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Mutations in the *vpu* and *env* Genes of HIV-1 Can Adversely Impact Infectivity: A Dissertation

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MUTATIONS IN THE *vpu* AND *env* GENES OF HIV-1 CAN ADVERSELY IMPACT

INFECTIVITY

A Dissertation Presented by

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Dedication

This thesis is dedicated to my Dad, Tobey C. Richards.

I miss you.

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I would like to thank:

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Abstract

The Human Immunodeficiency Virus (HIV) is able to infect CD4$^+$ T cells as well as macrophages. Macrophage-tropism has been linked to determinants in the envelope of HIV. These determinants allow envelopes to exploit low levels of CD4 for infection. Macrophages are an important reservoir of virus, especially during chronic infection, and are likely responsible for the bulk of virus produced after CD4$^+$ T cells have declined. Viral factors that may impact the ability to infect macrophages are worth studying because this cell type is so important in infection.

It was previously reported that the macrophage-tropic primary isolate AD8 was vpu-independent. The molecular clone YU-2, derived from brain tissue without culture, was also reported to be macrophage-tropic despite having a mutation in the *vpu* start codon. It was therefore possible that vpu-independent envelopes could evolve *in vivo*. To examine this possibility, I constructed chimeras containing wild type or defective *vpu* start codons, and gp160 sequences from AD8, YU-2 or SF162 (a vpu-dependent control). I also used full length AD8 and YU-2 with wild type or defective *vpu* start codons. I infected macrophages with equal amounts of virus, and measured viral output over two weeks. Viruses with defective *vpu* start codons were released to lower levels compared to their wild type *vpu* counterparts. In contrast to previous reports, the AD8 envelope is not vpu-independent for replication in macrophages. The YU-2 envelope is also not vpu-independent.

Macrophage-tropic envelopes from late stages of infection can be sensitive to antibodies that bind the CD4 binding site on gp120, implying that macrophage-tropic

envelopes have more exposed CD4 binding sites. Neutralizing antibodies may act as modulators of macrophage-tropism over the course of infection. Using chimeras containing gp120 sequences derived from the PBMC of four HIV⁺ patients, I examined the capacity for envelopes to infect macrophages. Three patients (MM1, 4, and 8) had macrophage-tropic envelopes before and after developing autologous neutralizing antibodies. Three patients (MM1, 4, and 23) developed heterologous antibodies against IIIB, an easily neutralized T-cell line adapted strain of HIV-1. This data indicates that macrophage-tropism in these patients is not modulated by the presence of neutralizing antibodies.

The macrophage-tropism of envelopes tends to segregate depending on the tissue origin of the virus. Envelopes from two separate tissues from the same patient exhibit very different infectivity characteristics. The B33 envelope, from brain tissue, is very infectious and is macrophage-tropic, while the LN40 envelope, from lymph node tissue, is weakly infectious and is not macrophage-tropic. Replacing the entire gp41 of LN40 with that of B33 restores some infectivity to LN40. The cytoplasmic domain of gp41 contains many motifs important for assembly and infectivity. To examine which motifs are responsible for the weak infectivity of LN40, I made chimeras of gp41, as well as point mutations in gp41. The LN40 chimera containing the entire gp41 of B33 restored the most infectivity. Point mutations in the palmitoylation site, Pr55^{gag} binding region, and dileucine motif at the C-terminus also restored infectivity when combined. Determinants in the gp41 cytoplasmic domain are responsible for the weak infectivity of

LN40; however, it is possible that there are contributing determinants in gp120, such as the ability to use low levels of CD4.

Here, I examined how changes in the *vpu* and *env* genes of HIV-1 can impact infectivity, especially infectivity of macrophages. Changes that adversely impact the virus' ability to infect macrophages may also impact the overall course of disease. However, the data here show that retaining the ability to infect, and replicate in, macrophages give HIV an advantage. I speculate that retaining the ability to infect macrophages gives the virus a reservoir for later in disease, when CD4$^+$ T cells have been depleted, as well as way of avoiding neutralizing antibodies. This work further defines the importance of macrophages in HIV-1 infectivity and disease.

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Preface

Data in Chapter III originally appeared in "Effects of *vpu* start codon mutations on replication in macrophages", KH Richards and PR Clapham, *Journal of General Virology*, 2007 Oct;88(Pt 10):2780-92.

Data in Chapter IV on construction of HXBc2/ patient chimeras and neautralization assays originally appeared in "Development of the antibody response in acute HIV-1 infection". M. Aasa-Champan *et al*, *AIDS*, 2004 Feb 20;18(3):371-81. Used with permission.

Chapter I: An Introduction to AIDS and HIV

A Short History of HIV and AIDS

In 1981, a US physician reported several cases of homosexual men with immunological dysfunction [112]. These patients' illnesses were characterized by CD4$^+$ T cell decline, opportunistic infections and rare cancers. This collection of symptoms was named Acquired Immune Deficiency Syndrome (AIDS).

In 1983, Barré-Sinoussi *et al*. reported the isolation of a new human retrovirus from patients diagnosed with AIDS [17]. This virus was eventually named Human Immunodeficiency Virus (HIV). Two types of HIV have been discovered: HIV-1 and HIV-2. HIV-2 was first reported in 1986 in West African subjects [50].

An estimated 25 million people worldwide have died of AIDS since the beginning of the epidemic 25 years ago. In 2007, 2.5 million people were newly infected with HIV. The majority of people living with HIV is in sub-Saharan Africa; however numbers are rising in Asia and North America. Worldwide, 33.2 million people are currently living with HIV [258][209][271].

Phylogeny, Origins and Epidemiology of HIV

HIV belongs to the Retroviridae family, genus lentivirus [16]. The retroviridae family is characterized by the ability to convert the viral genomic single-stranded RNA to double stranded DNA via the enzyme reverse transcriptase. Lentiviruses typically cause slow disease progression and primarily target lymphocytes and macrophages [244].

HIV-1 and HIV-2 have distinct genomic differences, discussed in more detail below. Simian Immunodeficiency virus (SIV) was discovered in primates, but usually does not cause AIDS-like illness in their natural hosts [64].

HIV-1 is divided into 3 groups: Main (M), Outlier (O), and Non-M, Non-O (N). The M group is divided further into 9 subtypes: A, B, C, D, F, G, H, J, and K [181][62]. The M group also contains a number of circulating recombinant forms (CRFs), which appear to be recombinants of the 9 subtypes [167].

HIV-1 and HIV-2 likely arose from separate zoonosis events. HIV-2 is genetically closely related to SIV from sooty mangabey monkeys (SIV_{smm}) [124][103]. HIV-1 groups M and N are genetically related to SIV from chimpanzees (SIV_{cpz}) [102][56]. SIV_{cpz} seems to be a combination of SIV from greater spot-nosed monkeys (SIV_{gsn}) and SIV from red-capped mangabeys (SIV_{rcm}) [11]. Group O viruses are genetically related to SIV from gorillas (SIV_{gor}) [261].

Subtype A viruses are prevalent in Africa, and can be found in North Africa, the Middle East, Eastern Europe, Central and South Asia. Subtype B viruses are prevalent in North America, the Caribbean, South America, Western Europe and Australia. Subtype B can also be found in Eastern Europe and Asia. Subtype C viruses are prevalent in South Asia, and can be found in Africa. Over half of all new infections worldwide have been identified as subtype C. CRFs can be found in North Africa and the Middle East (CRF AG), East Asia (CRF BC), and South Asia (CRF AE). Most CRFs can also be found in Africa. Subtypes D, F, G, H, J, and K can also be found in Africa, along with viruses belonging to groups O, and N [136].

Genome Organization

HIV-1, HIV-2, and SIV are complex retroviruses. Their genomes encode the prototypical *gag*, *pol*, and *env* genes, along with several other genes [271][209][120]. These additional genes are *vif*, *vpr*, *rev*, *tat*, and *nef* [119][52]. HIV-1 also contains the *vpu* gene [53][247]. HIV-2 and SIV contain the *vpx* gene [137]. *gag*, *pol*, and *env* are structural genes; they are translated into polyproteins that must be cleaved by either viral or cellular proteases before maturation.

The remaining proteins are translated from spliced mRNAs. *Tat*, *rev*, and *nef* are the first genes transcribed and expressed during viral replication, followed by *vif*, *vpu*, *vpr*, *env*, *gag*, and *gag-pol*. *Tat* and *rev* are regulatory genes; *vif*, *vpr*, *vpu*, and *nef* are accessory genes [119][84].

The entire viral genome is flanked by Long Terminal Repeats, or LTRs. These areas are not translated, but contain many *cis*-acting elements that help direct production of viral proteins. These elements include the transactivation response region (TAR) stem, the Poly A site, the primer binding sequences (pbs) stem, and the core encapsidation signal (Ψ). These elements regulate the elongation of viral RNA, splicing, RNA dimerization, packaging and reverse transcription of the viral genome [19][179][180][212][28][108][259]. The topography of the HIV-2 LTRs is different from that of HIV-1 [177][98][157].

