAC levels, thus promoting expression from aberrant, extrachromosomal DNA that would normally be transcriptionally repressed. Alternatively, Vpr may simply be acting

as a required transcriptional activator for unintegrated DNA, though it is unclear why differences in transcriptional output was not observed from integrated LTRs in MDDCs infected with SIV_{sm}/SIV_{mac}/HIV-2 in the presence or absence of Vpr. Together, these data show a conserved role for Vpr in maintenance of unintegrated DNA during infection. Expression from the episomal DNA may be a critical source for low-level viral replication that maintains tissue reservoirs in infected individuals, even in the presence of HAART.

CHAPTER THREE

Introduction

Vpr is a well-studied HIV-1 accessory protein whose sequence and function(s) is conserved through primate lentiviral evolution (177, 196). Though Vpr has been ascribed a number of functions, the most thoroughly characterized of which is its ability to induce G_2 cell cycle arrest, the mechanisms responsible for inducing G2 arrest and the consequences of the G2 cell cycle arrest on viral replication and fitness are still poorly understood (206, 207). Induction of G2 cell cycle arrest by Vpr is dependent on its interaction with the $DCAF^{CRL4}$ ubiquitin ligase complex, components of which have been shown to be involved in or regulate DNA replication and DNA damage repair (221, 224, 413, 414). In the presence of Vpr, the $DCAF^{CRL4}$ complex associates with a number of DNA-damage repair proteins, including the SLX4com, which is involved in Holliday junction repair, UNG2, which is part of the base-excision repair pathway that removes uracils misincorporated into DNA, and HLTF, a DNA helicase involved in chromatin remodeling (228, 230, 236, 238, 239). Interestingly, these interactions are not conserved across primate lentiviral Vprs, nor are they necessary for Vpr-mediated cell cycle arrest, indicating that these interactions may not be responsible for maintenance of Vpr-mediated DNA damage response and G_2 arrest *in vivo* during infection or that additional, unidentified cofactors are involved in Vpr function (237, 239, 379).

The reasoning behind Vpr-mediated cell cycle arrest has remained equally unclear. It has been suggested that G_2 arrest increases virus production, since the viral-LTR appears to be most active in G_2 phase (203). Additionally, G_2 arrest would allow

cellular resources to be diverted from cell division to viral production, allowing the virus to replicate more efficiently (203). But in cell lines and cycling $CD4^+$ T cells, Vpr is dispensable for infection (345). Vpr expression is lost in virus serially passaged $CD4^+$ T cell lines *in vitro*, suggesting the function of Vpr may be more complex than what can be delineated from *ex vivo* or *in vitro* infections (216). Another, contending hypothesis is that G_2 arrest is the outcome of a Vpr-controlled DNA-damage response (DDR). Recent studies have demonstrated that Vpr orthologs from diverse primate lentiviruses can activate the DDR in human cells, suggesting that activation of DDR is a conserved function of lentiviral Vpr alleles (237). It has been proposed that Vpr may intentionally induce double-strand breaks in the host genome to initiate DDR, resulting in both G_2 arrest and suppression of an antiviral interferon response, though the mechanism remains unclear (236). Alternatively, it has also been suggested that Vpr modulates signaling through either ATM or ATR, both of which are DDR initiator kinases (382, 383), to induce pro-inflammatory responses, thus enhancing virus replication and spread.

My recent work has used primary human MDDCs, which are susceptible to infection at a low but measurable level, as a model for infection (184). In this model, I see a robust restriction to infection in the absence of Vpr that is unique to MDDCs and potentially resting $CD4^+$ T cells (185). In my work, I note that this restriction occurs post-integration in a single round of infection and is alleviated by Vpr-association with the $DCAF^{CRL4}$ complex. In my current work, I looked at infection of MDDCs with diverse primate lentiviruses to determine if my observations with HIV-1 were conserved across primate lentiviral evolution, specifically amongst those that encode Vpx. Vpx, similar to

Vpr, is a small accessory protein encoded by primate lentiviruses in the SIV_{sm}/SIV_{mac}/HIV-2 lineage (176). It is incorporated into the virion, similar to Vpr, through association with the same p6 region of Gag and also associates with the same $DCAF^{CRL4}$ complex to counteract host cell restriction (194, 225–227). The reason for the high level of overlap between Vpx and Vpr function is due to the origin of Vpx. Vpx is thought to have originated from a duplication event of Vpr, after which the host cell targets of the two proteins diverged; Vpr initiates a DDR by targeting an unknown host factor while Vpx targets SAMHD1 for proteasomal degradation (176–180, 183, 415). SAMHD1 is a dNTPase that reduces dNTP pools in macrophages, DCs, and resting $CD4^+$ T cells, hampering reverse transcription (179, 180, 182, 183). Presence of SAMHD1 is thought to be the main reason HIV-1 is poorly infectious in MDDCs (182).

Surprisingly, I found that infection of human MDDCs with HIV-2, SIV_{sm}, or SIV_{mac} -ΔVpr viral isolates had no effect on infection when compared to WT viruses. Rather, I found that infection with ΔVpr-HIV-2 or -SIV_{mac} induces robust type I IFN production from productively infected MDDCs that is absent or decreased in WT virus infection. Type I IFN production was induced at a post-reverse transcription step and was prevented upon initiation of infections in the presence of integration inhibitor or infection with SIV_{mac} Vpr mutants that do not induce G_2 cell cycle arrest. Finally, type I IFN induction could be blocked upon initiating infection of SIV_{mac} ΔVpr viruses in the presence of inhibitors to NF-κB signaling pathway. Together, my data suggests a conserved role for Vpr in harnessing the DDR pathway to prevent viral sensing that occurs during integration of the viral dsDNA into the host genome.

Results

Vpr expression does not affect single round replication of SIV_{mac}.

I have previously reported a robust, single round block to HIV-1 infection of MDDCs in the absence of Vpr. I wanted to assess whether this restriction was present during infection with primate lentiviruses that encode Vpx, (179, 180). Previous studies have demonstrated that human MDDCs can be efficiently transduced by SIV_{mac} lentivectors (416) suggesting an absence of species-dependent restrictions to SIV_{mac} infection of human cells. I infected human MDDCs with single-cycle, VSV-G pseudotyped, GFP reporter SIV_{mac}, (SIV_{mac}-GFP Δenv/G) -WT, -ΔVpr, -ΔVpx, or -ΔVpr/ΔVpx at MOI = 1 to determine the individual and cumulative effects of Vpr and Vpx on viral infectivity in MDDCs. I hypothesized that, similar to HIV-1, the SIV_{mac}-GFP Δenv/G ΔVpr virus would be poorly infectious in human MDDCs. SIV_{mac}-GFP Δenv/G -ΔVpx and -ΔVpr/ΔVpx were used as negative controls that I assumed would be poorly infectious, since Vpx is known to enhance infectivity in MDDCs by targeting SAMHD1 for degradation. Surprisingly, I observed slightly enhanced infection levels upon infection with SIV_{mac}-GFP Δenv/G ΔVpr virus compared to WT virus. As expected, Vpx-deletion completely ablated infection of human MDDCs by SIV_{mac} (Fig. 20).

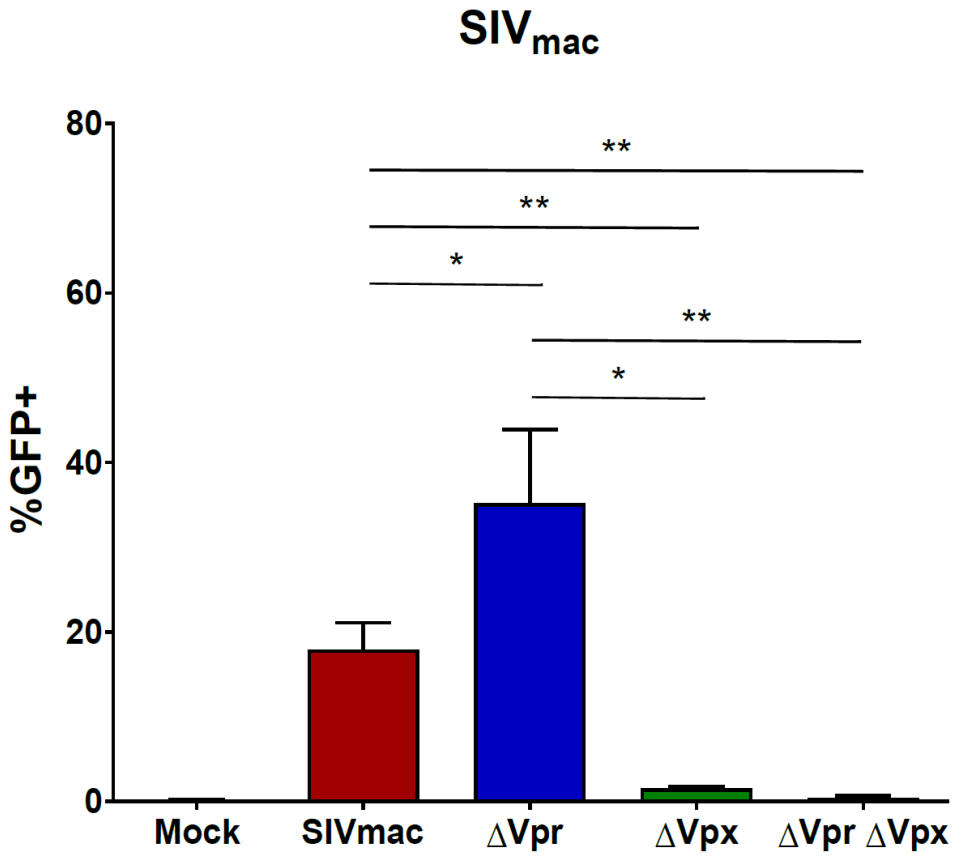


Figure 20. Vpr does not affect infection of human MDDCs with primate lentiviruses that encode Vpx.

MDDCs were infected at MOI = 1 with SIV_{mac}-GFP Δenv/G -WT, -ΔVpr, -ΔVpx, or -ΔVpr/ΔVpx for three days. GFP expression was assessed by FACS analysis. Data is the mean +/- SEM of infections of six independent donors. Significance calculated using a paired student's T test where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

SIV_{mac} Vpr regulates type I IFN induction during infection of MDDCs.

Numerous studies have described that infection with Vpx-encoding lentiviruses results in proteasomal degradation of SAMHD1 and robust enhancement of lentiviral reverse transcription in MDDCs (179, 180, 417–419). Reverse transcription is an error-prone process and can result in generation of aberrant dead-end viral DNA intermediates that can be subject to innate immune sensing. Furthermore, infection of MDDCs with Vpx-encoding HIV-2 virus particles, or HIV-1 infection in the presence of Vpx, results in cGAS-dependent sensing of viral RT-products (320, 420). Interestingly, previous studies have suggested that Vpr recruits structure specific endonuclease regulator, SLX4com that has been implicated in regulation of numerous DNA repair pathways and in Holiday junction resolution, and activation of endonuclease activity (236) to process non-productive reverse transcription intermediates and thus avoid innate immune sensing. I hypothesized that infection of MDDCs with Vpx-encoding SIV_{mac}-GFP Δenv/G viruses might result in increased level of reverse transcripts that in the absence of Vpr be detected by nucleic acid sensors in MDDCs. To test this, I first measured late-RT products during SIV_{mac}-GFP Δenv/G -WT, -ΔVpr, and -ΔVpx infection of two independent donors of MDDCs by qPCR at 18 and 42 hours post infection. I observed an increase in late RT-products in the absence of Vpr at both 18 h and 42 h post infection (Fig. 21A and B). Note that the primer-probe combination (U5-Gag region) used for the quantification of viral DNA detects all forms of viral dsDNA forms including viral integrants.

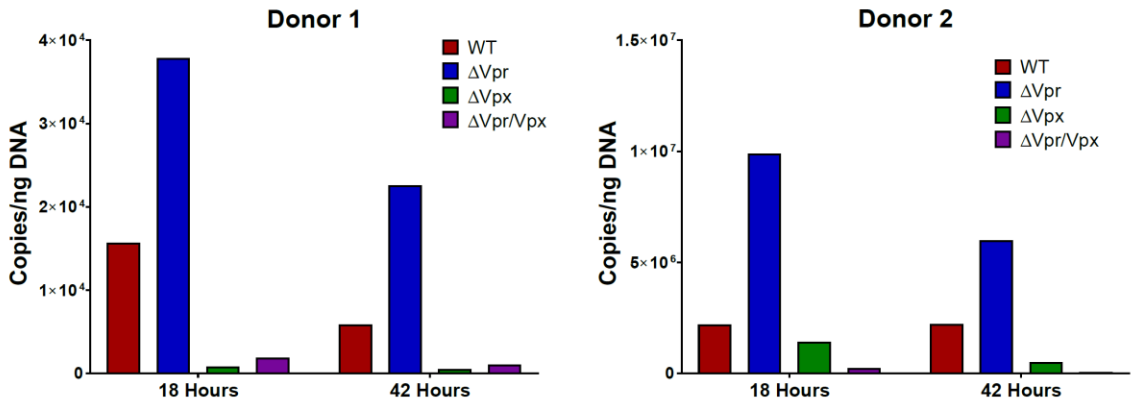


Figure 21. SIV_{mac} Vpr modestly increases the amount of late RT products during infection of MDDCs.

MDDCs were infected at MOI = 3 with SIV_{mac}-GFP Δenv/G -WT, -ΔVpr, -ΔVpx, or -ΔVpr/ΔVpx. Viral stocks had been pre-treated with DNase to reduce background plasmid contamination from virus stocks used for infections. Cells were harvested for DNA at either 18 or 42 hours post infection. Late RT products were quantified via qPCR as compared to a known standard of plasmid DNA. Two respective donor infections are shown.

The effect of HIV-1 Vpr on type I IFN regulation during infection has been extensively studied in the literature, with varying results. Opposing studies have shown specific downregulation of type I IFN in the presence of HIV-1 Vpr or, alternatively, induction of a type I IFN response due to HIV-1 Vpr expression (236, 252, 256, 258, 259, 265, 372). I failed to detect induction of type I IFN responses in MDDCs infected with HIV-1/WT or ΔVpr viruses (Fig. 9). Since SIV_{mac} Vpr did not modulate MDDC infection like HIV-1 Vpr, I hypothesized that modulation of type I IFN response by SIV_{mac} Vpr might be divergent as well. MDDCs were infected with SIV_{mac} Δenv/G -WT, -ΔVpr, -ΔVpx or -ΔVpr/ΔVpx viruses (MOI =3), and cell-free supernatants were harvested on day 3 post infection. The amount of type I IFN released in cell-free supernatants was measured using a previously described bioassay (369). For quantification, secreted IFN was compared to a known standard of IFNα. I observed a significant increase in type I IFN secretion in MDDCs infected with SIV_{mac}ΔVpr virus as compared -WT infection (Fig. 22). Both -ΔVpx, or -ΔVpr/ΔVpx viruses were non-infectious on MDDCs (Fig. 20) and did not result in production of type I IFN (Fig. 22).