Schematics of the genome organizations of HIV-1 and HIV-2 are in Fig. 1.1
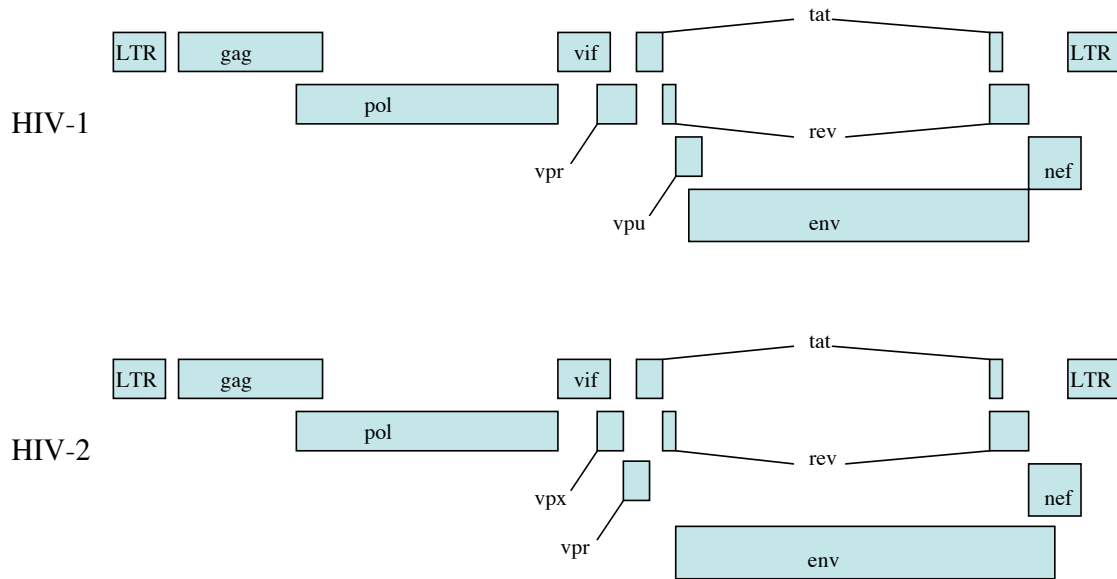
Fig. 1.1 Schematics of the genome organizations of HIV-1 and HIV-2.

The HIV-1 Life Cycle

Productive infection of cells by HIV-1 occurs in a stepwise process [84]. This process is outlined below, and in Fig. 1.2.

Step 1: Viral entry into target cell via CD4 and a coreceptor (either CCR5 or CXCR4). Viral and plasma membranes fuse.

Step 2: Partial uncoating of the virus capsid, and reverse transcription of the viral genome.

Step 3: Transport of the pre-integration complex (PIC) to the nucleus.

Step 4: Integration of the viral DNA into the host cell chromosomal DNA.

Step 5: Transcription of the viral genome into mRNA; viral mRNA transported to the cytoplasm.

Step 6: Envelope synthesized in the endoplasmic reticulum; gag and gag-pol synthesized in the cytoplasm.

Step 7: Viral proteins transported to cell's plasma membrane.

Step 8: Condensation of gag, gag-pol, and viral genomic RNA at the cell membrane (surface in most cell types; however, in macrophages, this step may occur at late endosomal membranes).

Step 9: Virus particles released from cell membrane.

Step 10: Processing of gag to create mature virus particles.

Steps that are important to this thesis will be discussed in more detail in the appropriate sections.

(1) Virus binds CD4 and CCR5; viral and plasma membranes fuse

(2) Partial uncoating of virus capsid; reverse transcription

(3) Transport of PIC to nucleus

(4) Integration into chromosome

(5) Transcription into mRNA; mRNA transported to cytoplasm

(6) Envelope synthesized in ER; gag, gag-pol synthesized in cytoplasm

mRNAs

proteins

Virion RNA   *Viral proteins*

(7) Viral proteins and genomic RNA transported to cell surface

(8) Condensation of proteins and RNA

(9) Virus particles bud from cell membranes

(10) Virus particles mature and become infectious

Fig. 1.2 The HIV-1 life cycle. Numbers above correspond to steps described in the text.

Pathogenesis and Tropism of HIV-1

HIV can be transmitted in three ways: 1) sexual contact with an infected individual, 2) contact with the blood of an infected individual through a wound (open cuts, intravenous needle use), and 3) vertically transmitted from infected mother to child (during childbirth or breastfeeding). Once inside the body, HIV-1 infects CD4$^+$ T helper cells, macrophages, and some populations of dendritic cells [292][222]. Dendritic cells in mucosal membranes can carry HIV to the lymph nodes, where the virus can be passed to circulating T cells [106][151]. Immune cells in the gut-associated lymphoid tissue (GALT) are rapidly depleted in the first weeks of infection [35][265][33]. A gradual decline in the numbers of CD4$^+$ cells in the periphery is seen over the course of disease. HIV infection in the gut results in leakage of bacterial products into the blood, contributing to general activation of the immune system [34]. Chronic activation of the immune system provides activated CD4$^+$ T-cells as targets for HIV, which leads to activation induced cell death (AICD) [49][10]. These situations likely cause the decline of CD4$^+$ cells seen over the course of infection. Death from AIDS, or a variety of opportunistic infections, usually occurs 10 years post infection, unless antiretroviral treatment is used (see the section **Treatment of HIV**).

Many infected individuals also develop HIV Associated Dementia (HAD) late in disease. The mechanisms of HIV entry into the central nervous system are unclear, although evidence points to infected monocytes crossing the blood-brain barrier as being the culprits [47][110][162][240].

Upon acute infection with HIV-1, there is a spike in viral replication, which then levels out for a number of years before increasing again later in disease [74]. HIV-

specific antibodies can be detected 4-8 weeks after infection (Fig 1.3) [1][200][205]. Once these antibodies are detected, the individual is considered seroconverted.

As was mentioned above, HIV infects cells using CD4 (a member of the immunoglobulin superfamily) present on T-cells and macrophages as its main receptor [144][182][63]. HIV also requires a coreceptor to gain entry to cells. HIV uses either CCR5, or CXCR4 as the coreceptor [48][71][73][90]. Isolates that are only able to use CCR5 are called R5-tropic; isolates that use only CXCR4 are called X4-tropic. Isolates able to use both CCR5 and CXCR4 are called dual-tropic, or R5X4, viruses. Most HIVs are R5-tropic, with up to 50% switching to X4-tropic during late infection. The switch from R5-tropism to X4-tropism is associated with accelerated progression to AIDS and death [54][253]. Scarlatti *et al*. followed children with progressive HIV-1 infection. Viruses isolated from the asymptomatic stages of infection generally were R5-tropic; viruses isolated from the symptomatic stages were X4-tropic. Children in which the switch to X4-tropism occurred early had poor prognoses [220].

In cell culture, some viral isolates are CD4-independent, meaning that they do not need to use CD4 to enter cells; they are able to use only a coreceptor. The *in vivo* significance of this observation is unclear, as these CD4-independent viruses tend to also be sensitive to neutralizing antibodies [153][126][148][82].

Macrophages are important for the establishment, persistence, and pathogenesis of HIV infection. Macrophages infected with HIV-1 are resistant to cytopathic effects, allowing them to become important reservoirs of virus [266]. Macrophages in the submucosal tissues may be the initial targets for HIV-1, allowing for dissemination of

virus throughout the immune system [266]. Perivascular macrophages are a likely reservoir of HIV-1 in the brain, leading to infection of microglia of monocyte/ macrophage lineage [91] [109] [251] [281] [57]. Infected macrophages may also play a role in viral escape from antiretroviral therapy.

Because macrophages express low levels of CD4 and CCR5, a virus must be able to utilize low levels of these receptors in order to infect. Primary X4-tropic viruses tend to infect macrophages less efficiently compared to R5-tropic viruses [72]. Non-macrophage-tropic R5 viruses are prevalent in immune tissue and blood, while R5 macrophage-tropic viruses are readily found in brain tissue, especially in HIV[+] patients with dementia [203][111]. R5 viruses from late stages of disease have been reported to be more macrophage-tropic [113][162][257].

In vivo, HIV-1 targets activated T-cells, as well as resting/non-dividing T-cells. During the acute phase of infection, HIV-1 mainly targets activated T-cells, which provides the bulk of HIV-1 particles [292]. During the chronic phase, resting T-cells and macrophages are responsible for the persistence of the virus in the immune system, acting as viral reservoirs [49]. They are also responsible for dissemination to the central nervous system and the GI tract [47][110][162][240][135][195].
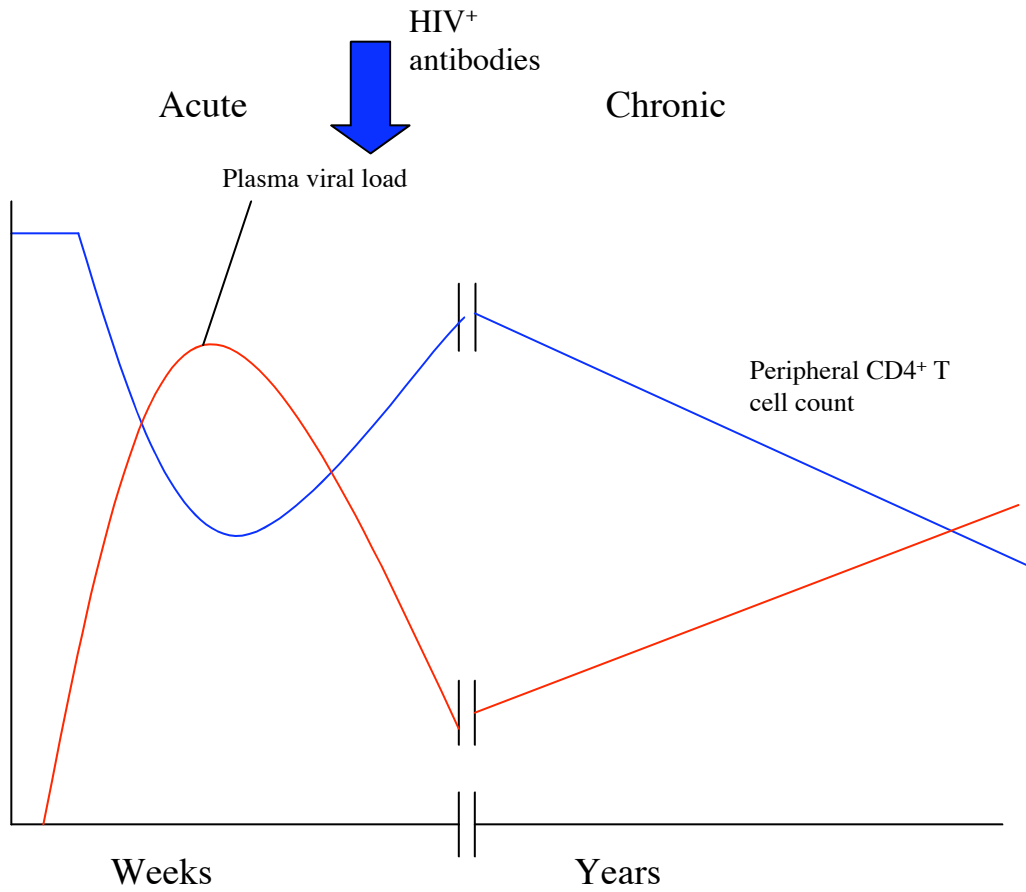
Fig. 1.3 Course of HIV infection. Acute infection is characterized by a drop, then rise, in CD4$^+$ T-cells, and a spike in viral load over several weeks. Chronic infection is characterized by a slow decline of CD4$^+$ T-cells, and a slow increase in viral load over several years.

The Genes of HIV-1

As mentioned above, HIV-1 is a complex retrovirus. Its 9 genes are classified into three types: 1) structural genes, 2) regulatory genes, and 3) accessory genes. Structural genes encode proteins that are responsible for structural elements of the virus particle. These genes include *env*, *gag*, and *pol*. Regulatory genes are responsible for regulating viral gene expression. These genes include *tat* and *rev*. Accessory genes were once considered non-essential for viral replication in cell culture. However, some of these genes have been found to be important in cultures of some cell types, as well as *in vivo*. The accessory genes are *nef*, *vif*, *vpu*, and *vpr*.

These genes are summarized in Table 1.1. The *env* and *vpu* genes will be discussed in greater detail in sections **The Envelope of HIV-1** and **The Vpu Gene and Protein of HIV-1**, respectively.

Table 1.1 The Genes of HIV-1

Gene	Protein Characteristics	Primary Functions	Location in the virus particle	References
Tat	Nuclear protein	Increases the steady-state levels of viral RNA by forming a more processive RNA pol II transcription complex	Not applicable	[65] [92] [294] [61]
Rev	Nucleolar phosphoprotein containing a nuclear localization signal (NLS)	Exports HIV mRNAs out of the nucleus into the cytoplasm for translation; does this by binding Rev Response Element (RRE) on viral pre-mRNAs.	Not applicable	[18] [89] [171] [172] [173]
Nef	Membrane-associated phosphoprotein	Downregulation of surface molecules CD4, MHC-I, MHC-II, CD3 T-cell receptor complex, CD28; enhancement of virus infectivity, modulation of cellular activation pathways	Within capsid	[20] [67] [104] [115] [143] [231] [223] [249] [250]
Vif	Protein containing many domains for interacting with cellular proteins; forms homodimers	Interacts with APOBEC3G (restricts viral replication by converting cytosines to uracils during reverse transcription); sends APOBEC3G to the ubiquitin-proteosome pathway for degradation	Not applicable	[55] [118] [155] [170] [174] [176] [183] [235] [236] [289] [291]
Vpu	Membrane-associated protein	Downregulated CD4 from the ER, enhances virus release	Not applicable	[229] [279] See **The Vpu Protein of HIV-1**
Vpr	Small protein	Stimulate LTR-driven gene expression, promote transport of PIC to nucleus, arrest infected cells in G2, induce apoptosis, modulate mutation rates during reverse transcription	Within capsid	[52] [290] [12] [94] [121] [142] [168]

Table 1.1 con't The Genes of HIV-1

Env	Glycoprotein; initially synthesized as a polyprotein gp160	Interaction with CD4 and CCR5/CXCR4 to gain entry into target cells	In surface plasma membrane, as trimer spikes	[6] [295] [276] See **The Envelope of HIV-1**
Gag	Initially synthesized as a polyprotein Pr55^{Gag}; cleaved by viral protease (PR) during budding from the cell surface into matrix (MA), capsid (CA), nucleocapsid (NC) and p6.	Structural protein MA: targets Pr55^{Gag} to cell membranes CA: forms protective core around viral genomic RNA; promotes virus assembly NC: encapsidates viral genomic RNA into virions p6: recruits Tsg101 to promote virus release	MA: just inside viral lipid bilayer CA: center of virus particle, cone around viral genomic RNA NC: complexed with viral genomic RNA p6: location unknown	[7] [8] [97] [101] [105] [123] [130] [160] [178] [189] [196] [197] [214] [215] [268] [282]
Gag-pol	Initially synthesized as a polyprotein Pr160$^{Gag-Pol}$; autocatalyzes itself to free protease (PR); PR then cleaves the remaining polyprotein into reverse transcriptase (RT) and integrase (IN)	Structural protein PR: viral protease RT: converts viral genomic RNA into DNA upon uncoating in the cytoplasm IN: integrates viral DNA into the host cell genomic DNA	PR: between MA and CA RT and IN: within capsid	[13] [37] [60] [100] [138] [270]

The Envelope of HIV-1

The *env* gene encodes the envelope polyprotein gp160. gp160 is cleaved into two proteins, gp120 and gp41, by cellular furin/ furin-like proteases **[116]. gp120 is heavily glycosylated, and is found on the surface of virus particles [81] [159] [295]. gp41 is inserted in the virus plasma membrane via a transmembrane domain, and has a cytoplasmic domain with many motifs responsible for envelope assembly and trafficking [75] [76] [267] [221]. gp120 and gp41 are non-covalently linked on the virus particle surface and arranged in trimers [122] [80]. These trimers are called envelope spikes.**

gp120 has two domains, an inner domain and an outer domain [152][283]. In the CD4-bound form, the outer domain is connected to the inner domain by a 4-stranded antiparallel β-sheet called the bridging sheet (see Fig.1.4a). Areas called the variable (V) loops are also present. There are 5 variable loops in gp120, designated V1 to V5 [243]. The stems of the V1/V2 loops make up part of the bridging sheet (Fig. 1.4a) [152] [285].

The CD4 binding domain residues line the cavity between the inner and outer domains [152]. The bridging sheet and the V3 loop, located on the outer domain, are responsible for binding the coreceptor (either CCR5 or CXCR4) [152] [285] [25] [242]. Keeping the CD4 binding site recessed protects many important sites from being recognized by the immune system [125] [82]. The variable loops' sequences can evolve quickly, providing rapid escape from any neutralizing antibodies targeted to these immunogenic regions.

Analysis of V3 loop sequences has shown that CXCR4-usage is associated with increased basic residues, fewer N-linked glycosylation sites, and increased positively-

charged residues [66] [93] [125] [208]. Studies have also shown that the V1/V2 loop is involved in coreceptor tropism; however, the specific residues and/or motifs involved are poorly defined [283]. Recently, Huang *et al.* reported that the transmembrane domain of gp41 also has a role in coreceptor tropism [131].

The ectodomain of gp41 (the section of the protein immediately before the transmembrane domain) contains 2 heptad repeats, HR1 and HR2. HR1 and HR2 are coiled-coil domains. They pack together in antiparallel to create the 6-helix bundle important for membrane fusion (Fig. 1.5b) [39] [42] [275]. Further discussion of functional domains in gp41 is in Chapter V.

Fusion of the virus membrane and the target cell membrane occurs in a stepwise process. First, gp120 binds CD4. This causes conformational changes to occur in gp120, exposing residues in the bridging sheet responsible for coreceptor binding [248] [218] [44]. Next, the coreceptor is bound by the bridging sheet and the V3 loop [152] [242]. Conformational changes then occur in gp41 that allow the fusion peptide in gp41 to insert into the target cell membrane. Finally, the 6-helix bundle brings the viral and cell membranes together, allowing them to fuse, and the viral core is released into the cytoplasm of the cell (Fig. 1.6) [149] [96] [41] [221] [70] [76] [75].
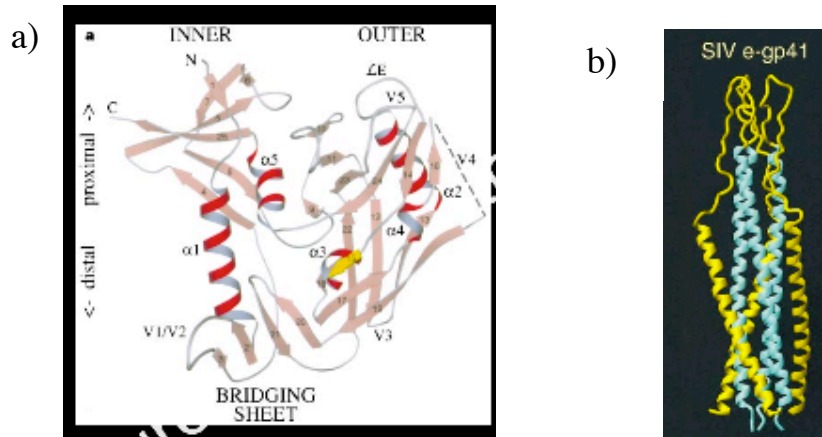
Fig. 1.4 Ribbon diagrams of gp120 and gp41. a) gp120 , b) gp41 ectodomain of SIV. Reproduced from [152] and [39].
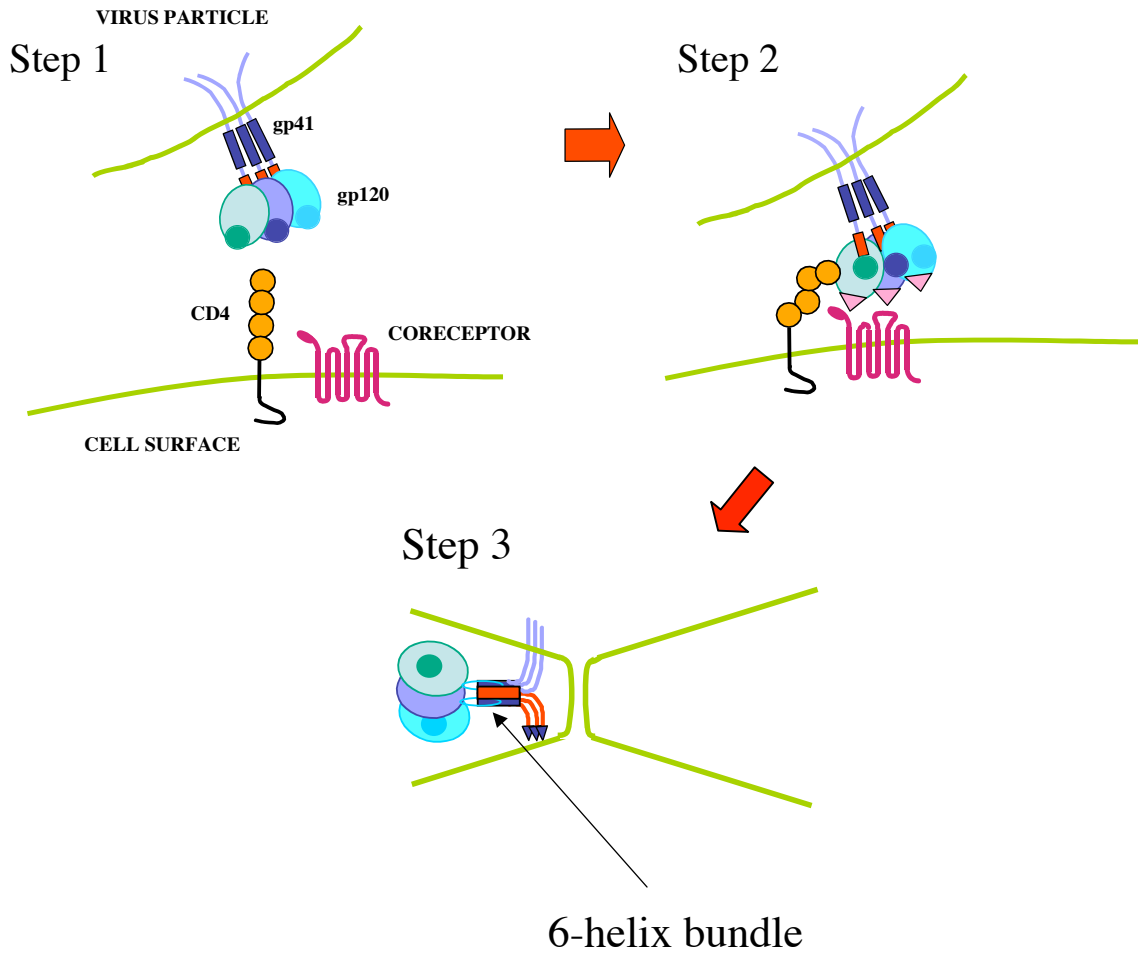
Fig. 1.5 Fusion of the HIV and host cell plasma membranes. Step 1: Virus particle approaches CD4^{+} coreceptor^{+} target cell. Step 2: gp120 interacts with CD4, causing conformational changes that expose the coreceptor sites; gp120 then interacts with the coreceptor. Step 3: Conformational changes occur in gp41, allowing the fusion peptide to insert into the target cell membrane. The 6-helix bundle forms, bringing the viral and cell membranes together for fusion.