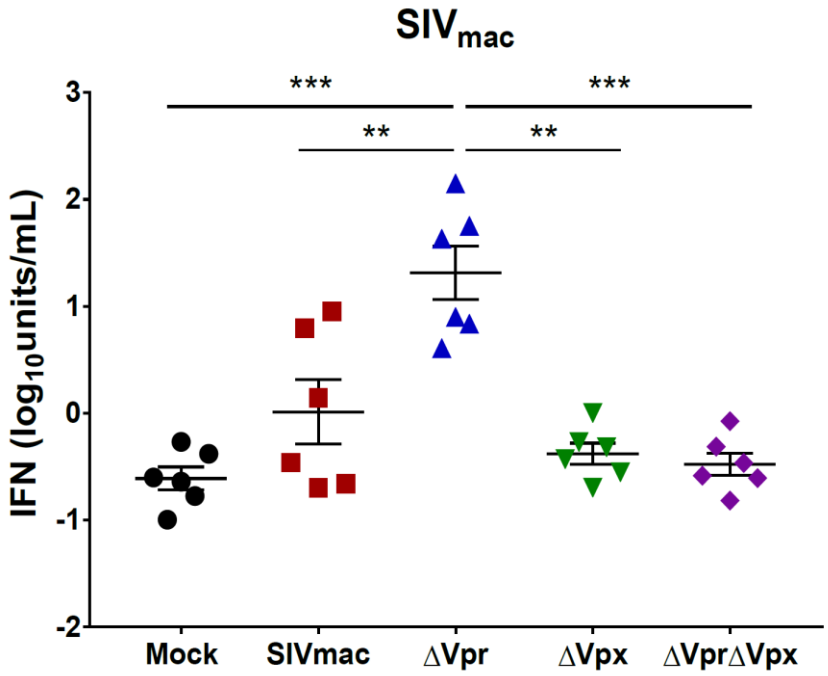


Figure 22. SIV_{mac} Vpr suppresses type I IFN production during infection of MDDCs.

MDDCs were infected at MOI = 3 with SIV_{mac}-GFP Δenv/G -WT, -ΔVpr, -ΔVpx, or -ΔVpr/ΔVpx. Supernatants were harvested at day three post infection and type I IFN was quantified using a sensitive bioassay as compared to a standard of IFNα. Briefly, HEK 293 cells containing an ISRE-driven luciferase reporter were incubated with cell culture supernatants or a standard curve of recombinant IFNα-containing growth media for 21 hours before quantification of luciferase expression. Data is representative of the mean +/- the SEM for infections of six independent donors. Data was log transformed to normalize the distribution and significance was calculated using a paired student's T test where *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Host sensing of lentivirus integration is blocked by SIV_{mac} Vpr.

Since infection with SIVmacΔVpr viruses resulted in increased amounts of viral DNA in MDDCs at early times post infection (Fig. 21), I determined if type I IFN production was the result of differential RT-product accumulation and sensing in the absence of Vpr. To test this further, MDDCs were infected with SIV_{mac} Δenv/G -WT, -ΔVpr, -ΔVpx or -ΔVpr/ΔVpx viruses (MOI =3), in the presence or absence of the RT-inhibitor tenofovir and the integrase inhibitor raltegravir to block different stages of the viral life cycle, and determine the step of the virus life cycle that is subject to host sensing and innate immune activation. I hypothesized that if induction of type I IFN occurs upon sensing of reverse transcripts, only tenofovir will block type I IFN production. Alternatively, if host sensing of SIV_{mac}ΔVpr virus replication occurs after completion of reverse transcription, both inhibitors (tenofovir and raltegravir) will be able to block type I IFN secretion. Interestingly, tenofovir pre-treatment of MDDCs which efficiently blocked viral infection, was able to completely abrogate type I IFN secretion (Fig. 23A, B), suggesting that SIV_{mac} reverse transcripts might be sensed in MDDCs in the absence of Vpr.

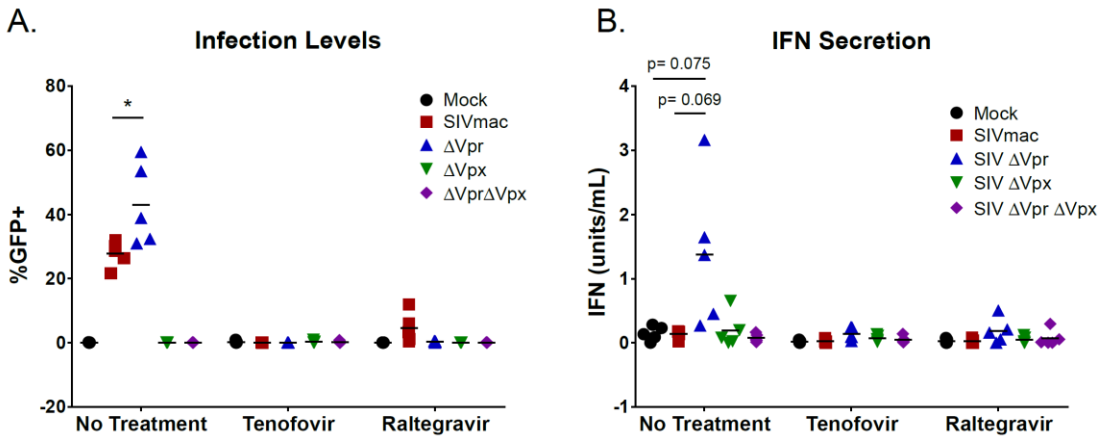


Figure 23. SIV$_{mac}$ ΔVpr triggers innate immune sensing at a post-reverse transcription step in MDDCs.

(A-B). MDDCs were infected at MOI = 3 with SIV$_{mac}$-GFP Δenv/G -WT, -ΔVpr, -ΔVpx, or -ΔVpr/ΔVpx for three days. Parallel infections were treated with the RT-inhibitor tenofovir or the integration inhibitor raltegravir. Cells were analyzed for GFP expression (A) and type I IFN production in the supernatants was quantified using a bioassay (B). Data is representative of the mean +/- SEM of infections of five independent donors. Significance was calculated using a paired student's T test where *p<0.05, **p<0.01.

All integrases have a characteristic catalytic core domain, the D,D35E motif (421–423), and hence can be inhibited by raltegravir. Raltegravir prevents lentiviral DNA from inserting itself into the host genome. Because integrase inhibitors are known to cause nuclear accumulation of unintegrated lentiviral DNA (Fig. 14 (395, 404)), I hypothesized that raltegravir pre-treatment might result in the accumulation of linear or circularized viral DNA forms that may exacerbate innate immune activation and type I IFN secretion in $SIV_{mac}\Delta$Vpr-infected MDDCs. Surprisingly, raltegravir pre-treatment, which inhibited productive infection of MDDCs by GFP-expressing $SIV_{mac}\Delta$Vpr Δenv/G virus (Fig. 23A) also completely abrogated production of type I IFN (Fig. 23B), suggesting that integration of lentiviral DNA into the host genome or post-integration steps of the viral life cycle were subject to host sensing mechanisms that result in production of type I IFN.

In order to tease apart the kinetics of induction of type I IFN in $SIV_{mac}\Delta$Vpr-infected MDDCs, reverse transcription was arrested at different times pre- and post-infection by addition of tenofovir. The percentage of GFP+ cells obtained at each time point (time of tenofovir addition) was then determined at day 3 post infection by FACS analysis. In addition, cell-free supernatants were harvested at day 3 and type I IFN production was quantified by a bioassay. Addition of tenofovir at 3 hours before addition of virus, or at 0, 3, or 9 hours post infection significantly inhibited type I IFN production in SIV_{mac}-GFP Δenv/G-ΔVpr infected cultures, confirming that neither virus entry, sensing of incoming viral RNA genome or initiation of reverse transcription result in viral sensing and type I IFN production (Fig. 24A, B). Addition of tenofovir at 18 hours

post infection blocked infection by ~50% (Figure 24A), but type I IFN production was robustly induced (Fig. 24B), suggesting that steps after completion of reverse transcription are subject to host sensing mechanisms.

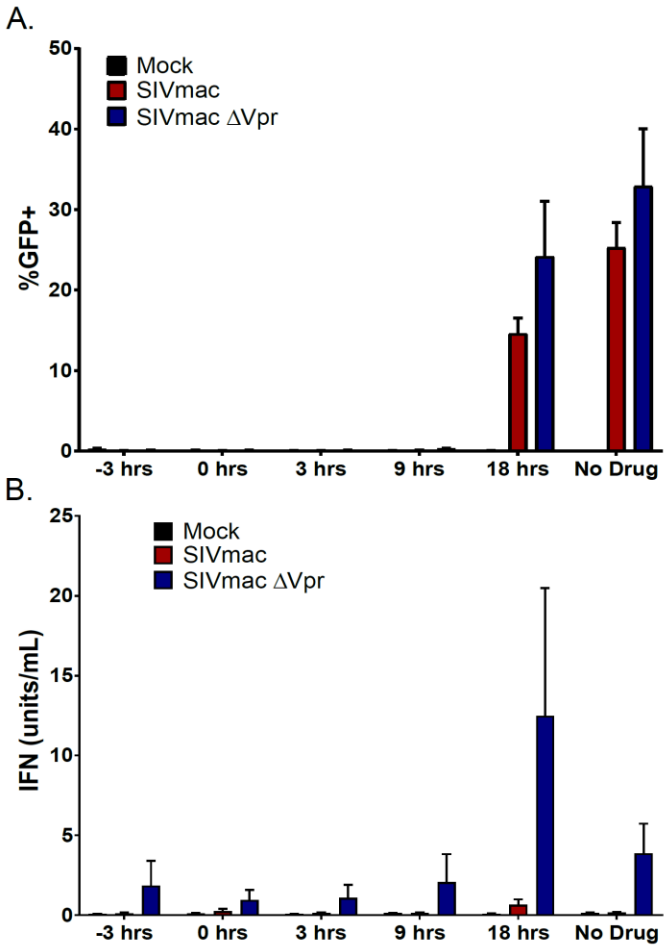


Figure 24. Completion of reverse transcription in MDDCs is necessary for host sensing of infection with SIV_{mac} ΔVpr.

(A-B.) MDDCs were infected at MOI = 3 with SIV_{mac}-GFP Δenv/G or SIV_{mac}-GFP Δenv/G ΔVpr. Tenofovir was added to block reverse transcription at 3 hours prior to infection, at the time of infection, or 3, 9 or 18 hours post infection. At day three post infection, cells were analyzed for GFP expression (A) or type I IFN secretion into the supernatants was quantified (B). Data is the mean +/- SEM of infections with five independent donors.

HIV-2 Vpr and SIV_{sm} Vpr also block IFN induction in human MDDCs.

I next wanted to characterize whether these results seen with SIV_{mac} could also be extended to other viruses in the same lentiviral lineage. These include SIV_{sm}, which causes non-pathogenic infections in its natural host, sooty mangabeys (424), and HIV-2, which can cause AIDS in humans. SIV_{mac} originated from multiple cross species transmissions of SIV_{sm} from sooty mangabeys to macaques that were co-housed in captivity (33). Similarly, HIV-2 is thought to be the result of a transmission event of SIV_{sm} to humans through preparation of game meat (17). MDDCs were infected with HIV-2 Rod9-GFP Δenv/G -WT, -ΔVpr, or -ΔVpx and SIV_{sm}-GFP Δenv/G -WT, -ΔVpr, or –ΔVpx viruses (MOI = 3), and cells were harvested at day 3 post infection for FACS analysis. Similar to SIV_{mac} infection of MDDCs, absence of Vpr had negligible impact on infection of HIV-2 (Fig. 25A) and SIV_{sm} (Fig. 25B) in MDDCs, while Vpx deletion completely ablated infection of both HIV-2 and SIV_{sm} (Fig. 25A, B).

I next determined if infections of MDDCs with HIV-2 or SIV_{sm} in the absence of Vpr could also induce type I IFN production similar to that observed with SIV_{mac}ΔVpr infections of MDDCs. MDDCs were infected at MOI = 3 with SIV_{sm} Δenv/G -WT, –ΔVpr or -ΔVpx and Rod9 Δenv/G -WT, – ΔVpr, or -ΔVpx in the presence or absence of tenofovir and/or raltegravir. Similar to what I observed with SIV_{mac}, type I IFN production was significantly increased in both HIV-2 ΔVpr and SIV_{sm}ΔVpr infections (Fig. 25C, D). Furthermore, type I IFN production in HIV-2 ΔVpr and SIV_{sm}ΔVpr-infected MDDCs was completely blocked upon pre-treatment with either tenofovir or raltegravir (Fig. 25C, D). Alternatively, infection with both HIV-2 Rod9-GFP Δenv/G-

WT and SIV_{sm} GFP Δenv/G- WT (encoding WT-Vpr) also induced low but detectable levels of type I IFN, suggesting that HIV-2 and SIVsm viruses are subject to additional Vpr-independent sensing mechanisms in human MDDCs (420) . Alternatively, Vpr alleles encoded by HIV-2 and SIV_{sm} might not be as efficacious as SIV_{mac} Vpr in suppressing virus sensing mechanisms in human MDDCs.

To determine if the findings observed with single-cycle HIV-2 viruses are also observed with infections of MDDCs with replication competent HIV-2, I infected MDDCs (MOI = 3) with VSV-G-pseudotyped Env-encoding replication competent HIV-2 Rod9 -WT or -ΔVpr and harvested cell-free supernatants at three days post infection. Tenofovir and raltegravir were again used as controls to ensure sensing is due to infection and not virus particle addition. Similar to what I observed with single-round infection analysis, infection of MDDCs with replication competent HIV-2 ΔVpr resulted in enhanced secretion of type I IFN that is reduced in WT HIV-2/Rod9 infection. Furthermore, infections of HIV-2 Rod9 -WT or -ΔVpr in the presence of RT or integration inhibitors reduced type I IFN production (Fig. 25E). Together, these data suggest that members of SIV_{sm}/SIV_{mac} /HIV-2 lineage encode Vprs that suppress sensing of lentiviral integration into host genome.