The Vpu Gene and Protein of HIV-1

The *vpu* gene is unique to HIV-1, and some SIVs [15] [58] [59]. *vpu* likely originated in SIV$_{cpz}$ [11]. There is no equivalent gene in HIV-2. 80 nucleotides of the 3' end of *vpu* overlap the 5' end of *env*. *vpu* and *env* are transcribed together on a bicistronic mRNA, and translation of vpu occurs via leaky scanning by ribosomes, while translation of env occurs via discontinuous ribosome-scanning [232] [9]. Mutations that disrupt the *vpu* reading frame result in increased translation of *env* [227] [245].

The *vpu* gene encodes a 16 kDa, 77-86 amino acid protein [53]. This protein has a transmembrane domain at the amino-terminus, and two α-helices in the carboxy-terminus in the cytoplasmic domain. These two domains have very distinct functions. The transmembrane domain increases the release of virus particles from the plasma membrane of the host cell [226]. The cytoplasmic domain degrades CD4 from the endoplasmic reticulum [278].

The transmembrane domain can form ion channels similar to the HA of influenza viruses [87] [229]. A single amino acid substitution in the transmembrane domain makes vpu sensitive to the ion channel blocker rimantadine [128]. This domain also is able overcome a cellular block present in HeLa cells and macrophages; but not present in HOS and 293T cells [190] [263] [12] [69] [139] [228]. UBP is a cellular protein that could possibly be indirectly involved in virus release. UBP binds to gag; vpu releases gag from UBP, allowing gag to be moved to the cell surface for incorporation into virus particles [40] [117].

Vpu enhances virion release from plasma membranes by overcoming host cell blocks. Several of these blocks have been identified. First, Hsu *et al.* found that vpu destroys the host cell antiviral ion channel TASK-1 [129]. Second, Neil *et al.* and Van Damme *et al.* reported that the cellular protein BST-2 (also known as tetherin) tethered HIV-1 particles to the cell surface in the absence of vpu [191][260]. BST-2/ tetherin is a membrane-associated protein with a cytoplasmic tail at the N-terminal, a transmembrane domain, a predicted extracellular coiled-coil domain, and a GPI membrane anchor at the C-terminal [191]. This protein is expressed in HeLa cells, which have a vpu-restrictive phenotype, but is not expressed in 293T cells, which do not have a vpu-restrictive phenotype. BST-2/tetherin is also expressed in terminally differentiated B cells, bone marrow stromal cells, and plasmacytoid dendritic cells [260]. It is unclear whether this protein is also expressed in macrophages.

Varthakavi *et al.* found that calcium modulating cyclophilin ligand (CAML) also restricts virus release in the absence of vpu. This cellular block is overcome by vpu as well as the envelope of HIV-2, and is likely the restrictive factor described in previous work with heterokayrons. CAML is an integral membrane protein expressed in HeLa cells, and is required for T cell survival; however, expression of this protein in macrophages is unclear [262].

The CD4 degradation function of vpu is well understood. First, the α-helices of vpu interact with the cytoplasmic tail of CD4 [30] [158] [269] [288]. Next, constitutively phosphorylated serines at positions 52 and 56 in vpu recruit cellular proteins β-TrCP, skp1, and the E3 ubiquitin ligase complex [230] [22] [175]. Finally, CD4 is

ubiquitinated, and retrotranslocated from the endoplasmic reticulum to the proteosome [225] [99]. Removal of CD4 from the endoplasmic reticulum prevents the formation of CD4-envelope complexes, allowing newly synthesized envelope to travel to the cell surface for incorporation onto budding virus particles [141] [278].

Treatment of HIV

Over ten years ago, Highly Active Antiretroviral Therapy (HAART) was introduced as a treatment option for people infected with HIV. Currently, five types of drugs are in wide use in patients: RT inhibitors, PR inhibitors, IN inhibitors, fusion inhibitors, and CCR5 antagonists. RT inhibitors are either nucleoside (NRTI) or non-nucleoside (NNRTI) inhibitors. NRTIs are nucleotide mimics without the 3'-OH group (chain terminators) [224]. They are incorporated into the growing DNA strand, preventing its further extension. NNRTIs inhibit DNA polymerization by binding the active site in RT [147]. PR inhibitors are generally synthetic compounds that are recognized by structure by PR [184]. IN inhibitors inhibit strand transfer during integration of the viral genome [88].

Fusion inhibitors interact with the HR1 domain after CD4 binding, preventing formation of the 6-helix bundle. Enfuvirtide (also known as T20) has been used for many years, especially as a salvage therapy [140] [154]. CCR5 antagonists block the coreceptor, preventing gp120 from binding. One example of these CCR5 antagonists is Maraviroc, which has been approved by the FDA for wide use [210] [280].

Unfortunately, viruses become resistant to these treatment options, and patients stop responding to these therapies. Gag processing is also an attractive target because it must

occur in a very specific order of steps. Currently, work in underway blocking the maturation of CA [4] [161] [293]. Gag-processing drugs are still in the experimental stages.

Since the HIV epidemic started over 25 years ago, the main goal has been to create an effective vaccine. Vaccine strategies that aim to induce neutralizing antibodies have been intensely studied. HIV-1 has many effective mechanisms for protecting itself from these neutralizing antibodies. gp120 is heavily glycosylated, making it poorly immunogenic. This glycosylation also protects the underlying amino acids from antibody binding. Effective antibodies to the variable loops exist; however, the variable loops frequently acquire sequence changes that help the virus evade the immune response. Other immunogenic epitopes are present in HIV-1 (i.e., gp41, the CD4 binding site, and the coreceptor binding sites), but these are typically hidden from antibodies [284] [38].

There are many obstacles to developing a vaccine for HIV. It is unlikely that there will be an effective vaccine soon. The best hope for defeating the HIV/AIDS epidemic is prevention and education.

Chapter II: Materials and Methods

Patient Envelope Sources

Viral gp120 sequences were derived from the PBMC of four homosexual men enrolled at St. Mary's Hospital STD Clinic, London, UK [1]. Viral gp160 sequences were derived from frozen brain and lymph node tissues from patients at autopsy at the University of Edinburgh [201]. Viral gp120 and gp160 sequences were amplified by PCR, as described previously [201] [1].

Construction of Molecular Clones

vpu$^{+/-}$ chimeras and full lengths

The molecular clone pNL4.3 was used to construct all chimeric viruses used in the experiments described here. p3'SF162 (containing the 3' half of the SF162 isolate genome) was used to provide the *vpu* gene for all chimeras [46]. The construction of *vpu$^-$* p3'SF162 (ATG to ATA start codon mutation) was described previously [69]. Because the *vpu* and *env* genes overlap, I constructed two sets of chimeric clones. One set of clones was constructed to contain chimeric *vpu* genes and complete *env* genes; the second set was constructed to contain chimeric *env* genes and complete *vpu* genes. Premature stop codons were introduced into the *nef* genes of some of these latter constructs (Fig. 3.1).

The first set of chimeric clones was constructed using unique BbsI and BlpI sites in p3'SF162. This allowed the introduction of different *env* genes into p3'SF162. *Vpu* and *env* genes were then subcloned into pNL4.3 via the EcoRI and XhoI sites. Chimeric

vpu+ and *vpu*- clones containing complete envelope genes from SF162, AD8 and YU-2 were constructed. This approach resulted in the NL4.3/AD8 and NL4.3/YU-2 chimeric clones containing chimeric *vpu* sequences (69 amino acids from SF162, 28 amino acids from AD8 or YU-2).