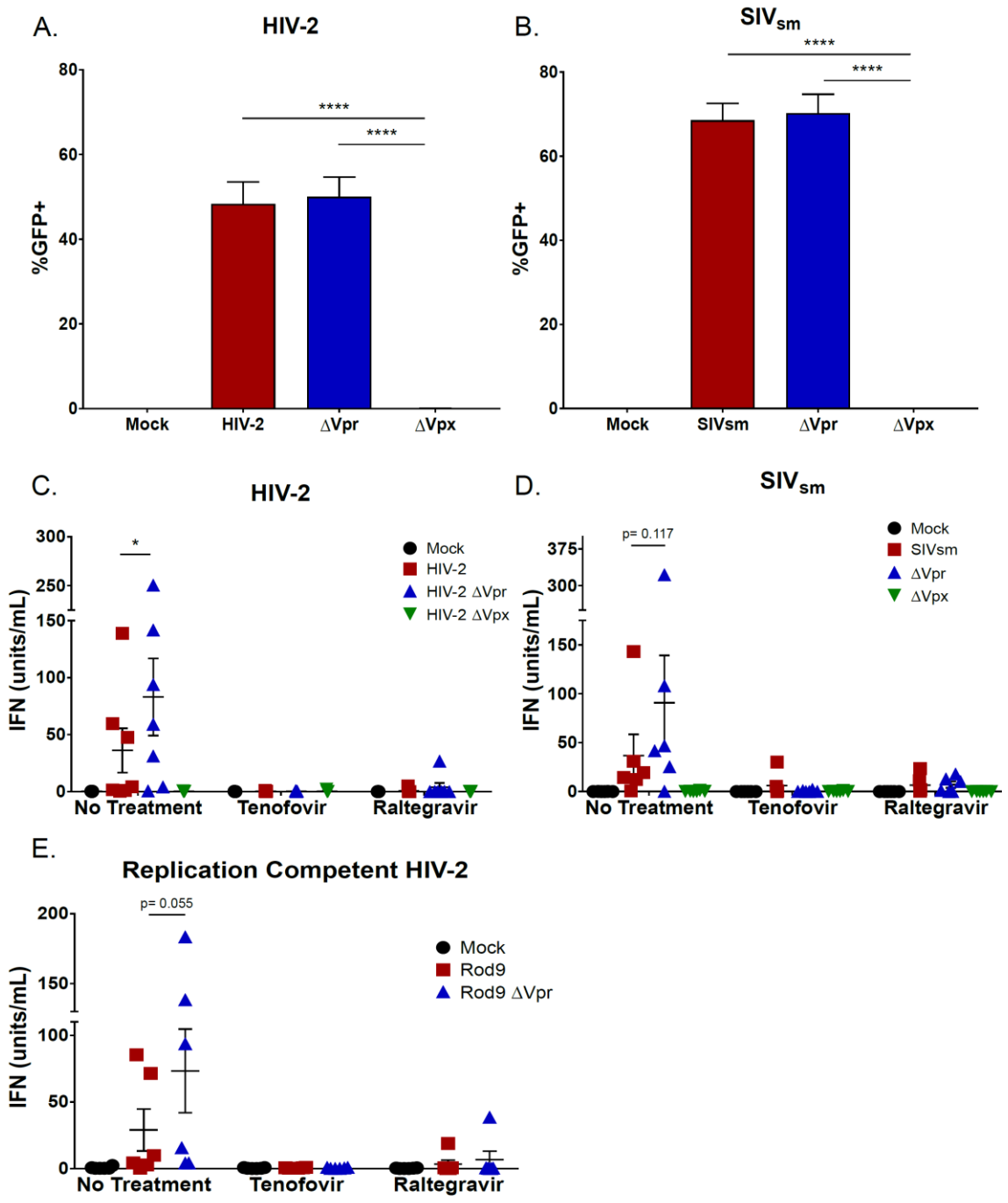

A.
HIV-2
%GFP+
80
60
40
20
0
****
****
Mock
HIV-2
ΔVpr
ΔVpx
B.
SIVsm
****
****
%GFP+
80
60
40
20
0
Mock
SIVsm
ΔVpr
ΔVpx
C.
HIV-2
IFN (units/mL)
300
250
150
100
50
0
*
Mock
HIV-2
HIV-2 ΔVpr
HIV-2 ΔVpx
No Treatment
Tenofovir
Raltegravir
D.
SIVsm
IFN (units/mL)
375
300
150
100
50
0
p= 0.117
Mock
SIVsm
ΔVpr
ΔVpx
No Treatment
Tenofovir
Raltegravir
E.
Replication Competent HIV-2
IFN (units/mL)
200
150
100
50
0
p= 0.055
Mock
Rod9
Rod9 ΔVpr
No Treatment
Tenofovir
Raltegravir

Figure 25. Vpr from Vpx-encoding lentiviruses antagonizes type I IFN signaling in MDDCs.

(A). MDDCs were infected at MOI = 3 with Rod9-GFP Δenv/G -WT, -ΔVpr, or -ΔVpx for three days and GFP was asses by FACS analysis. Data is the mean +/- SEM of infections with seven independent donors. (B). MDDCs were infected as in (A) with SIV_{sm}-GFP Δenv/G -WT, -ΔVpr, or -ΔVpx. Data is the mean +/- SEM of infections with six independent donors. (C). MDDCs were infected as in (A) with Rod9-GFP Δenv/G -WT, -ΔVpr, or -ΔVpx. Parallel infections were treated with tenofovir or raltegravir to block reverse transcription or integration, respectively. Supernatant from day 3 post infection was analyzed for type I IFNs using a quantitative bioassay. Data is the mean +/- SEM from seven independent donors. (D). MDDCs were infected as in (B) with SIV_{sm}-GFP Δenv/G -WT, -ΔVpr, or -ΔVpx in the presence of tenofovir or raltegravir. Supernatants were harvested at day 3 post infection for type I IFN quantification using a sensitive bioassay. Data is the mean +/- SEM of six independent donors. (E). MDDCs were infected at MOI = 3 with replication competent Rod9/G or Rod9 ΔVpr/G. Parallel infections were treated with the RT inhibitor tenofovir or the integration inhibitor raltegravir. Supernatants were analyzed at day 3 post infection for the presence of type I IFNs. Data is the summary of six infections with six independent donors. Significance calculated using a paired student's T test where *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Suppression of host sensing of lentiviral integration in MDDCs is NF-κB dependent and is correlated to the ability of Vpr to induce DDR.

G_2 cell cycle arrest is a conserved function of Vpr and is thought to be the outcome of Vpr-mediated regulation of the DDR. I hypothesized that ability of SIV_{sm}/SIV_{mac}/HIV-2 Vpr to block type I IFN induction is the result of Vpr-mediated degradation of a viral integration sensor, resulting in initiation of a DDR response. In support of this hypothesis, it has also been reported that many members of DDR pathways are under positive selection, which is a hallmark of retroviral restriction factors (410, 411). To determine if G_2 arrest/DDR induction function of SIV_{mac} Vpr correlates with its ability to suppress type I IFN production, I made several mutations to SIV_{mac} Vpr that had previously been characterized to block Vpr-mediated G_2 cell cycle arrest (377, 409). Work is underway to confirm differential regulation of the cell cycle by these Vpr mutants. I infected MDDCs with SIV_{mac} Vpr mutants -VprV21A and -VprS81A at MOI = 3 and analyzed supernatants for type I IFN production at day 3 post infection. All Vpr mutants were incorporated into virions at levels similar to WT-Vpr (Chapter 2) and were infectious in MDDCs, though VprV21A shows reduced infectivity (Fig. 26A). Interestingly, I found that infections of MDDCs with one mutant that has reduced cell cycle arrest capacity, VprS81A, results in enhanced type I IFN production, a phenocopy of the SIV_{mac} ΔVpr infection (Fig. 26B). Furthermore, the VprV21A mutant blocks type I IFN production, which I hypothesize correlates with increased cell cycle arrest capacity (Fig. 26B). Work to confirm this hypothesis is in progress.

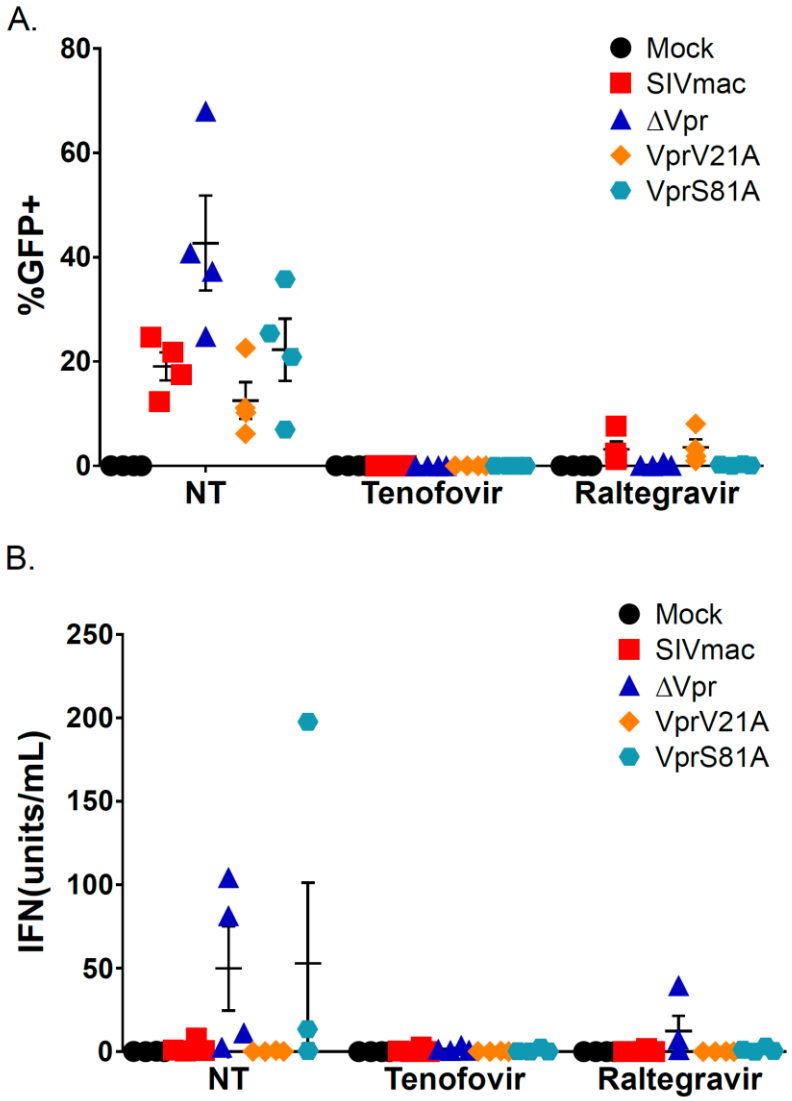


Figure 26. Vpr antagonism of innate immune sensing correlates with its ability to induce G_2 arrest.

(A-B) MDDCs were infected with SIV SIV_{mac}-GFP Δenv/G -WT, -ΔVpr, -VprV21A, or -VprS81A for three days. Parallel infections were treated with tenofovir or raltegravir. Cells were analyzed for GFP expression (A) and IFN secretion in infection supernatants (B). Data is the mean +/- SEM of infections with four independent donors.

Previously published work has demonstrated that treatment of cells with etoposide (induces DNA double strand breaks)-induced type I IFN responses in a IRF3-independent, NF-κB dependent manner (425). I hypothesized that a similar mechanism underlies lentiviral integration-induced type I IFN responses. To test this hypothesis, MDDCs were infected with SIV_{mac} GFPΔenv/G –WT or ΔVpr viruses in the presence of small molecule inhibitors that block pro-inflammatory signaling cascades. I utilized the inhibitors, BAY11-7082, an inhibitor of IκB-α that blocks NF-κB activation, (5Z)-7-Oxozeaenol, which inhibits TAK1, a signaling protein upstream of NF-κB, the NLRP3-inflammasome inhibitor, glybenclamide, and BX795, a TBK1 inhibitor, that blocks IRF3 activation (426). None of the inhibitors had any impact on cell viability at the concentrations tested (data not shown). Treatment with BAY11-7082, (5Z)-7-Oxozeaenol and BX795 enhanced infections of both WT and ΔVpr viruses, though differences were not statistically significant (Fig. 27A). Interestingly, both BAY11-7082 and (5Z)-7-Oxozeaenol that reduce NF-κB activation potently reduced type I IFN secretion from SIV_{mac}-GFP Δenv/G ΔVpr infected cells, while treatment with glybenclamide or BX795 had no effect on type I IFN secretion (Fig. 27B). Together, these experiments suggest that NF-kB activation is necessary for induction of type I IFN responses downstream of sensing of lentiviral integration into host genomes.

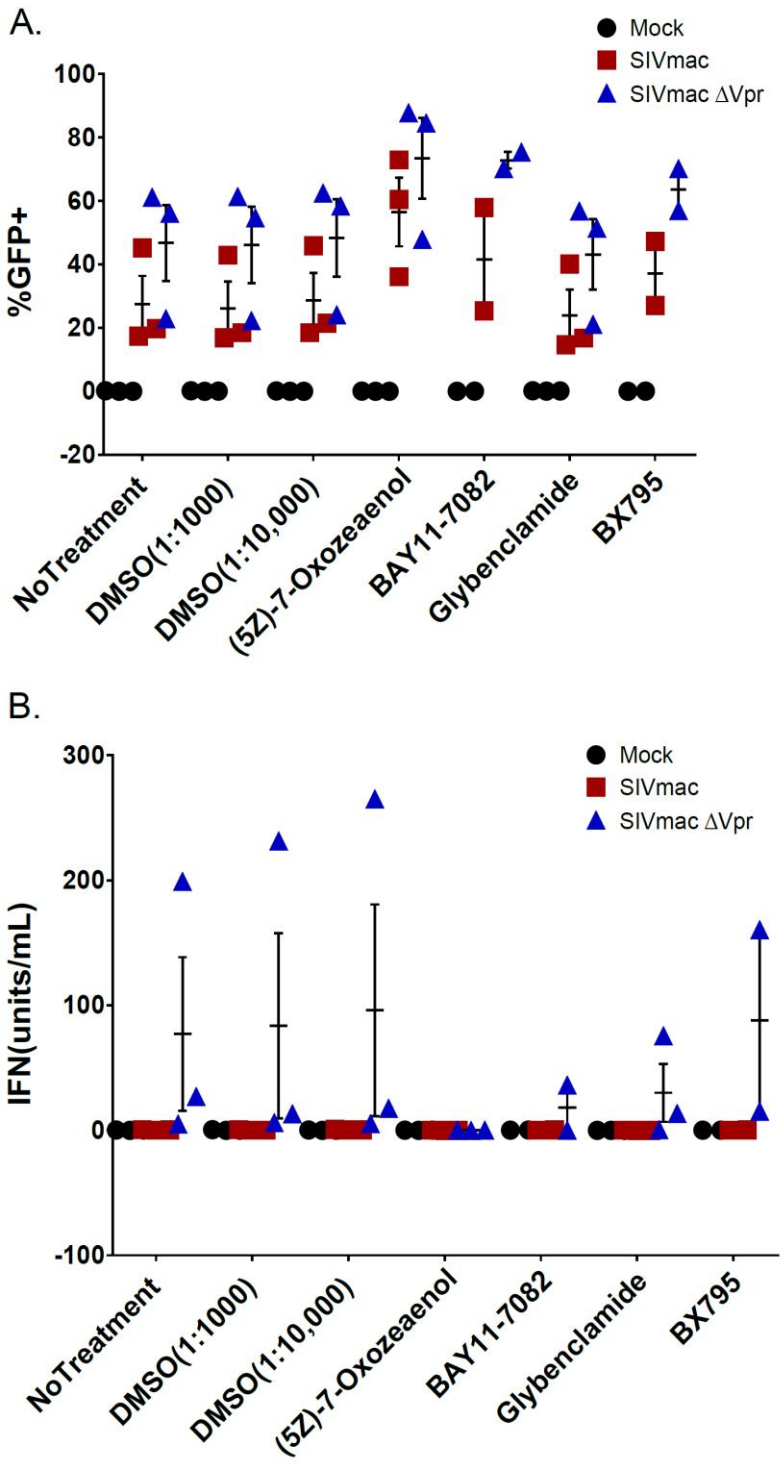

A.
Mock
SIVmac
SIVmac ΔVpr
%GFP+
100
80
60
40
20
0
-20
NoTreatment
DMSO(1:1000)
DMSO(1:10,000)
(5Z)-7-Oxozeaenol
BAY11-7082
Glybenclamide
BX795
B.
Mock
SIVmac
SIVmac ΔVpr
IFN(units/mL)
300
200
100
0
-100
NoTreatment
DMSO(1:1000)
DMSO(1:10,000)
(5Z)-7-Oxozeaenol
BAY11-7082
Glybenclamide
BX795

Figure 27. Type I IFN secretion from SIV_{mac} ΔVpr infected MDDCs is dependent on the NF-κB signaling cascade.

(A-B) MDDCs were infected at MOI = 3 with SIV_{mac}-GFP Δenv/G -WT, -ΔVpr, -ΔVpx, or -ΔVpr/ΔVpx in the presence of the inhibitors BAY11-7082 (1 μM), an IκB-α inhibitor, (5Z)-7-Oxozeaenol (1 μM), a TAK1 inhibitor, glybenclamide (50 μM), a NLRP3-inflammasome inhibitor and BX795 (0.1 μM), a TBK1 inhibitor. No treatment or equivalent concentrations of DMSO were used to confirm drug efficacy. Cells and supernatants were harvested on day 3 post infection to determine GFP expression (A) or type I IFN secretion (B). Data is the summary of infections with two or three independent donors.

Future Studies

Additional work to confirm that integration is the step of the viral life cycle that is sensed is ongoing. In the future, I plan on adding raltegravir to SIV_{mac} infected MDDCs at 0, 6, 9, and 18 hours post-infection, to further clarify the kinetics of the sensing mechanism. I predict that addition of raltegravir at 0, 6, and 9 hours will potently block IFN secretion, while raltegravir addition at 18 hours will neither block type I IFN production nor block viral gene expression. In the case that completion of virus integration takes longer than 18 hours, I will adjust my raltegravir addition times to better fit the SIV replication cycle. I also plan on using integrase catalytic site mutants to provide additional support for my hypothesis. I am currently in the process of making integrase-null (IntD116N) clones of SIV_{mac} Δenv/G -WT and -ΔVpr. I expect that neither D116N-WT nor D116N-ΔVpr (which are predicted to not integrate in the host genome) will trigger type I IFN production.

Work is still ongoing to demonstrate that Vpr-mediated regulation of DDR is preventing sensing of viral infection. I plan to use inhibitors that selectively block the DDR response in order to tie the DDR to viral sensing. I will use selective DDR inhibitors including caffeine, which blocks ATM and ATR mediated DDR sensing (383, 427). ATM and ATR are DNA-damage sensing kinases that broadly amplify signaling to recruit DNA repair machinery, ATM in response to double stranded breaks and ATR in response to single strand breaks or gaps (428–431). I will also use commercially available PARP-1 inhibitors, which block the ability of PARP-1 to detect and mark single strand breaks for repair (432–434). If viral sensing occurs through any of these pathways, I

would expect to ablate type I IFN production in the presence of inhibitors. I also plan on using chemical agents that induce DNA damage, like etoposide, which induces double strand breaks, to recapitulate the sensing I observe during integration in the absence of Vpr.

Additionally, I hope to show that HIV-1 Vpr antagonizes IFN production similar to SIV_{mac}/SIV_{sm}/HIV-2. I expect I may have to supplement HIV-1 infections of MDDCs with SIV_{mac} Vpx in order to achieve enough high levels of HIV-1 integration for detection by host sensing machinery. My future work in this area also involves repeating some of the experiments I have planned using DDR-regulating inhibitors, including caffeine, etoposide, and PARP-1 inhibitors with HIV-1 in the presence of SIV_{mac} Vpx. I also plan to measure RT-products and integrated proviruses in the presence of SIV_{mac} Vpx to better characterize the effect of Vpr on reverse transcription and integration in my system. Collectively, I hope to definitely show a conserved role for Vpr in targeting a DDR-pathway sensor that detects conserved patterns during primate lentiviral integration.

Currently, my data reveal a viral sensor that detects viral infection during or post integration. This sensor is antagonized by Vprs encoded by diverse primate lentiviruses in the SIV_{mac}/SIV_{sm}/HIV-2 lineage, and may also be antagonized by HIV-1 Vpr, though work is currently underway to determine this conclusively. Sensing occurs through an NF-κB-dependent mechanism. Vpr-mediated coordination of cell cycle arrest appears to be necessary to block IFN secretion in reaction to infection. Together, these data suggest the presence of a novel viral sensor that may play a crucial role in driving Vpr maintenance amongst primate lentiviruses.

DISCUSSION

The human evolutionary lineage has been riddled with exposure and infection by retroviruses, as evident by the 8% of our DNA that is composed of viral elements (435). Individual cellular defenses against infections have co-evolved with viral evasion in a race for survival (436). In this work, I identified a novel detection point for viral invasion, retroviral integration. Individual cellular host defenses have evolved to counteract viral infection, either by creating barriers that prevent conserved steps in the viral life cycle from occurring or by developing sensing mechanisms that detect infection (436, 437). These barriers to infection must be actively circumvented by viral proteins in order for a successful, spreading infection to occur. Lentiviral accessory proteins, including Vpr, are encoded specifically to counteract these host defense mechanisms, often referred to as restriction factors, and must evolve every time a species jump occurs (140). Restriction factors have undergone millions of years of positive selection to detect and counteract conserved stages of the retroviral life cycle (436). Restriction factors have been identified that block or detect most stages of the viral life cycle, including uncoating, reverse transcription, nuclear entry, and virion release, but until this work, viral detection at the step of integration has not been identified (336, 438, 439).

Vpr's role during infection has remained elusive to researchers for years. It has clear involvement with some sort of DDR, as evident by the conserved role in initiating G_2 cell cycle arrest, but outcomes or advantages of this arrest remain poorly defined (236, 355, 375). Expression of Vpr in cells induces DNA-damage foci which are the result of multiple DDR pathways including the Fanconi anemia (FA) pathway, the ataxia

telangiectasia mutated (ATM) and ataxia telangiectasia mutated Rad3 (ATR) pathways (236, 382, 383, 440). These pathways are all critical for coordination of DNA-damage repair, suggesting Vpr may act to directly induce DNA damage (236, 428, 441). Despite this evidence, interactions that would mediate Vpr-induced DNA damage remain poorly defined (237, 379, 442). We can take clues from pull down studies about the function of Vpr; Vpr associated with a number of DNA-damage repair proteins including UNG2, SLX4com, and HLTF (228, 235, 236, 238, 239). UNG2 is part of the base excision repair pathway that recognizes and removes misincorporated uracils or deaminated cytosines from DNA (443). The SLX4com is a key intermediary in the FA pathway, which coordinates proteins involved in nucleotide excision repair, homologous recombination and translesion synthesis in order to resolve Holliday junctions (444, 445). People with mutations in key regulators in the FA pathway have a hypermutable phenotype that significantly increases their risk for a variety of cancers (444, 445). SLX4 itself is a nuclear scaffold protein that binds and coordinates the activity of three different structure specific nucleases (444). Vpr, in the context of $DCAF^{CRL4}$ binds directly to SLX4, but has been shown to regulate the activity of fellow complex member MUS81/EME1, an endonuclease that cleaves Holliday junctions during repair (236, 444). It has been suggested that Vpr selectively activates MUS81/EME1 to create double strand breaks in the host DNA in order to induce G_2 arrest and prevent accumulation of viral DNA that would be subject to sensing (236). Finally, the most recently identified interactor with Vpr that has been discovered is HLTF, a protein that is involved with resolution of stalled replication forks (238, 239). In addition to these Vpr-$DCAF^{CRL4}$ interactors, the

DCAF^{CRL4} complex itself has been indicated as a regulator of DNA-damage repair, which poses the possibility that the Vpr-DCAF^{CRL4-} interaction may regulate the DDR independently of other host factors (413, 414, 446). Together, the extensive interactions of Vpr with host DDR pathway proteins suggest that Vpr plays a crucial role in regulating DNA-damage repair to the advantage of the virus.

Vpr is incorporated into virions and enters infected cells associated with the viral capsid (191–194, 339, 447). After entry, Vpr localizes to the nuclear membrane, shuttling in between the nucleus and cytoplasm, though some molecules also remain associated with the PIC (447, 448). The localization of Vpr supports the long held hypothesis that Vpr aids nuclear import, though it has become clear this is not the case (347, 449). My work in Chapter 1 reveals a role for virion associated HIV-1 Vpr in enhancing transcriptional output from the viral LTR. It is unclear whether this is the result of Vpr acting directly as a transcription factor or, instead, somehow modifying the integration site to make it more transcriptionally active. There is evidence to support both hypotheses. Vpr has been shown to degrade histone modifiers responsible for condensing DNA and reducing transcription (241, 242). Vpr is also capable of binding and transactivating the viral LTR, directly increasing production of viral transcripts (200–205). Similarly, in Chapter 2, I reported that presence of Vpr increases expression from unintegrated DNA. Again, this could be the result of Vpr acting directly on the viral LTR to promote transcription or, instead, modifying unintegrated DNA to increase its transcriptional competency. I propose that the effect of Vpr on chromatin structure is responsible for both observed effects of Vpr. Histones are loaded onto viral DNA rapidly

after entering the nucleus (412). Vpr-mediated degradation of HDACs may be critical for early, rapid transcription, both for integrated and unintegrated DNA (241, 242). I propose that in the absence of Vpr, viral DNA is rapidly silenced by chromatin modifications, potentially as a mechanism of host defense to prevent expression from foreign DNA in the nucleus (Fig. 28). In the presence of Vpr, chromatin modification is prevented, via direct engagement with the $DCAF^{CRL4}$ complex, allowing for transcription from LTR via the host PolII polymerase (Fig. 28). It is possible that chromatin remodeling is due to direct targeting of HDACs by the Vpr-$DCAF^{CRL4}$ complex, or it may be the outcome of general induction of DDR, which is known to relax chromatin structure in order for DNA repair to occur (241, 242, 450–452). This is only observed in cells where there is a block to integration, like the indirect block of low nucleoside pool in MDDCs or the artificial block of raltegravir or mutations in viral integrase. In more active, dividing cells that are far more permissive to infection, chromatin structure in in flux more often, due to events like cellular division, and other viral proteins like tat are sufficient to drive transcription. Alternatively, it is also possible that a common mechanism could be resulting in both the enhanced transcriptional output I characterized in Chapter 1 and 2 and the antagonism of viral sensing of integration I observe in the presence of Vpx, characterized in Chapter 3.

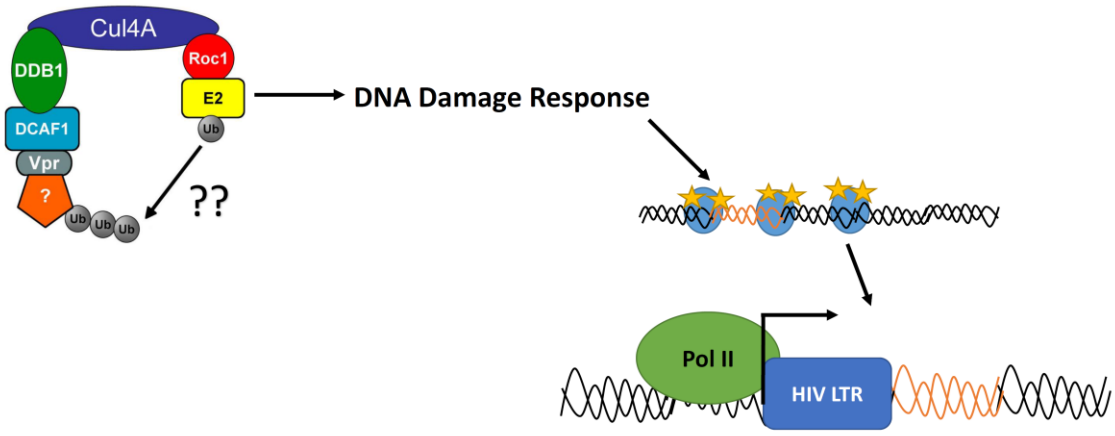


Figure 28. HIV-1 Vpr enhances viral transcription via modification of chromatin structure.

HIV-1 Vpr, in associates with the $DCAF^{CRL4}$ complex, results in modification of chromatin structure for enhanced recruitment of Pol II and increased LTR-driven transcription. It remains unclear if this is the effect of general induction of DDR, which is known to relax chromatin structure or due to $DCAF^{CRL4}$-mediated targeting of a host protein that regulates chromatin architecture, such as host HDAC proteins.