The second set of chimeric clones were constructed using the KpnI site downstream of the *vpu* gene. These clones contain complete SF162 *vpu* genes, and chimeric *env* genes. The leader sequences and first 12 amino acids of the *env* genes in these clones were derived from SF162. Premature stop codons were introduced by PCR mutagenesis of the *nef* gene at the XhoI site of the NL4.3/SF162, NL4.3/AD8 and NL4.3/YU-2 chimeric clones described above.

Full-length AD8 with *vpu*+ or *vpu*- (ATA at the start codon) were described by Theodore *et al*. [254]. Full length *vpu*- YU-2 (CTG at the start codon) was described by Li *et al*. [163]. The YU-2 *vpu* start codon was repaired by PCR mutagenesis.

HXBc2 containing patient gp120 sequences

Patient gp120 sequences were amplified by Aasa-Chapman *et al*. using PCR from PBMC as described previously [1]. With this PCR, BstEII and MluI sites were introduced into the patient sequences at the beginning and end of gp120, respectively. These sites were used to introduce the envelope sequences into the HXBc2 molecular clone.

B33 and LN40 chimeras and point mutations

B33 and LN40 gp160 sequences were amplified from patient tissues by PCR as described previously [201]. Chimeras exchanging the entire gp41 were generated using the MfeI site just upstream of the start of gp41, and the BlpI site downstream from

envelope. Chimeras exchanging the C-terminal half of gp41 were generated using the SalI site just upstream of the palmitoylation site, and BlpI. Point mutations were generated by PCR mutagenesis.

Production of virus stocks

Virus stocks were prepared by calcium chloride transfection of HEK 293T cells [203]. *Vpu$^{+/-}$* chimeras and full length clones were co-transfected with a vector expressing the vesicular stomatitis virus (VSV) G protein. HXBc2 chimeras containing either patient gp120 sequences, or reference gp120 sequences were transfected alone. B33wt, LN40wt, and variants of each were co-transfected with pNL4.3 *env$^-$* to make pseudotype virus stocks. pNL4.3 *env$^-$* has a stop codon early in envelope, but expresses all other HIV proteins, including Tat. All stocks were clarified by low speed centrifugation 48 hours post-transfection, and were frozen at -152°C.

Cell Culture: Cell Lines and Primary Macrophages

HEK 293T [77], NP2 [241], TZM-bl and RC49 cells [207] were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 10 µg/mL gentamicin (Invitrogen). Cells were maintained at 37°C, 5% CO_2.

Macrophages prepared by elutriation [234] [107] were provided by the University of Massachusetts Medical School, Center for AIDS Research Cell Culture Core. Macrophages were also prepared from PBMC by adherence [237] [238] [239]. Briefly, PBMC were prepared from whole blood by Ficoll-Paque density gradient centrifugation. 5×10^6-5×10^7 PBMC were placed in 150cm^3 Petri dishes in DMEM containing 10% heat inactivated human plasma, and incubated at 37^0C for 3 hours. Plates were gently washed

with DMEM three times, and DMEM, 10% human plasma was added. Plates were incubated overnight, washed again the next day, then incubated for six days at 37^0C. After six days, adherent macrophages were washed three times with EDTA, and gently scraped off the plates with a cell scraper. Macrophages were resuspended to 2.5×10^5 cells/mL in DMEM, 10% human plasma, seeded in 48-well trays and incubated overnight at 37^0C. Macrophages were infected the following day.

Infectivity Assays

NP2, TZM-bl, or RC49 cells were seeded in 48-well trays at 4×10^4/mL the day before infection. The cells were infected with serial tenfold dilutions of virus for 3 hours at 37^oC. Virus was removed, fresh media was added and cells were incubated for 48 hours (in the case of TZM-bl cells) or 72 hours (in the cases of NP2 and RC49 cells). Cells were fixed and stained *in situ* for *lacZ* expression (TZM-bl; see below for more detail) or p24 antigen (NP2, RC49. and macrophages). After staining, cells were assessed for focus-forming units (ffu) as described below.

To assess infection of macrophages by *vpu*$^{+/-}$ chimeras and full length clones, macrophages in 48-well trays were infected with 100 µl viral stock, containing 25-50pg reverse transcriptase (RT), of the VSV G pseudotyped viruses or other doses as described. Macrophages were spinoculated by centrifugation at 1000 rpm for 45 minutes [193]. After centrifugation, infected macrophages were incubated at 37^oC for 3 hours. Virus inoculum was removed, and cells were washed twice with fresh media. Supernatants were harvested immediately following washing, then at approximately 3 day intervals for 2 weeks. Two weeks post-infection, the infected macrophages were

fixed and stained for intracellular p24 antigen. Harvested supernatants were assessed for RT activity by RT-ELISA (Cavidi Tech Inc. Sweden).

To assess infection of macrophages by HXBc2 chimeras, macrophages in 48-well trays were infected with serial tenfold dilutions of virus. Macrophages were spinoculated and incubated as described above. Virus inoculum was removed, fresh media was added, and cells were incubated for one week. At the end of one week, macrophages were fixed and stained for *in situ* p24. After staining, cells were assessed for ffu.

Single-round infectivity assay

I examined whether the AD8 envelope conferred a vpu-independent phenotype when viral replication was limited to a single round. Macrophages were infected with high doses of virus (1600 pg in 100 μl) by spinoculation as described above. After incubation at 37°C for four hours cells were washed and, 10 μM of the reverse transcriptase inhibitor, indinavir sulfate (NIH AIDS Research and Reference Program) was added to cells to prevent subsequent rounds of replication. Supernatants were harvested at 24, 48, 72 and 96 hours and infections were fixed after 96 hours. RT activity of the supernatants was assessed by RT-ELISA.

In situ staining for *lacZ* expression in TZM-bl cells

TZM-bl cells carry the β-galactosidase gene controlled by an HIV LTR that is expressed following infection and tat expression [272]. For viral titrations on these cells, PBS containing 0.5 mg/mL X-Gal, 3 mM potassium ferricyanide, 3 mM ferrocyanide and 1 mM magnesium chloride was added directly to cells following fixation with 0.5% gluteraldehyde in PBS at 4°C. Areas of infected HeLa TZM-BL cells were regarded as

foci of infection and were counted by light microscopy [203]. The titers (in ffu/mL) were calculated.

In situ immunostaining for p24 antigen and envelope
      Transfected 293T, infected NP2 cells, RC49 cells, or macrophages were fixed with cold (-20^0C) 1:1 methanol: acetone, washed with PBS, then immunostained for p24 or envelope. For p24 staining, supernatants containing monoclonal antibodies 365 and 366 for p24 (UK Centre for AIDS Research) diluted 1:40 in 1% fetal calf serum, 0.05% sodium azide in PBS were placed on cells, and incubated for 1 hour at room temperature. For envelope staining, anti-gp41 Chessie 8 monoclonal antibody was used as described for p24 staining. The cells were washed twice in 1% FBS, 0.05% sodium azide PBS. Secondary antibody (goat anti-human conjugated to β-galactosidase) diluted 1:400 in 1% FBS, 0.05% sodium azide PBS, was added, and incubated for 1 hour at room temperature. Cells were washed once in 1% FBS, 0.05% sodium azide PBS, then twice in PBS. PBS containing 0.5 mg/mL X-Gal, 3 mM potassium ferricyanide, 3 mM ferrocyanide and 1 mM magnesium chloride (PBS-X-GAL) was then added to the cells. Cells were incubated at 37ºC for 3 hours. Infected cells stained blue. The titers (in ffu/mL) were calculated.

Cell:cell fusion assays
      HEK 293T cells transfected (described above) with _env_⁺ pSVIIIenv and pNL4.3 _env_⁻ (at 4×10^5/mL) were cocultivated TZM-bl cells (seeded at 8×10^4/mL in 48-well trays the day before). Syncytia formed 4-8 hours after mixing the two cell types. Cells were fixed and stained with methanol containing 1% methylene blue and 0.25% basic fuchsin for 10 minutes. Stained cells were then rinsed with PBS.

Reverse Transcriptase (RT)-ELISAs

Virus stocks and supernatants from macrophage infections were assessed for reverse transcriptase activity (RT) by RT-ELISA (Cavidi Tech Inc) as was described previously [83]. Briefly, samples of inactivated virus were placed in wells containing a solution containing bromo-deoxyuridine triphosphate and pre-coated with poly-A primer, and incubated overnight at 33ºC. Plates were washed the next day with buffer containing 0.75% Triton X-100, and 0.25% of the buffer solution provided with the kit. Tracer solution (containing bromo-deoxyuridine triphosphate binding antibody conjugated to alkaline phosphate) was placed in the wells, and incubated at 33ºC for 90 minutes. Wells were washed again, substrate was added, and wells were incubated at room temperature for 30 minutes. Alkaline phosphatase activity was measured at 405 nm. The standard curve was plotted, and the amount of RT in the samples was calculated (as pg/mL) using the KC Junior program (BioTek Technologies).

gp120 ELISAs

High binding polystyrene (Costar) plates were coated with sheep anti-gp120 antibody (D7324; International Enzymes, Inc.) at 5 μg/mL in PBS, and incubated overnight at 4ºC. Plates were washed 2 times with PBS/ .05% Tween 20, then blocked with PBS/ 3% BSA for 1 hour at room temperature. Plates were washed 4 times with PBS/ .05% Tween 20. Samples and standard (HIV-1 LAVB recombinant gp120; Protein Sciences Corporation) were serially diluted in PBS/ 1% BSA/ .02% Tween 20, and added to wells. Plates were incubated at room temperature for 2 hours, then washed 10 times with PBS/ .05% Tween 20. Anti-gp120 monoclonal antibody (b6; provided by D. Burton at The Scripps Research Institute) was added to the wells at .4 μg/mL in PBS/ 1% BSA/

.02% Tween 20, and plates were incubated at room temperature for 2 hours. Plates were

washed 10 times with PBS/ .05% Tween 20. Goat anti-human IgG F(ab')$_2$ horseradish

peroxidase conjugated (31312; Pierce) diluted 1:5000 in PBS/ 1% BSA/ .02% Tween 20

was added to the wells, and plates were incubated at 37° C for 1 hour. Plates were washed

4 times with PBS/ .05% Tween 20, then once with water. Substrate (3,3´,5,5´-

tetramethylbenzidine, also known as TMB; Pierce) was added to wells, and plates were

incubated at room temperature for 30 minutes. Plates were read at 450 nm, standard

curves were plotted, and the amount of gp120 (in ng/mL) was calculated using the KC

Junior program (Bio-Tek Technologies).