Vpr is maintained across all known primate lentiviruses and its ability to induce G_2 arrest, presumable through interactions with the DDR pathway is also conserved (141, 176, 177, 196, 207, 208, 237, 415, 436). It has been shown that a number of proteins involved in DDR pathways are under positive selection, though the reasoning for this is somewhat unclear (410, 411). Positive selection is a process by which viral infection drives host species diversification of proteins that restrict the virus (453, 454). Many of these genes, including BRCA1 and BRCA2, are integral for genome stability and mutations can confer significantly increased susceptibility to various cancers (410). It has been proposed by others that the only logical reason for selecting for genome instability is to counteract a greater threat, like viral invasion into the host genome, like occurs during retroviral integration (410). I believe that there is an interplay between Vpr-orthologs in primate lentiviruses and the host DDR response which has resulted in positive selection of proteins that are critical for genome integrity, despite the potential deleterious effects of non-sense mutations in these genes. My results in Chapter 3 suggest Vpr mediates antagonism of host sensor of viral integration and I propose that this host sensor is a DDR pathway protein that detects retroviral integration products (Fig. 29).

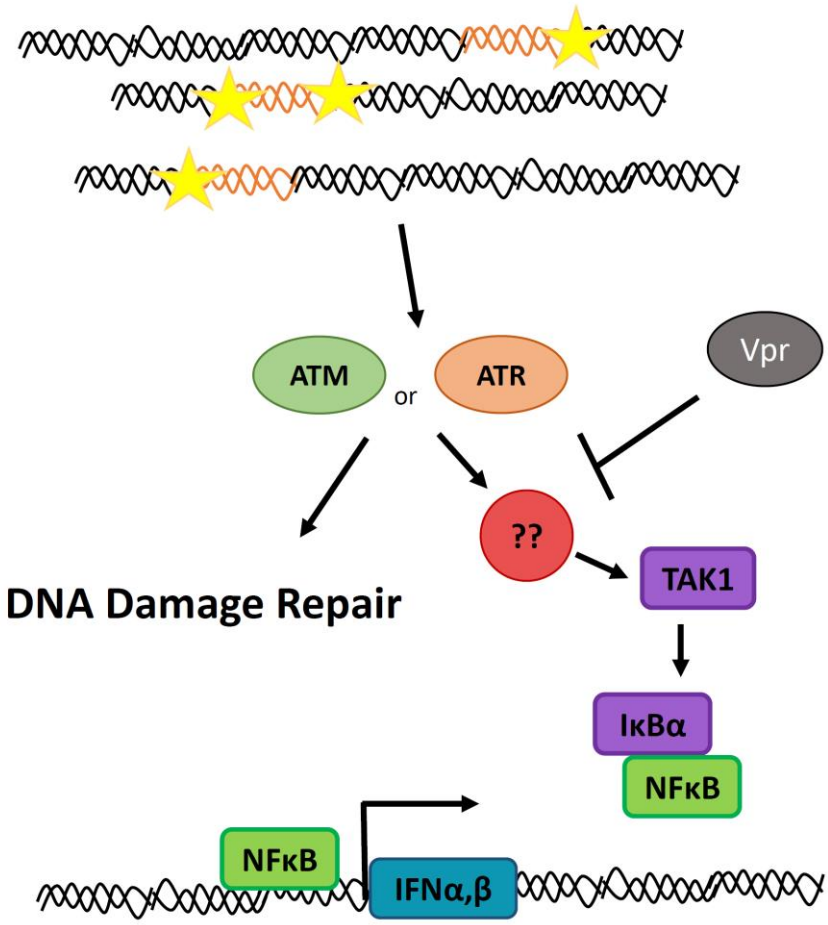


Figure 29. SIV_{sm}/SIV_{mac}/HIV-2 Vpr suppress integration-induced production of type I IFN.

SIV_{sm}/SIV_{mac}/HIV-2 Vpr blocks DDR-induced signaling pathways triggered by integration of Vpx-encoding primate lentiviruses. This block occurs only in pathways that lead to production of type I IFN and not pathways involved in integration site repair, allowing for successful infection without production of antiviral IFNs. In this model, ATM or ATR are the proposed DNA-damage sensors that signal through a TAK1-dependent pathway to trigger IFN.

Integration offers an unconventional target for viral sensing, since double-stranded DNA in the nucleus resembles normal host DNA. Unpublished data from Hisashi Akiyama in my lab has revealed a Vpr-independent late infection sensor that detects *de novo* synthesized viral RNA (Akiyama, unpublished data). Based on work he has done to characterize this viral sensor, I believe that Vpr-mediated antagonism of IFN occurs earlier in infection, pre-viral RNA production but post integration (Akiyama, unpublished data, Fig. 23, 25). Integration occurs through a conserved mechanism for all known retroviruses, creating a pattern that may be susceptible to sensing by host DNA repair machinery. In all cases, the end result is a two nucleotide, 5' flap and a 3' gap in the DNA (110). For HIV-1 this gap is five nucleotides, though the exact length varies slightly between viruses (109, 110). These structures are dissimilar from other naturally occurring patterns of DNA damage and must be repaired before DNA replication in order for cellular division to occur (109, 110). The consequence of a lack of repair in cycling cells is DNA damage-induced cell death, which occurs before the virus can successfully create new progeny. This would be detrimental to viral spread and persistence in a host. In non-cycling cells, like MDDCs, I propose that the outcome of this DNA-damage is viral sensing and IFN production. This outcome, like DDR-induced cell death, would be detrimental to viral spread via the creation of an antiviral environment. Both outcomes would result in strong selective pressure for maintenance of a viral protein to block sensing of integration to allow for viral propagation.

Human DNA already encodes a number of proteins to counteract both current and ancient retroviruses due to persistent exposure to and infection by retroviruses over our

evolutionary history (436, 453). One such protein is SAMHD1, a dNTPase that lowers the nucleoside pool in cells, restricting reverse transcription (179, 180, 389). In addition to its anti-HIV-1 activity, SAMHD1 has been reported to regulate retroelements within the human genome (455, 456). SAMHD1, along with a handful of other proteins, prevents LINE-1 and LINE-1-related retrotransposition in human cells (455). Naturally occurring mutations in SAMHD1 domains responsible for regulating LINE-1 elements result in Aicardi-Goutières Syndrome, an inflammatory disorder characterized by massive type I IFN production, similar to what I see during infection of MDDCs with ΔVpr virus in the presence of Vpx (455). Vpx causes SAMHD1 levels to drop and remain low for at least 5 days post exposure (457). Under these conditions, reverse transcription occurs much more efficiently, similar to what occurs with retroelements in AGS cells (457). Until now, research on SAMHD1 deficiency-induced IFN has been limited to retroviral transcription, ignoring the potential for integration-mediated sensing (119). With the potential deleterious effects of retroelement transposition, a mechanism to detect cells in which a mass of integration events is occurring would be advantageous for detection and clearance of cells in which retrotransposition is going unchecked (119).

In my studies, all Vpx-encoding viruses tested induced IFN in the absence of Vpr expression. HIV-1, the only virus tested that does not encode a Vpx gene, did not induce any measurable type I IFN in human MDDCs (Miller, unpublished data). HIV-1 is normally poorly infectious in MDDCs, due to high expression of the restriction factor SAMHD1 (179, 180). I believe that the requirement for Vpx in viruses sensed during infection of human MDDCs is not a direct one. Vpx enhances reverse transcription in

these cells, which are normally far less permissive to infection, increasing the number of integration events and thus increasing the likelihood of detection of such events (418). In the absence of Vpx, integration events are rare, so the ability to measure a response to integration in a population of cells diminishes. Work is currently underway to determine if HIV-1 Vpr regulates sensing of integration, similar to SIV_{mac}/SIV_{sm}/HIV-2.

Interestingly, the Vpr from SIV_{sm} is the least effective at counteracting sensing in human MDDCs, suggesting that species-specific evolution of Vpr is necessary for function. SIV_{sm} is the most ancient of the lentiviruses tested and the ancestor that gave rise to both SIV_{mac} and HIV-2 (17). Co-evolution between virus and host restriction is a defining characteristic of long term exposure to viral infection and suggestive of a conserved mechanism for detecting viral infection throughout primate evolution (436, 453). Additionally, published data suggests that SIV_{sm} Vpr has reduced ability to induce G_2 arrest in human cells (208). It is also non-pathogenic in its natural host, whereas SIV_{mac} and HIV-2 infection result in progressive, AIDS-like disease in macaques and humans, respectively (17). It is possible that, similar to G_2 arrest in human cells, SIV_{sm} Vpr is unable to fully antagonize sensing of integration and IFN production in human MDDCs. Alternatively, IFN production from incomplete antagonism of sensing could be a mechanism by which viral infection is controlled in sooty mangabeys.

Collectively, my work in Chapter 3 strongly suggests that Vpr is acting to selectively regulate the DDR to allow for successful integration in the absence of viral sensing. I hypothesize that this function is the result of selective regulation of an ATM- or ATR-triggered DDR response (Fig. 29). ATM, ATR and DNA protein kinase (DNA-

PK) are the three initiator kinases responsible for regulating the induction of DDR pathways (458). ATM and DNA-PK initiate a response to DNA double strand breaks, while ATR initiates a response to DNA single-strand breaks (458). While it is easy to assume a single strand break response would be more relevant to lentiviral integration, proteins involved in NHEJ have been shown to be critical for successful integration (380). Interestingly, cells deficient in NHEJ factors also are incapable of making 2-LTR circles, a form of unintegrated DNA that is competent for gene expression (109, 459). It is possible, as proposed by Li, *et al*, that circularization of unintegrated DNA is important to prevent pro-apoptotic signals that result from detection of linear viral DNA in the nucleus resembling double strand breaks (460). Additionally, it has been shown that Vpr can induce an ATM or ATR response, and induction of ATR independently of Vpr expression results in S/G_2 cell cycle arrest (380, 382, 383, 461). It has been suggested that activation of these pathways may even enhance integration, though that remains somewhat controversial (462–464). ATM and ATR both signal through NF-κB and can result in induction an IFN response (425), similar to what I observe during infection of MDDCs in the absence of SIV_{sm}/SIV_{mac}/HIV-2 Vpr. I propose that Vpr is selectively regulating the ATM/ATR responses to allow for repair of integration but block DNA-damage induced NF-κB-dependent IFN production (Fig. 29). I hypothesize that this occurs through direct, $DCAF^{CRL4}$-mediated degradation of host protein involved in the IFN signaling pathway, but my current work has yet to prove this (Fig. 29). In the absence of Vpr, host machinery will repair integration-induced DNA damage, but antiviral IFNs will be produced which ultimately will restrict viral spread (335, 367). I

hypothesize that in cycling cells, the outcome of integration in the absence of Vpr, instead of IFN, is increased susceptibility to DNA-damage induced apoptosis.

It remains unclear whether similar mechanisms are involved in HIV-1 Vpr enhancement of infection of MDDCs, the ability of diverse Vprs to increase expression of unintegrated DNA and SIV_{sm}/SIV_{mac}/HIV-2 Vpr antagonism of sensing of viral infection in MDDCs. The primary data supporting this hypothesis is from my work with both HIV-1 and SIV_{mac} Vpr mutants. Though work to characterize G_2 arrest capacity of the SIV_{mac} Vpr mutants is in progress, it is interesting that the same mutations that preserve expression of unintegrated DNA (Chapter 2) also prevent IFN production during infection (Chapter 3). Taken a step further, HIV-1 Vpr mutations that block association with the $DCAF^{CRL4}$ complex, which are subsequently unable to induce G_2 arrest are also unable to enhance expression of unintegrated DNA (Chapter 2) and increase expression from the viral LTR during MDDC infection (Chapter 1). Additionally, Vprs that are less efficient at inducing G_2 arrest in human cells, namely SIV_{sm} Vpr, are also less efficient at blocking IFN induction. The commonality could be that Vpr-engagement with DNA-damage machinery, the result of which is induction of G_2 arrest, is necessary for all observed effects of Vpr I have reported in this work. Further work is underway to better understand the mechanisms of all of these processes, which should bring clarity to this question.

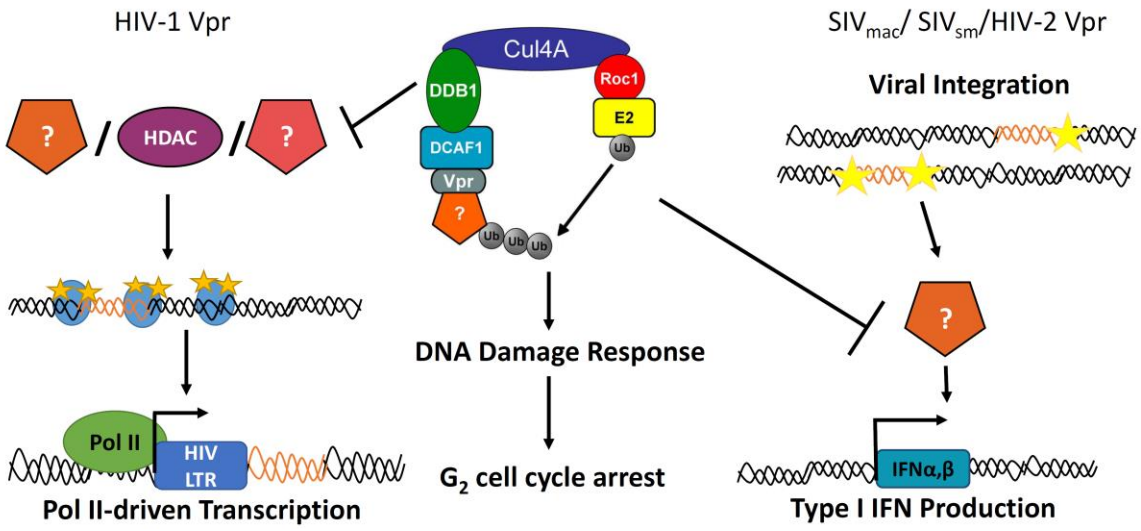


Figure 30. HIV-1 and SIV_{mac}/SIV_{sm}/HIV-2 Vpr induce a DNA damage response via the $DCAF^{CRL4}$ complex in order to enhance infection.

HIV-1, HIV-2, SIV_{sm}, and SIV_{mac} Vprs all function to induce a DNA damage response during infection. This culminates in the G_2 cell cycle arrest that has been the well characterized effect of Vpr expression. HIV-1 does this in order to remodel chromatin in cells with high barriers in to infection, like resting cells or in the presence to a block to integration. HIV-2/SIV_{mac}/SIV_{sm} all encode Vpx, which increases infectivity in cell types that normally restrict HIV-1 infection, like MDDCs and resting $CD4^+$ T cells. Because of this, the virus can readily integrate and becomes susceptible to detection during integration site repair. Vpr from these viruses, in addition to increasing expression of unintegrated DNA when there is a block to integration, blocks DDR-triggered innate immune signaling that would result in type I IFN production. Whether these functions are entirely separate, like depicted above, or more intricately linked through a common Vpr-$DCAF^{CRL4}$ target has yet to be clarified.

At this time, I propose a model by which HIV-1 Vpr and HIV-2/SIV_{sm}/SIV_{mac} Vprs have divergent functions (Fig. 30). HIV-1 Vpr regulates chromatin modification to enhance transcriptional output from both integrated and unintegrated DNA (Fig. 30). It appears that HIV-2/SIV_{sm}/SIV_{mac} Vpr may also perform this role, but due to the presence of Vpx, it is only observed when there is a block to integration (Fig. 30). HIV-2/SIV_{sm}/SIV_{mac} Vprs also act to prevent sensing of integration in MDDCs (Fig. 30). HIV-1 Vpr may also have this function as well, but at this time I have not been able to identify MDDC sensing of viral integration due to the low infectivity of HIV-1 on MDDCs. Together, this work strives to better understand the role of Vpr during infection. Future studies should focus on clarifying the mechanism of action for the observed effects of Vpr. It is my hope that with continued studies, we as a field can fully understand what interactions Vpr has with host cell machinery and why it is maintained throughout primate lentiviral evolution.

METHODS

Common buffers and reagents

Buffers

Phosphate buffered saline (PBS): Tissue culture grade PBS was purchased from Invitrogen (catalog #14190-250). Non-tissue culture PBS was made as a 10x solution in water: 1.36 M sodium chloride, 0.026 M potassium chloride, 0.0176 M monopotassium phosphate, 0.1M sodium phosphate dibasic heptahydrate in nanopore water

PEB: 2mM EDTA, 0.5% BSA in PBS (Invitrogen, catalog #14190-250)

4% paraformaldehyde (PFA) in PBS: Boston bioproducts (catalog #BM-155)

6x DNA loading dye: 0.5 mM EDTA, 30% glycerol, 0.125% bromophenol blue, 0.125% xylene cyanol FF

Western blot running buffer (5x): 1.52% tris base, 7.2% glycine, 0.5% SDS in nanopure water; dilute 1:5 in nanopure water before use

Western blot transfer buffer: 39 mM glycine, 48 mM tris base, 0.037% SDS, 20% methanol in nanopore water

ELISA wash buffer: 0.2% tween-20 (Fisher, catalog #BP337-500) in PBS (Invitrogen, catalog #14190-250)

6x SDS loading dye: 0.3% Bromophenol blue, 3.33% SDS, 1.67% β-mercaptoethanol

Triton X lysis buffer: 50 mM Tris hydrochloride pH 8.0, 150 mM sodium chloride, 5 mM EDTA, 1% Triton X-100, 1 protease inhibitor tablet in 10 mL nanopure water

Media

Unless otherwise indicated, all cells were cultured in either Roswell Park Memorial Institute (RPMI) medium (Invitrogen, catalog #11875-119) with10% heat inactivated fetal bovine serum (FBS) (Invitrogen, catalog #2022-01-30) and 1% penicillin/streptomycin (P/S) (Fisher, catalog #SV30010) (R10) or Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, catalog #11965-118) with 10% FBS and 1% pen/strep (D10). Unless stated otherwise, adherent cells were lifted for passaging or seeding using trypsin-EDTA (0.25%) (Invitrogen, catalog # 25200056).

Plasmids

HIV-1 plasmids

HIV-1 proviral plasmids Lai/YU2 env, Lai/Bal env, Lai-luc Δenv (Env deficient HIV-1 containing a luciferase reporter gene in place of *nef*), Lai-GFP Δenv (Env deficient HIV-1 containing GFP in place of *nef*) and the HA-Vpr expression plasmid have been previously described and were obtained from Dr. Michael Emerman at the Fred Hutchinson Cancer Research Institute (347, 465). Proviral Lai (CXCR4-tropic) clones containing Vpr mutations, F34I, W54R, and H71R and frame-shift mutation in Vpr (ΔVpr) have been described previously and were also obtained from the Dr. Michael Emerman (201, 235, 347, 466). These Vpr mutations were transferred to Lai-YU2 env, Lai-luc Δenv or Lai-GFP Δenv proviral plasmids using Apa I and Sal I restriction sites or the Nhe I and Sal I restriction sites. To create proviral clones encoding Vpr-Q65R mutation, the Apa I – Sal I fragment of Lai-YU2 env was subcloned into pSL1180 cloning vector (Stratagene) and site directed mutagenesis was preformed using a kit

(QuikChange II, Aligent Technologies, catalog #2005235) and the following primers: 5'-GCCATAATAAGAATTCTGCGACAACTGCTGTTTATCCATTTC-3' and 5'-GAAATGGATAAACAGCAGTTGTCGCAGAATTCTTATTATGGC-3'. The mutated fragment was ligated back into Lai-YU2 env, Lai-luc Δenv or Lai-GFP Δenv using Apa I-Sal I restriction sites. The point mutation Vpr-R90K was derived by sub-cloning the Sal I – BamH I fragment of both Lai-luc Δenv into pSL1180 (Stratagene) and via site directed mutagenesis (QuikChange II, Aligent Technologies, catalog #2005235) using the following primers: 5'-CGTTACTCAACAGAGGAGAGCAAAAAATGGAGCCAGTAGATCCTAGAC-3' and 5'-GTCTAGGATCTACTGGCTCCATTTTTTGCTCTCCTCTGTTGAGTAACG-3'. The mutated fragment was ligated back into Lai-luc Δenv and Lai-GFP Δenv using Sal I – BamH I restriction sites. Integrase-null (catalytic mutant, D116N) clones of Lai-luc Δenv -WT and -ΔVpr were created by ligating the Nhe I – Sal I fragment of Lai-luc Δenv into pSL1180 (Stratagene) and via site directed mutagenesis (QuikChange II, Aligent Technologies) with the following primers: 5'-GCCAGTAAAAACAATACATACAAACAATGGCAGCAATTTCACCAG-3' and 5'-CTGGTGAAATTGCTGCCATTGTTTGTATGTATTGTTTTTACTGGC-3'. Clones were confirmed via sequencing (Genewiz) and sub-cloned back into Lai-luc Δenv and Lai-luc Δenv ΔVpr using Nhe I and Sal I restriction sites to create Integrase-deficient (Lai-luc Δenv/D116N and Lai-luc Δenv ΔVpr/D116N.

HIV-2 plasmids

Single cycle HIV-2 proviral plasmids, Rod9-GFP Δenv and Rod9-GFP Δenv ΔVpx, were gifts of Dr. Masahiro Yamashita at the Aaron Diamond AIDS Research Center. Replication competent HIV-2 proviral plasmid, Rod9, was obtained from Dr. Geoffrey Gottlieb, University of Washington (467). The ΔVpr mutation was created by subcloning the Bcl I – Hind III fragment of Rod9 into pSL1180 (Stratagene) and conducting site directed mutagenesis (QuikChange II, Aligent Technologies, catalog #2005235) using the following primers: 5'-CAGGTCTGGTCTAAGGGCTTAAGCACCAACAGAGC-3' and 5'-GCTCTGTTGGTGCTTAAGCCCTTAGACCAGACCTG-3'. ΔVpr mutation was ligated back into Rod9 using Bcl I - Hind III restriction sites or into Rod9-GFP Δenv using Avr II – BsmB I restriction sites.

SIV_{sm} Plasmids

Env-deficient GFP expressing SIV proviral plasmid (SIV_{sm}-GFP Δenv) was a gift from Dr. Welkin Johnson, Boston College. The viral clone contains the *gag-pol* region, as well as *vif, vpx,* and the majority of *vpr* from the E543 isolate of SIV_{sm} and has been previously described (101, 468). I ligated the Sph I – Bcl I fragment of SIV_{sm}-GFP Δenv into pSL1180 (Stratagene) and introduced mutations using site directed mutagenesis (QuikChange II, Aligent Technologies, catalog #2005235) to abrogate Vpr or Vpx expression. Primers to mutate the start codon of Vpr to a stop codon were as follows: 5'-CCTCCAGGACTAGCATAAATAGGCAGAAAGACCTCCAGAAG-3' and 5'-CTTCTGGAGGTCTTTCTGCCTATTTATGCTAGTCCTGGAGG-3' and primers to

introduce a premature stop codon into Vpx were as follows: 5'-CCTGGGAATACTGGCATGAATGAAATGGGAATGTC-3' and 5'-GACATTCCCATTTCATTCATGCCAGTATTCCCAGG-3'. Clones were confirmed via sequencing (Genewiz) and fragments containing mutated Vpr or Vpx sequences were ligated back into SIV_{sm}-GFP Δenv using Sph I – Bcl I restriction sites to generate SIV_{sm}-GFP Δenv ΔVpr or SIV_{sm}-GFP Δenv ΔVpx proviral plasmids.

SIV_{mac} plasmids

Env-deficient, SIV_{mac}-GFP Δenv was obtained from Dr. Welkin Johnson, Boston College, and has been previously described (101, 468). It is a proviral clone based on the SIV_{mac239} isolate. Replication competent proviral plasmids, SIV_{mac239} ΔVpr, SIV_{mac239} ΔVpx, and SIV_{mac239} ΔVpr/Vpx were obtained from the NIH AIDS Research and Reference Reagent Program (contributed by Dr. Ronald C. Desrosiers). Restriction fragments containing the Vpr or Vpx inactivating mutations (ΔVpr, ΔVpx, or ΔVpr/Vpx) were transferred into SIV_{mac}-GFP Δenv using the Kas I and Sph I restriction sites. In order to make mutations to SIV_{mac} Vpr, Sph I and BstB I restriction sites were used to subclone the Vpr portion of SIV_{mac}-GFP Δenv into pSL1180 (Stratagene). Site directed mutagenesis (QuikChange II, Aligent Technologies, catalog #2005235) was conducted using the following primers for VprHRG: 5'-CGAGCGCTCTTCATGGCTTTCGCAGGCGCCTGCATCCACTCC-3' and 5'-GGAGTGGATGCAGGCGCCTGCGAAAGCCATGAAGAGCGCTCG-3', for V21A: 5'-GGATGAATGGGTAGCGGAGGTTCTGGAAG-3' and 5'-

CTTCCAGAACCTCCGCTACCCATTCATCC-3' and for S81A: 5'-GGATGCATCCACGCCAGAATCGGCC-3' and 5'-GGCCGATTCTGGCGTGGATGCATCC-3'. Mutations to ablate *tat* production were made in the same subclone using the following primers: 5'-GACATGGAGACACCCTAGAGGGAGCAGGAGAAC-3' and 5'-GTTCTCCTGCTCCCTCTAGGGTGTCTCCATGTC-3'. The Sph I – BstB I fragment was ligated back into both SIV_{mac}-GFP Δenv and SIV_{mac}-GFP Δenv ΔVpr. To create integrase-null SIV_{mac}-GFP Δenv -WT and -ΔVpr, the BamH I – Bcl I portion of SIV_{mac}-GFP Δenv was cloned into pSL1180 (Stratagene) and site directed mutagenesis (QuikChange II, Aligent Technologies, catalog #2005235) was conducted using the following primers: 5'-GGGACTTGGCAAATGAATTGTACCCATCTAGAGGG-3' and 5'-CCCTCTAGATGGGTACAATTCATTTGCCAAGTCCC-3'. The BamH I – Bcl I fragment containing the mutation in *integrase* was ligated back into both SIV_{mac}-GFP Δenv -WT and -ΔVpr. All mutations were confirmed by sequencing (Genewiz). To create the wild type SIV_{mac} Vpr and Vpr-mutant (VprHRG, -VprV21A, and -VprS81A) expression constructs, Vpr orf was PCR amplified using the following primers, 5'-AGGCAGAATTCGAAGAAAGACCTCCAG-3' and 5'-AGCACTCGAGTTATAGCATGCTTCTAG-3' were Phusion DNA Polymerase (Fisher, catalog #F530L). PCR-amplified fragments were spin column-purified using the QIAquick PCR purification kit (QIAGEN, catalog #28104), digested with EcoR I and Xho I restriction enzymes and ligated into pME18S-Flag eukaryotic expression plasmid in frame with a N-terminal Flag epitope.

SIV3+ Plasmids

SIV3+ plasmid was generously provided by Dr. Andrea Cimarelli, Centre Internationale de Recherche en Infectiologie of Lyon (416). ΔVpr, ΔVpx, and ΔVpr/Vpx versions of SIV3+ were generated by ligating the Ale I – Pac I portions of SIV_{mac}-GFP Δenv -ΔVpr, -ΔVpx, or -ΔVpr/Vpx into SIV3+. Corresponding Ale I – Pac I fragment of SIV_{mac}-GFP Δenv (WT) was also ligated into SIV3+ to create an expression plasmid with identical Vpr and Vpx protein sequences.

Cells and viruses

Cells

TZM-bl, HeLa and HEK 293T cells have been described previously (122, 327, 469). All were cultured in D10. TZM-Bl cells were obtained from NIH AIDS Reference Reagent Program (contributed by Dr. John Kappes). HeLa cells were obtained from the lab of Rachael Fearns (Boston University School of Medicine). HEK293T and THP-1 cells were obtained from ATTC and cultured in D10 and R10 media repectively. HEK293 ISRE-luc cell line was obtained from Dr. Junzhi Wang (National Institute for the Control of Pharmaceutical and Biological Products, China) and Dr. Xuguang Li (University of Ottawa, Canada) and express luciferase under the control of an IFN-inducible promoter carrying the IFN-stimulated response element (369). Cells were cultured in D10 containing 2 µg/mL puromycin (Fisher, catalog #A1113802).

Viral Preparations

All replication competent viruses used in these studies were derived using calcium phosphate mediated transient transfection of HEK 293T cells, as described previously (330). HIV-1, HIV-2, SIV_{mac} or SIV_{sm} vectors were generated from HEK293T cells via co-transfection of the Δenv-viral clone with a CMV-driven VSV-G expression plasmid. HEK 293Ts were seeded the day before transfection at a density of 2.5-3.0x10^5/mL in 6-well tissue culture plates (Fisher, catalog #08-772-1B) or 10 cm tissue culture dishes (Fisher, catalog #08-772E). Transfections were achieved by mixing plasmid DNA (3 µg total per well of a 6-well plate or 12 µg total per 10 cm tissue culture dish) with 0.25 M (anhydrous) calcium chloride solution. A 2x BBS solution (50 mM BES, 280 mM sodium chloride, 1.5 mM disodium phosphate in water, filtered through a 0.45 µM syringe filter (Fisher, catalog # 09-754-21) was added to the DNA-containing calcium chloride solution, which was then vortexed and incubated for a minimum of 15 minutes before addition to HEK293T cells. Cells were washed the following morning once with PBS to remove residual transfection reagent. Cell-free supernatant were harvested at 2 days post transfection.