Ultracentrifugation of virus particles

       HEK 293T cells were transfected as described above. Forty-eight hours post

transfection, the supernatant was removed from the cells, and spun at 5000 rpm in a

Beckman-Coulter Ultracentrifuge (Optima L-90K, SW40 rotor). The supernatant was

filtered through a .45 μm or .22 μm syringe filter, layered onto a 20% sucrose cushion,

then spun at 22,000 rpm for 2 hours at 4°C in a Beckman-Coulter Ultracentrifuge

(Optima L-90K, SW40 rotor). The supernatant (including the sucrose) was poured off,

and the virus particles were resuspended in either Laemmli buffer for SDS-PAGE and

Western blotting, or DMEM for use in infectivity assays, RT ELISAs, gp120 ELISAs.

SDS-PAGE and Western blotting

       Virus particles (isolated as described above) were resuspended in 2x Laemmli

buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, .004% bromphenol blue, .125 M

Tris HCl; Sigma), then loaded onto an 8% SDS-PAGE gel. Viral proteins were

transferred to PVDF membranes, and probed for gp41 (with Chessie 8; NIH AIDS Research and Reference Reagent Program) and p24 gag (with 183-H12-5C; NIH AIDS Research and Reference Reagent Program). Bound antibodies were detected by horseradish peroxidase chemiluminescence (Pierce).

Chapter III: Effects of *vpu* start-codon mutations on HIV-1 replication in macrophages: No evidence of rescue by envelope

Introduction

The Human Immunodeficiency virus type 1 (HIV-1) *vpu* gene encodes a 77-86 amino acid, ≈16 KDa protein [53]. Vpu is predominantly expressed in the endoplasmic reticulum and the Golgi [198] [264], but also colocalizes with markers for recycling endosomes [264]. The vpu protein contains a transmembrane domain at the amino terminus and 2 cytoplasmic α-helices at the carboxyl terminus.

Over 80 nucleotides at the 3' end of *vpu* overlap the 5' end of *env* in the HIV-1 genome, and the two genes are transcribed together on a bicistronic mRNA [232]. Translation of the vpu protein occurs via leaky scanning by ribosomes, while the translation of the env glycoprotein occurs via discontinuous scanning by ribosome [233] [9]. Mutations that disrupt the *vpu* reading frame were reported to result in increased translation of *env* [227] [245].

Vpu has two distinct functions during viral replication: 1) increased release of virus particles from plasma membranes, and 2) degradation of intracellular CD4. The transmembrane domain is responsible for virus release from host cell plasma membranes [226] [229]. This domain forms oligomers that act as ion channels [87] [229]. A single amino acid substitution in the TM domain was shown to render vpu activity sensitive to the ion channel blocker rimantadine, causing a decrease in the release of virus particles from infected cells [128]. Vpu was reported to interact with a host cell ion channel,

TASK-1, which may have anti-viral activity disrupted by vpu [129]. Vpu has also been reported to associate with a member of the tetratricopeptide family called Vpu Binding Protein (UBP) [40]. UBP was shown to bind to vpu and p55 gag [40]. Overexpression of UBP diminished the release of virus particles, suggesting that vpu may remove UBP from gag in order to facilitate its transport to the cell surface [40] [117].

Vpu enhances virion release from plasma membranes by overcoming host cell blocks. Several of these blocks have been identified. First, Hsu *et al.* found that vpu destroys the host cell antiviral ion channel TASK-1 [129]. Second, Neil *et al.* and Van Damme *et al.* reported that BST-2 (also known as tetherin) tethered HIV-1 particles to the cell surface in the absence of vpu [191][260]. BST-2/ tetherin is a membrane associated protein with a cytoplasmic tail at the N-terminal, a transmembrane domain, a predicted extracellular coiled-coil domain, and a GPI membrane anchor at the C-terminal [191]. This protein is expressed in HeLa cells, which have a vpu-restrictive phenotype, but is not expressed in 293T cells, which do not have a vpu-restrictive phenotype. BST-2/tetherin is also expressed in terminally differentiated B cells, bone marrow stromal cells, and plasmacytoid dendritic cells [260]. It is unclear whether this protein is also expressed in macrophages.

Varthakavi *et al.* found that calcium modulating cyclophilin ligand (CAML) also restricts virus release in the absence of vpu. This cellular block is overcome by vpu and the envelope of HIV-2, and is likely the restrictive factor described in previous work with heterokayrons. CAML is an integral membrane protein expressed in HeLa cells, and is

required for T cell survival; however, expression of this protein in macrophages is unclear [262].

The cytoplasmic region of vpu downregulates CD4. This region recruits cellular proteins that ubiquitinate CD4 and induce its degradation in a multi-step process. First, the vpu α-helix interacts with the cytoplasmic tail of CD4 [29] [269] [288] [158]. Second, constitutively phosphorylated serines at residues 52 and 56 recruit the cellular proteins β-TrCP, skp1, and the E3 ubiquitin ligase complex [230][22] [175]. Finally, these proteins ubiquitinate CD4, triggering CD4 translocation from the endoplasmic reticulum to the proteosome for degradation [225] [99] [225]. The removal of CD4 from the secretory pathway by vpu limits the formation of CD4: envelope complexes in the endoplasmic reticulum, allowing more efficient envelope trafficking through the secretory system [279] [141]. The viral release function of vpu, rather than CD4 degradation, was shown to be more important for replication in macrophages, which typically express low levels of CD4 [228] [156] [187] [14].

Like vpu, nef has been reported to downregulate CD4. However, while vpu removes CD4 from the endoplasmic reticulum, nef downregulates CD4 from the plasma membranes of infected cells via clathrin coated pits [204] [205] [114]. The requirement of nef for HIV-1 replication in macrophages is controversial [36] [250].

HIV-2 and most SIVs lack a *vpu* gene, yet are fully functional in its absence. Determinants in the HIV-2/SIV envelope have been reported to confer virus release [31] [134]. In the HIV-2 envelope, these determinants have been proposed to be a GYXXθ motif in the cytoplasmic tail, and an uncharacterized region in the ectodomain of gp41

[2]. The GYXXθ region in the gp41 cytoplasmic tail has been shown to recruit adapter protein 2 (AP-2) complex, and that this was required to maintain the enhanced virus release function [192]. The determinants in SIV envelopes that may enhance virus release have not been reported.

It is possible that HIV-1 envelopes may also evolve to overcome a lack of functional *vpu* in a similar fashion to HIV-2/SIV envelopes, and that increased envelope expression in the absence of vpu may provide an advantage *in vivo*. The envelope of the AD8 isolate was reported to be vpu-independent because it conferred virus release from transfected HeLa cells and replicated in macrophages in the absence of vpu [227]. YU-2, which possesses a mutated *vpu* start codon, was cloned directly from the brain tissue of an infected individual with neurological complications, [163]. YU-2 was reported to infect macrophages efficiently, despite its *vpu* start codon mutation [163]. Mutations in the *vpu* start codon also occur in about 1% of sequences derived from primary isolates in the HIV databases. I hypothesized that *vpu* start codon mutations and the evolution of vpu-independent envelopes to compensate may occur *in vivo*.

Here, I compared the capacity of AD8 and YU-2 envelopes that lack functional *vpu* genes to replicate in macrophages. These results show that the elimination of vpu function severely affected virion release and virus replication for both AD8 and particularly for YU-2 in macrophages. Neither the AD8 nor YU-2 envelopes were able to rescue macrophage replication for *vpu*-minus chimeric viruses. I also confirmed that decreased virion release in vpu-defective infections of macrophages is due to a defect in viral release exacerbated by inefficient viral spread.

Results

Construction of vpu^{+/-} chimeric viruses

Our lab previously described *vpu*⁺ and *vpu*⁻ chimeras of SF162 and NL4.3 [69]. These constructs have complete *vpu* and *env* genes from SF162 in the background of NL4.3. The *vpu*⁻ construct contained a single mutation in the *vpu* start codon (ATG to ATA mutation) that eliminated vpu function. I constructed additional chimeric viruses carrying envelope sequences from HIV-1 AD8 and YU-2. Two strategies were used to prepare chimeras because the *vpu* and *env* genes overlap. The first set of chimeric clones was constructed with full-length AD8 or YU-2 *env* genes and chimeric *vpu* genes. These allowed us to evaluate the function of complete *env* genes, including the leader sequence, in the absence of functional *vpu*.

The second set of chimeric clones was constructed with a complete SF162 *vpu* gene and chimeric *env* genes. These viruses contain the *env* leader sequence from SF162, which allowed us to test a potential role for this region in vpu-independence. This second set of chimeric clones included some constructs that carried a premature stop codon in *nef*, which enabled us to examine the possible effect of nef on virus release. Figure 3.1 shows the structure of the genomes for both sets of chimeric clones. I also used virus derived from full length AD8 and YU-2 molecular clones with and without functional *vpu* genes to evaluate the role of vpu for viral replication in macrophages.