Virus harvest and concentration

Virus-containing cell supernatants were harvested 2 days post-transfection, filtered through a 0.45 µm filter (Fisher, catalog # 09-754-21) to clear cell debris, and stored at -

80°C until further use. For some experiments, virus particles were concentrated by ultracentrifugation on a 20% sucrose cushion [24,000 rpm at 4˚C for 2 h with a SW28 rotor (Beckman Coulter)] (470). The virus pellets were resuspended in PBS (Invitrogen, catalog #14190-250), aliquoted and stored at -80 °C until use.

Virus Titration- $p24^{gag}$ ELISA

The capsid content of HIV-1 was determined using an in-house $p24^{Gag}$ ELISA. 96-well, clear, flat-bottom, immunolon, nonsterile ELISA plates (Fisher, catalog #12-565-136) were coated with 100 µL HIV-Ig (50 mg/mL, NIH AIDS Research and Reference Reagent Program, catalog #3957) in PBS overnight at 37ºC. Standard and samples were diluted in assay diluent that consists of 10% normal calf serum (NCS) (Invitrogen, catalog #26170043), 0.5% Triton X-100 (Fisher, catalog #BP151-500) in PBS (Invitrogen, catalog #14190-250). Recombinant $p24^{gag}$ protein (Advanced BioScience Laboratories, Inc. Lot #B-53) standard was diluted serially (2-fold dilutions, 4 ng – 0.0625 ng) in assay diluent. Samples or p24gag standards (100 µL volume) were added to HIV Ig-coated, 96-well plate for 2 hours at 37ºC. After incubation, the plate was washed 5 times with ELISA wash buffer (250 µL). Primary anti-$p24^{gag}$ antibody (Clone 183-H12-5C), grown and prepared in lab from an anti-p24 hybridoma cell line (NIH AIDS Research and Reference Reagent Program, catalog #1513) was diluted to the appropriate working concentration in assay diluent, and added to the ELISA plate for 1-3 hours at 37ºC or overnight at 4ºC. After incubation, the plate was washed five times with 250 µL ELISA wash buffer. Secondary goat anti-mouse-HRP (1:70,000 dilution in assay diluent,

Sigma, catalog # A2554-1ML) was added to the ELISA plate for 1 hour at 37ºC. Plate was washed 5 times with 250 µL wash solution and 100 µL of 1:1 mixture of TMB 2-Component Microwell Peroxidase Substrate (Seracare, catalog #KPL 50-76-00) was added for 30 minutes at room temperature. Reaction was stopped with 50 µL 4N sulfuric acid (Fisher, catalog # SA818-1) and analyzed for absorbance at 450 nm.

TZM-bl Titration

The infectious titer was determined via infecting TZM-bl cells, as described previously (331, 469). Briefly, $1x10^4$ TZM-bl cells were seeded per well in a 96 well, flat bottom plate (Fisher) the afternoon before infection. Cells were infected in triplicate with a series of viral dilutions in 100 µL D10 containing polybrene (10 µg/mL final, Fisher, catalog #TR-1003-G) for 48 hours. Cells were fixed with a solution of 0.2% gluteraldehye (Fisher) and 1% formaldehyde (Calbiochem) in PBS for 5 minutes after which cells were washed once with PBS (Invitrogen, catalog #14190-250). Staining solution was freshly made and consisted of 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride, and 0.4 mg/mL X-gal (Fisher, catalog # FP2500040; stock at 40 mg/ml in DMSO) in PBS. TZM-bl cells were stained for infection for a minimum of 1 hour at 37 ºC. The number of blue cells per well, indicating tat-driven transcription of β-galactosidase, were counted for an appropriate dilution (one containing 5-30 positive signals) and reported as infectious particles (IP) per ml.

Infection Readouts

Viral replication in MDDCs and DC- T cell co-cultures was determined by measuring $p24^{Gag}$ content in cell culture supernatants at indicated days post infection by an ELISA (331). Infection of MDDCs using luciferase reporter virus was analyzed using Bright-Glo Luciferase System (Promega, catalog #E2620) (332). Cells were lysed in 50 µL Glo Lysis Buffer (Promega, catalog #E2661). Lysates were either stored at -20ºC or 25 µL was combined with 25 µL BrightGlo luciferase (Promega, catalog #E2620) and chemiluminescence production was measured.

Isolation of primary human immune cells

PBMC Isolation

PBMCs were isolated from de-identified leukopacks obtained from NYBiologics. Briefly, leukocyte mixture was divided between 4 conical tubes and volume brought up to 30 mL with unsupplemented RPMI (Invitrogen, catalog #11875-119). Leukocyte/RPMI mixture was floated on top of 14 mL of Ficoll Paque Plus (Fisher, catalog #45-001-750) and centrifuged at 1400 RPM for 30 minutes. The leukocyte interface of Ficoll and media was harvested and washed four times with unsupplemented RPMI (Invitrogen, catalog #11875-119). Cells were either frozen ($2x10^8$/vial in freezing media) or used for monocyte/$CD4^+$ T cell isolation. Freezing media consisted of 50% FBS (Invitrogen, catalog #2022-01-30), 40% unsupplementedRPMI (Invitrogen, catalog #11875-119) and 10% tissue culture grade DMSO (Sigma, catalog #D2650).

Bead Isolation

Monocytes or $CD4^+$ T cells were positively isolated using antibody coated beads against CD4 (T cells) or CD14 (monocytes) (Miltenyi, catalog #130-045-101 and #130-050-201, respectively). PBMCs were washed once with PEB buffer and resuspended in 100 µL PEB buffer and 10 µL beads per $1x10^7$ cells for 15 minutes at 4ºC. Magnetic isolation columns (Miltenyi, catalog number #130-042-401) were applied to the Miltenyi sorting magnet and rinsed with 3 mL PEB. Cells were washed once with 50 mL PEB. PBMCs were added to magnetic column(s) ($2x10^8$ PBMCs in 2.5 mL PEB per column) and allowed to pass through the column via gravitational flow. Flow through was collected for further isolation if necessary. Columns were washed 3 times with 3 mL PEB. Column(s) were then removed from the magnet and 5 mL PEB was plunged through them to remove bound cells.

Dendritic Cell Differentiation and CD4+ T cell activation

Monocytes were cultured in 3.75 ng/mL IL-4 (Becton Dickinson, catalog # BD554605) and 10 ng/mL GM-CSF (Miltenyi, catalog #130-093-866) in R10 for 6 days for differentiation into MDDCs or in 20 ng/mL M-CSF (Peprotech, catalog #300-25B), 10% human AB serum, heat inactivated (Corning, MT35060CI), 1% P/S in RPMI (Invitrogen, catalog #11875-119) for 5 days for differentiation into macrophage differentiation. $CD4^+$ T cells were stimulated with phytohemagglutinin (PHA) (Invitrogen, catalog #10576015) for 2 days, washed and cultured in 50U/ml IL-2 (NIH AIDS Reference Reagent Program; contributed by Dr. Maurice Gately, Hoffman-Roche) containing R10 media. Purity has

been validated for this technique at over 90% using FACS analysis for MDDC or $CD4^+$ T cell markers.

HIV Inhibitors:

In indicated experiments, cells were pretreated with the reverse transcription inhibitor zidovudine (AZT, 10 µM, NIH AIDS Research and Reference Reagent Program, catalog #3485), reverse transcription inhibitor efavirenz (1 µM in DMSO, NIH AIDS Research and Reference Reagent Program, catalog #4624), integrase inhibitor raltegravir (30 µM or 60 µM in DMSO, NIH AIDS Research and Reference Reagent Program, catalog #11680 or Selleckchem, catalog #50-615-1) or reverse transcription inhibitor tenofovir (10µM or 40µM, NIH AIDS Research and Reference Reagent Program, catalog #10198) for 30 minutes at 37°C prior to infection and maintained for the duration of the cultures. Cells were treated with the protease inhibitor indinavir (1 µM, NIH AIDS Research and Reference Reagent Program, catalog #8145) post-virus exposure.

Signaling Pathway Inhibitors

In indicated experiments, cells were pretreated with the TAK1 inhibitor (5Z)-7-Oxozeaenol (1 µM, Calbiochem, catalog #499610), the NLRP3 inhibitor Glybenclamide (50 µM, InvivoGen, catalog #tlrl-gly), the IκBα inhibitor BAY11-7082 (1 µM, InvivoGen, catalog #11B14-MM), or the TBK1 inhibitor BX795 (0.1 µM, Sigma, catalog #SML0694).

Quantitative Western blotting

Cell Processing

To detect Gag, Env, and Vpr in cell and virus particle lysates, 2-5x10^6 cells were lysed in 50-100 µL Triton X lysis buffer. Lysates were mixed with 6x loading dye and heated at 100 ºC for 5 minutes to denature the proteins. Input was normalized to equivalent amounts of cell-associated Gag content or 100-150 ng $p24^{Gag}$ concentrated virus equivalents (as determined by quantitative ELISA). Concentrated virus was lysed directly in 6x loading dye and heated at 100 ºC for 5 minutes to lyse and denature the proteins.

SDS PAGE Gel

SDS-PAGE gels were poured by hand and composed of 10% (Gag and Env detection) or 12.5% (Gag and Vpr detection) acrylamide (acrylamide 40% solution, bis-acrylamide 37.5:1, Fisher, catalog #BP1410-1). The separating gel consisted of 10% or 12.5% acrylamide (acrylamide 40% solution, bis-acrylamide 37.5:1, Fisher, catalog #BP1410-1), 0.375 M Tris hydrochloride, pH 8.8, 0.012% ammonium persulfate (Fisher, catalog #A682-500), 0.1% TEMED (Fisher, catalog #17919) in water. Stacking gel consisted of 4.5% acrylamide (acrylamide 40% solution, bis-acrylamide 37.5:1, Fisher, catalog #BP1410-1), 0.125 M Tris hydrochloride, pH 6.8, 0.012% ammonium persulfate (Fisher, catalog #A682-500), 0.1% TEMED (Fisher, catalog #17919) in water. Gels were run at

100 V until the loading dye approached the bottom of the gel (~2-4 hours). SDS-PAGE running buffer is described above.

Transfer

Resolved proteins were transferred to Whatman Nitrocellulose Membrane (0.45 µm paper, Fisher, catalog #45-004-002). using a semi-dry transfer apparatus run at 70 mA for a single gel or 150 mA to transfer two gels at once for 1 hour. Transfer buffer is described above.

Antibodies and Detection

Blots were blocked with a 1:1 mix of PBS/Li-Cor Odyssey Blocking Buffer (Fisher, catalog #NC9877369) in PBS (Invitrogen, catalog #14190-250) for 1-2 hours at room temperature or overnight at 4ºC. Blots were probed with rabbit anti-gp120 (a gift from Dr. Nancy Haigwood, Oregon National Primate Research Institute) and mouse anti-$p24^{Gag}$ (clone p24-2, NIH AIDS Research and Reference Reagent Program, catalog #6457), followed by goat anti-mouse IgG DyLight 680 (Pierce, catalog #35518) and goat anti-rabbit IgG DyLight 800 (Pierce, catalog #SA5-10036). To determine Vpr incorporation, a polyclonal rabbit anti-Vpr antibody (clone 1-50, NIH AIDS Research and Reference Reagent Program, catalog #11836) was used followed by goat anti-rabbit IgG DyLight 700. To quantify SIV_{mac} Gag, monoclonal rabbit anti-$p17^{Gag}$ was used (clone KK59, NIH AIDS Research and Reference Reagent Program, catalog #2320). To

detect Flag-SIV_{mac} Vpr and Vpr mutants, monoclonal mouse anti-Flag was used (Sigma, catalog #F3165). Primary antibody was incubated 2 hours at room temperature or overnight at 4ºC and secondary antibody was incubated for 1-3 hours at room temperature. All antibodies were prepared in a 1:1 mixture of PBS/Li-Cor Odyssey Blocking Buffer in PBS (Fisher, catalog #NC9877369). Blots were washed 3 times for 5 minutes with PBS-T (0.05% tween-20 in PBS). The membranes were scanned with an Odyssey scanner (Li-Cor).

Quantitative RT-PCR

For the quantitation of IFNβ and IP-10 mRNA, MDDCs (2-4x10^6 cells) were mock infected or infected with Lai-YU2 or Lai-YU2ΔVpr (MOI = 2). At 48 h post infection, cells were harvested for RNA isolation using RNAeasy (QIAGEN, catalog # 74104) RNA isolation kits and cDNA was synthesized using oligo dT primers and Superscript III RT (Invitrogen, catalog #18080-051). cDNA corresponding to 200 ng of RNA was analyzed by qRT-PCR using SYBR green (Fisher, catalog # FERK0241) to quantify mRNA levels for IFNβ (forward primer: 5'-ATTCTAACTGCAACCTTTCG-3' and reverse primer: 5'-GTTGTAGCTCATGGAAAGAG-3'), IP-10 (forward primer: 5'-TCATTGGTCACCTTTTAGTG-3' and reverse primer: 5'-AAAGCAGTTAGCAAGGAAAG-3') and GAPDH (forward primer: 5'-AGGGATGATGTTCTGGAGAG-3' and reverse primer: 5'-CAAGATCATCAGCAATGCCT-3'). The ΔΔCT value relative to GAPDH in the mock-infected cultures was set to 1, and the data from the infected cultures reported as fold

enhancements. To determine the extent of *de novo* viral transcription, the number of *tat-rev-nef* multiply spliced transcripts was determined by qRT-PCR using SYBR green (Fisher, catalog # FERK0241) as described previously (363), with the following primer set: forward primer, 5'-GCGACGAAGACCTCCTCAG-3' and reverse primer, 5'-GAGGTGGGTTGCTTTGATAGAGA-3'. The data were normalized to GAPDH levels. As a control, MDDCs were treated with AZT (10μM, NIH AIDS Research and Reference Reagent Program) for 30 min prior to infection and drug levels were maintained during the course of infection.

Quantification of viral RT-products and 2-LTR circles

In order to quantify viral RT-products or 2-LTR circles, MDDCs were spinoculated at indicated MOI at 2300 RPM for 1 h and then incubated 2 h at 37ºC. Cells were washed twice with PBS (Invitrogen, catalog #14190-250) and cultured for indicated amount of time before lysis and DNA extraction using the DNeasy kit (QIAGEN, catalog #51304). For HIV-1: MDDCs were infected with either Lai-YU2 or Lai-YU2 ΔVpr. For RT-products, the following primers: 5'-TGTGTGCCCGTCTGTTGTGT-3' and 5'-GAGTCCTGCGTCGAGAGAGC-3' and probe: 5′-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3′ was used. For 2-LTR circles, the following primers: 5'-AACTAGGGAACCCACTGCTTAAG-3' and 5'-TCCACAGATCAAGGATATCTTGTC-3' and probe: 5′-(FAM)-ACACTACTTGAAGCACTCAAGGCAAGCTTT-(TAMRA)-3′ were used.