I used the SF162 *vpu* in all the chimeric viruses in this study because its amino acid sequence is most closely related to the consensus sequences of primary isolates compared to the *vpu* sequence from NL4.3. The SF162 envelope was used as a vpu-

dependent control because it has consistently been shown to be dependent upon

functional vpu [69] [139] [228]. Immunostaining of transfected 293T cells for envelope

showed that all constructs produce envelope (not shown).
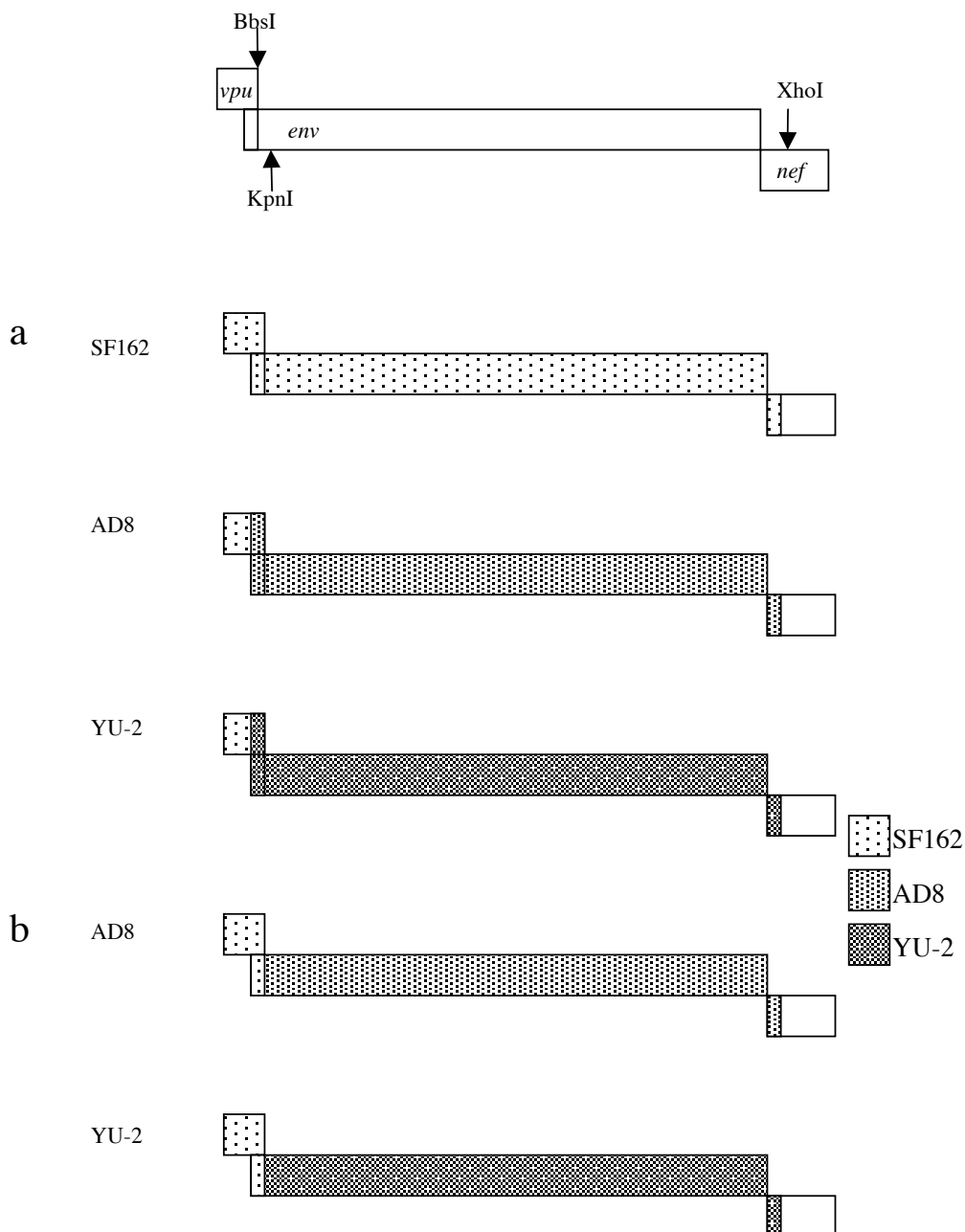
Fig. 3.1 Chimeric clones containing envelopes derived from SF162, AD8 and YU-2. a) Chimeras containing complete env genes. The AD8 and YU-2 clones contain chimeric vpu genes. b) Chimeras containing complete SF162 vpu genes. The AD8 and YU-2 clones contained chimeric env genes, where the leader sequence was derived from SF162. *Vpu* and *env* sequences were introduced into the pNL4.3 background via the EcoRI and XhoI sites.

Infectivity of VSV G+ Viruses

Virus stocks for each of the viral clones described above were prepared by calcium chloride transfection of plasmid DNA into 293T cells. Plasmid DNA encoding viral or chimeric clones was co-transfected with a VSV G expression vector to create VSV G$^+$ virions (see Materials and Methods). Incorporation of VSV G onto emerging virus particles subsequently conferred more efficient infection of primary macrophages.

HIV-1 virion release from 293T has been reported to be independent of vpu [3][216]. However, to confirm that the infectivity of our *vpu$^+$* and *vpu$^-$* viruses was not influenced by production from 293T cells, I compared the number of infectious and physical virus particles in each virus stock. Physical virus particles were measured as reverse transcriptase (RT) activity by RT-ELISA (see Materials and Methods); infectious virus particles were measured by titrating virus stocks onto NP2 parental cells (CD4$^-$ CCR5$^-$) [241]. Infectivity: RT ratios were calculated as an estimate of the infectivity per virus particle.

Table 3.1 shows the infectivity: RT ratios for each pair of *vpu$^+$* and *vpu$^-$* viruses. The ratios for each pair are generally very close; for six out of the ten pairs, the difference was less than two-fold (NL4.3/SF162 *vpu$^+$/$^-$ nef$^-$*, NL4.3/AD8 *vpu$^+$/$^-$*, NL4.3/AD8 *vpu$^+$/$^-$ nef$^+$*, NL4.3/ AD8 *vpu$^+$/$^-$ nef$^-$*, AD8 *vpu$^+$/$^-$*, and YU-2 *vpu$^+$/$^-$*). For *vpu$^+$/$^-$* pairs with a greater than two-fold difference, three of the four have a higher *vpu$^-$* ratio (NL4.3/SF162 *vpu$^+$/$^-$ nef$^+$*, NL4.3/YU-2 *vpu$^+$/$^-$*, and NL4.3/YU-2 *vpu$^+$/$^-$ nef$^+$*). One pair (NL4.3/YU-2 *vpu$^+$/$^-$ nef$^-$*) had a nearly ten-fold difference between the *vpu$^+$* and *vpu$^-$* virus.

Table 3.1 Infectivity of VSV G$^+$ virus stocks for macrophage infections

Virus	Infectivity (ffu/ml)	RT (pg/ml)	Ratio infectivity: RT (ffu/pg)
NL4.3/SF162 vpu^+ nef^+	4.6x10^5	47,833	9.6
NL4.3/ SF162 vpu^- nef^+	8.9x10^5	36,441	24.4
NL4.3/SF162 vpu^+ nef^-	4.9x10^5	72,742	6.7
NL4.3/SF162 vpu^- nef^-	2.2x10^5	39,920	5.5
NL4.3/AD8 vpu^+	3.7x10^5	45,572	8.1
NL4.3/AD8 vpu^-	4.4.x10^5	102,204	4.3
NL4.3/AD8 vpu^+ nef^+	1.6x10^5	27,466	5.8
NL4.3/AD8 vpu^- nef^+	4x10^5	107,466	3.7
NL4.3/AD8 vpu^+ nef^-	1.9x10^5	65,675	2.9
NL4.3/AD8 vpu^- nef^-	1.4x10^5	27,187	5.1
Full length AD8 vpu^+	1.1X10^5	58,095	1.9
Full length AD8 vpu^-	1.3X10^5	33,272	3.9
NL4.3/YU-2 vpu^+	4.7x10^5	135,521	3.5
NL4.3/ YU-2 vpu^-	3.6x10^5	47,966	7.5
NL4.3/YU-2 vpu^+ nef^+	1.4x10^6	134,316	10.4
NL4.3/YU-2 vpu^- nef^+	9x10^6	123,851	72.7
NL4.3/YU-2 vpu^+ nef^-	3.5x10^6	120,368	29.1
NL4.3/YU-2 vpu^- nef^-	5.1x10^5	139,771	3.6
Full length YU-2 vpu^+	1.1x10^4	2,136	5.1
Full length YU-2 vpu^-	6.9x10^4	9,781	7.1

Replication of vpu$^+$ and vpu$^-$ HIV-1 viruses in macrophages

Virus stocks were equalized by RT activity, and primary macrophages were infected with equal amounts of VSV G$^+$ replication competent viruses as described in the Materials and Methods. Virus release resulting from multiple rounds of infection and replication was measured over two weeks by RT-ELISA.

I first tested *vpu$^+$* and *vpu$^-$* AD8 viral clones for replication in macrophages (Fig. 3.2a). The envelope of AD8 was reported to compensate for the lack of functional vpu by enhancing virus release in HeLa cells [227]. Virus release by the *vpu$^-$* AD8 virus was variable depending on the experiment (not shown), but was consistently released at lower levels than AD8 *vpu$^+$*. Fig. 3.2a shows the maximum amount of released virions detected in one of several experiments, reaching 60% of *vpu$^+$* AD8 release (Fig. 3.2a, left panel).

YU-2, cloned directly from brain tissue without culture, also contains a *vpu* start codon mutation yet retains the ability to infect macrophages [277] [163]. This raised the possibility that the YU-2 envelope was vpu-independent. However, YU-2 *vpu$^+$* replicated considerably more efficiently than *vpu$^-$* YU-2 in macrophages (Fig. 3.2a, right panel).

I next tested the *vpu$^+$* and *vpu$^-$* NL4.3/AD8 chimeras containing either full-length AD8 envelopes or full-length SF162 *vpu* genes as described above. In both cases, *vpu$^+$* NL4.3/AD8 chimeras were consistently released at higher levels than the corresponding NL4.3/AD8 *vpu$^-$* chimeras (Fig. 3.3a, 3.3b), regardless of the strategy used to construct the chimeric viruses. Similarly, NL4.3/YU-2 *vpu$^+$* was consistently released from macrophages at higher levels than NL4.3/YU-2 *vpu$^-$*, again regardless of whether the

chimeric viruses carried full length or chimeric vpu or envelope sequences (Fig. 3.3a, 3.3b).

Fig. 3.4 shows *in situ* immunostaining for p24 at different times after infection of macrophages. Both *vpu*⁺ and *vpu*⁻ viruses infected macrophages, as can been seen by the presence of stained cells. Wells of macrophages infected with *vpu*⁻ viruses, however, have fewer infected cells than wells infected *vpu*⁺ viruses 96 hours post infection.