For SIV_{mac}: MDDCs were infected at MOI = 3 with SIV_{mac}-GFP Δenv/GFP -WT, -ΔVpr, -ΔVpx, or -ΔVpr/Vpr. To quantify RT-products, the following primers: 5'-TTGGGAAACCGAAGCAGG-3' and 5'-TCTCTCACTCTCCTTCAAGTCCCT-3' and probe: 5'-(FAM)-AAATCCCTAGCAGATTGGCGCCTGAA-(TAMRA)-3' was used. Maxima Probe 2X qPCR master mix (Fisher, catalog #K0261) was used with primer/probes to quantify DNA products. Cycle conditions were as follows: 95ºC, 10 min initial denaturation, followed by 40 cycles as follows: denature at 95ºC, 15 sec; anneal at 60ºC, 30 sec; extension at 72ºC, 30 sec.

Quantification of viral integration

To determine the number of proviral integrants, MDDCs ($3x10^6$ cells) were infected with virus (MOI = 3) for 2 h at 37˚C, washed with PBS (Invitrogen, catalog #14190-250) twice and cultured for 72 h before cells were lysed for DNA extraction with a DNeasy kit (QIAGEN, catalog #51304). As a background control, MDDCs were treated with 10 µM AZT (NIH AIDS Research and Reference Reagent Program) for at least 30 min prior to infection. Quantitative *Alu*-PCR was performed using 20 ng of DNA with the following primer sets, as described previously (371). For the first step, the following primers were used: *Alu*-forward 5′-GCCTCCCAAACTGCTGGGATTACAG-3′ and Gag-reverse 5'-GCTCTCGCACCCATCTCTCTCC-3'. For the second step, the following primers were used: R-U5-F: 5′-GCCTCAATAAAGCTTGCCTTGA-3′ and R-U5-R: 5′-TCCACACTGACTAAAAGGGTCTGA-3′ with the following probe: R-U5-Probe: 5′-

FAM-CCAGAGTCACACAACAGACG-TAMRA-3′. The data were normalized to a standard curve generated from infected HEK293 cell DNA (370, 371).

Splicing Assay

The assay for spliced viral RNAs has been described in detail and was preformed in conjunction with Ann Emery in the Swanstrom Lab at University of North Carolina, Chapel Hill (374). Briefly, cDNA primers with an internal random sequence block (Primer ID; (373)) were designed to be within the *env* intron, to measure the 4 kb size class of spliced viral RNAs, or spanning the D4/A7 splice junction to measure the 1.8 kb size class. The reverse primer for the 4-kb size class was: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**NNNNNNNNNNNNNN**GTACGCTAATACTTGTAAAGATTGCAGTACATGTACTACTT-3' and the reverse primer for the 1.8 kb size class was: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**NNNNNNNNNNNNNN**CAGTCTGAGCTGGGAGGTGGGTTGC-3'. Whole cell RNA from infected cells was purified and used in a cDNA reaction. After removal of the cDNA primers, PCR was carried out using a downstream primer encoded in the cDNA primer tail and a forward primer placed just upstream of the D1 major donor site in the 5' noncoding region, 5'-GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG**NNNN**TGCTGAAGCGCGCACGGCAAG-3'. PCR products were sequenced using the MiSeq platform, and sequence reads with the same Primer ID were collapsed into a single read (to correct for skewing during PCR since each unique Primer ID tag represents a separate viral mRNA

template). Data were processed using customized scripts that are available on request. The number of unique Primer IDs for each spliced product was used to determine the relative level of splicing from each splice donor to each splice acceptor in the viral genome with the exception of splicing events to the *nef* splice acceptor A7.

IP-10 measurements

Secreted IP-10 in MDDC culture supernatants was measured using a commercially available ELISA kit (Becton Dickinson, catalog # 550926), according to directions provided. Briefly, 96-well, clear, flat-bottom, immunolon, nonsterile plates (Fisher, catalog #12-565-136) were coated overnight at 4ºC with 100 µL of Capture Antibody in 0.1 Sodium Carbonate, pH 9.5. Wash buffer (0.05% Tween-20 in PBS) was prepared fresh each use. Plate(s) were washed 3 times with 250 µL wash buffer before application of samples/standards. Standards ranged from 500-7.81 pg/mL and were made fresh from frozen stocks for each application in assay diluent. Assay diluent, composed of 10% normal calf serum (NCS) (Invitrogen, catalog #26170043), 0.25% Triton X-100 (Fisher, catalog #BP151-500) in PBS (Invitrogen, catalog #14190-250), for the dilution of samples and standards was made in bulk and stored at 4°C for up to 6 months. Plates were incubated at room temperature with 100 µL of samples and standard for 2 hours and then washed five times with 250 µL wash buffer. Detection antibody was mixed with secondary anti-detection-HRP in 10% FBS/PBS and added in 100 µL for 1 hour at room temperature. Plate was washed 7 times with 250 µL wash solution and 100 µL 1:1 mixture of TMB 2-Component Microwell Peroxidase Substrate (Seracare, catalog #KPL

50-76-00) was added for 30 minutes at room temperature. Reaction was stopped with 50 µL 4N sulfuric acid (Fisher, catalog # SA818-1) and analyzed for absorbance at 450 nm.

IFN Bioassay

Secreted levels of bioactive type I IFN in infected MDDC supernatants was measured using a HEK293 ISRE-luc cell line which expresses luciferase under the control of an IFN-inducible promoter carrying the IFN-stimulated response element (369). Briefly, HEK293 ISRE-luc cells ($8x10^4$) were incubated with 20 µL or 50 µL MDDC culture supernatants for 21 hours. Cells were lysed in 50 µL BrightGlo lysis buffer (Promega, catalog #E2661). Luciferase activity in the cell lysates analyzed with Bright-Glo Luciferase System (Promega, catalog #E2620), as described above by combining 25 µL and 25 µL BrightGlo luciferse. Serial dilutions of recombinant interferon alpha ranging from 200-0.39 units/ml (PBL Interferon Source, catalog #11100-1) were added to cells in 50 µL in each experiment for generating a standard curve.

FACS

Cells were analyzed using either LSRII or FACSCalibur (Becton Dickinson) instruments with the help of Boston University Flow Cytometry Core who provided instrumentation and technical support.

Extracellular FACS staining

Cells were pre-chilled at 4ºC for 30 minutes prior to staining. Antibody solution was added at indicated dilution in 2% NCS (Invitrogen, catalog #26170043) in PBS (catalog #14190-250) for 30 minutes at 4ºC. Cells were washed once with 2% NCS/PBS and fixed in 4% PFA (Boston bioproducts, catalog #BM-155) for at least 30 minutes at 4ºC.

Intracellular FACS staining

Intracellular FACS staining was performed for cell-internal proteins post-fixation. Briefly, cells were permeabilized using Perm/Wash Buffer (1x in water, Becton Dickinson, catalog #554723) for at least 15 minutes at room temperature. Cells were stained at indicated dilution of antibody in Perm/Wash buffer for 30 minutes at 4ºC. Cells were washed once with Perm/Wash and resuspended in 2% NCS/PBS.

Antibodies

Intracellular fluorescence-activated cell sorter (FACS) analysis for $p24^{Gag}$ was done using FITC-conjugated anti-$p24^{Gag}$ monoclonal antibody (KC57; Beckman Coulter, catalog # 6604665) at a 1:25 dilution in 25 µL. Surface staining for CD11c was done using APC-conjugated anti-CD11c (Clone B-ly6, Becton Dickinson, catalog # 559877) using 3 µL/sample in 50 µL staining buffer. Surface staining for DC-SIGN (CD209) was conducted using FITC-conjugated anti-CD209 (Clone DCN46, Becton Dickinson, catalog # BD551264) using 3 µL/sample in 50 µL staining buffer.

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CURRICULUM VITAE

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EDUCATION

2011-2017	Doctor of Philosophy, Pathology and Laboratory Medicine, Focus in Immunology. Boston University School of Medicine, Boston, MA. Dissertation Research conducted with Dr. Suryaram Gummuluru, Ph.D. Dissertation: Role of viral protein R in infection of human dendritic cells by primate lentiviruses.
2007-2011	Bachelor of Science, Biochemistry with Honors Gonzaga University, Spokane, WA. Senior Thesis: Synthesis of Substituted Pyrimidines as Inhibitors of Beta-Carbonic Anhydrase Honors Thesis: Consumed by the Consumption: A look at the social aspects of tuberculosis

GRANTS AND FUNDING

2012-2014	Competitive Appointment to NIH-T32 Immunology Training Program. Boston University School of Medicine, Boston, MA.

PUBLICATIONS

Miller, C.; Akiyama, H.; Agosto, L.; Emery, A.; Ettinger, C.; Swanstrom, R.; Henderson, A.; Gummuluru, S. Virion associated Vpr alleviates a post-integration block to HIV-1 infection of dendritic cells. *Journal of Virology.* Accepted. Published online ahead of print, April 2017.

Kijewski, S.D.G.; Akiyama, H.; Feizpour, A.; **Miller, C.**; Ramirez, N.; Reinhard, B.M.; Gummuluru, S. Access of HIV-2 to CD169-Dependent Dendritic Cell-Mediated Trans Infection. *Virology.* August 2016.

Feizpour, A., Yu, X., Akiyama, H., **Miller, C**., Edmans, E., Gummuluru, S. and Reinhard, B. Quantifying Lipid Contents in Enveloped Virus Particles with Plasmonic Nanoparticles. *Small.* Nov. 2014.

Akiyama, H., **Miller, C**. Patel, H., Hatch, S.C., Archer, J., Ramirez, N., and Gummuluru, S. Virus particle release from glycosphingolipid-enriched microdomains is essential for dendritic cell-mediated capture and transfer of HIV-1 and henipaviruses. *Journal of Virology.* May 2014.

ORAL PRESENTATIONS

Miller, C., Akiyama, H., Nodder, S., Ettinger, C., and Gummuluru, S. Vpr enhances expression from unintegrated DNA in human cells. Accepted for oral presentation May 2017. Cold Spring Harbor Conference on Retroviruses, Cold Spring Harbor, NY.

Miller, C., Akiyama, H., Agosto, L., Emery, E., Swanstrom, R., Henderson, A., and Gummuluru, S. Vpr enhances HIV-1 infection of dendritic cells by alleviating a post-integration block to virus replication. May 2016. Cold Spring Harbor Conference on Retroviruses, Cold Spring Harbor, NY.

Miller, C., Akiyama, H., Agosto, L., Emery, E., Swanstrom, R., Henderson, A., and Gummuluru, S. Vpr enhances HIV-1 infection of dendritic cells by alleviating a post-integration block to virus replication. May 2016. Henry I Russek Student Achievement Day, Boston University School of Medicine. Boston, MA.

Miller, C., Akiyama, H., Ramirez, N., Kijewski, S., and Gummuluru, S. HIV-1 Vpr is necessary for gp120 production and incorporation into myeloid cell-derived progeny virions. May 2014. Cold Spring Harbor Conference on Retroviruses, Cold Spring Harbor, NY.

Miller, C., Carlson, M., Knapp, N., Sachet, M., Langfield, A., Holden, J., Cronk, J., Watson, J., and Warren, S. Lead generation for potential allosteric inhibitors of beta-carbonic anhydrase and synthesis of substituted pyrimidine inhibitors. Nov. 2010. M J Murdock Charitable Trust Research Conference. Linfield College. McMinnville, OR.

POSTER PRESENTATIONS

Miller, C; Akiyama, H; Agosto, L; Henderson, A; and Gummuluru, S. Essential role for Vpr in productive infection of dendritic cells by HIV-1. February 2016. Conference on Retroviruses and Opportunistic Infections. Boston, MA.

Miller, C; Akiyama, H; Kijewski, S; Agosto, L; Henderson, A; and Gummuluru, S. The role of viral protein R in HIV-1 infection of dendritic cells. May 2015. Henry I Russek Student Achievement Day, Boston University School of Medicine. Boston, MA.

Miller, C.; Carlson, M.; Sachet, M.; Cronk, J.; Watson, J. and Warren, S. Synthesis of Substituted Pyrimidines as Inhibitors of Beta Carbonic Anhydrase in *Escherichia coli.* Nov. 2010. M J Murdock Charitable Trust Research Conference, Linfield College. McMinnville, OR.

Knapp, N.; Holden, J.; Carlson, M..; Warren, S.; Sachet, M.; **Miller, C**.; Watson J.; Langfield, A.; Cronk, J. Lead generation for potential allosteric inhibitors of beta-carbonic anhydrase. June 2010. Joint 65th Northwest and 22nd Rocky Mountain Regional Meeting of the American Chemical Society. Pullman, WA.

HONORS AND AWARDS

2017	Trainee Support Award. Boston/Providence Center for AIDS Research, Boston, MA.
2016	Trainee Support Award. Boston/Providence Center for AIDS Research, Boston, MA.
2016	Graduate Medical Sciences Travel Award. Boston University School of Medicine, Boston, MA.
2016	Henry I Russek Student Achievement Award, First Prize. Boston University School of Medicine, Boston, MA.
2016	Young Investigator's Scholarship. Conference on Retroviruses and Opportunistic Infections, Boston, MA.
2015	Henry I Russek Student Achievement Award, Second Prize. Boston University School of Medicine, Boston, MA.
2011	Cum Laude. Gonzaga University, Spokane, WA.
2007-2011	Competitive appointment to the Gonzaga Honors Program. Gonzaga University, Spokane, WA.

TEACHING EXPERIENCE

Fall 2015	Group Discussion Leader for Module III in the Foundations of Biomedical Science class for the Graduate Medical Science program at Boston University (FS703).
Spring 2015	Tutor, Quiz Proctor and Discussion Group Leader for Immunology for the Masters in Medical Sciences and the Doctor of Medicine programs at Boston University (PA510, MS131).
Spring 2014	Lecturer for Overview of the Immune System Lectures I-III in the Basic Medical Sciences class for the Boston University Physician Assistant program (PS701).

ORGANIZATIONAL EXPERIENCE

2014-2017 Coordinator of leukocyte unit ordering and receiving for 10+ research labs at Boston University School of Medicine