I tested the possibility that the known vpu-dependent envelope could be forced to become vpu-independent when passaged multiple times through primary macrophages. A chimeric virus based on the NL4.3/SF162 described above with 50 amino acids removed from the beginning of *vpu* (NL4.3/SF62 delta *vpu*) was constructed. I infected macrophages with a high dose of this chimeric virus pseudotyped with VSV G. One week post-infection, supernatants were removed and used to infect fresh macrophages. This was repeated 4 times for a total of 5 passages. RT activity in the supernatants used for each infection was quantified. After the initial passage, the amount of virus in the supernatants was too low to effectively infect macrophages (Fig. 3.5).

The experiments described in Figs. 3.2, 3.3, and 3.5 were carried out multiple times on different batches of macrophages. The graphs shown here are representative of multiple experiments.

In summary, I found that the full-length *vpu*⁻ AD8 consistently replicated with lower efficiency in macrophages compared to *vpu*⁺ AD8. Using chimeric viruses, I was unable to show that the AD8 envelope could compensate for the lack of a functional vpu. In addition, neither the full length YU-2 nor its envelope compensated for the lack of a

functional vpu. I was unable to determine if the SF162 envelope can become vpu-independent due to low levels of virus output from macrophages.
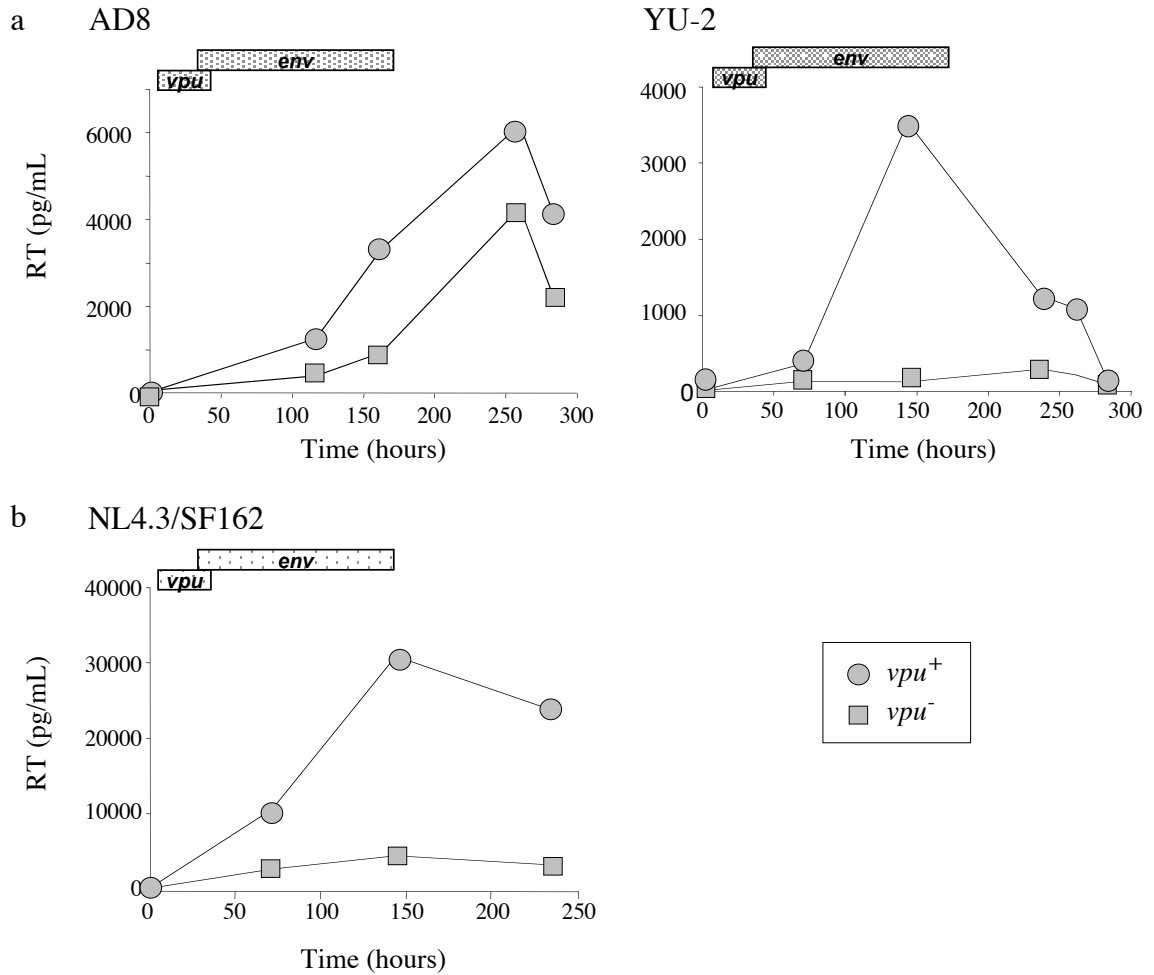
Fig. 3.2 Effect of *vpu* mutations on virion release from macrophages. Macrophages were spinoculated with equal amounts of virus, and incubated for 2 weeks. (a) AD8 and YU-2. (b) NL4.3/SF162. In all cases, *vpu*+ viruses were released from macrophages at higher levels than *vpu*- viruses. These graphs are representative of multiple assays.

a. Full length env, chimeric vpu
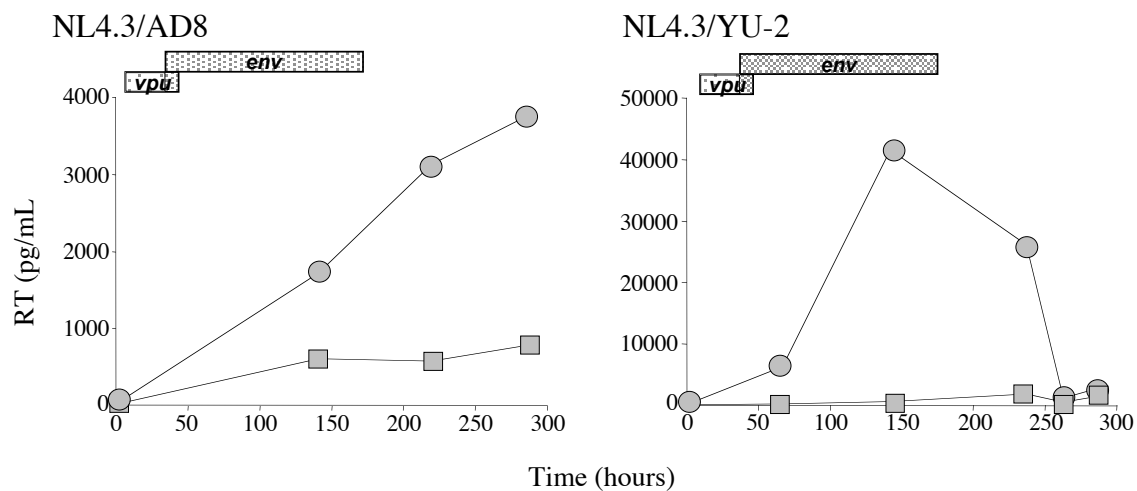
NL4.3/AD8

NL4.3/YU-2

b. SF162 vpu, chimeric env

NL4.3/AD8

NL4.3/YU-2

vpu^+

vpu^-
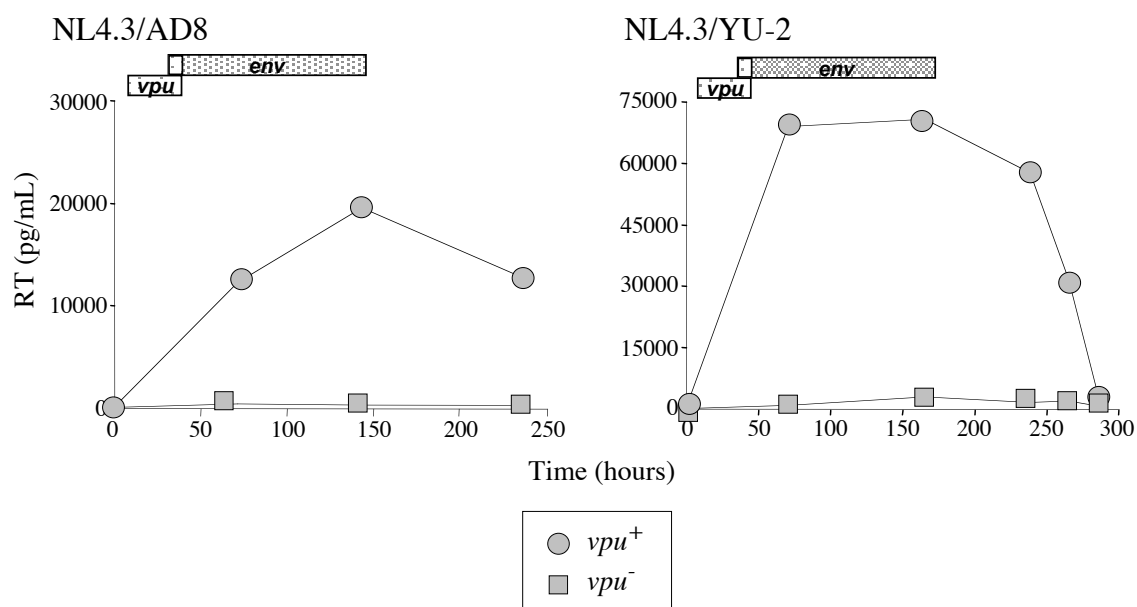
Fig. 3.3 Effect of *vpu* mutations on virion release from macrophages using chimeric constructs. Macrophages were spinoculated with equal amounts of virus and incubated for 2 weeks. (a) NL4.3/AD8 and NL4.3/YU-2 constructs carrying full-length *env* and chimeric *vpu* genes. (b) NL4.3/AD8 and NL4.3/YU-2 constructs carrying full-length SF162 *vpu* genes and chimeric *env* genes. In all cases, vpu^+ viruses were released from macrophages at higher levels than vpu^- viruses. These graphs are representative of multiple assays.
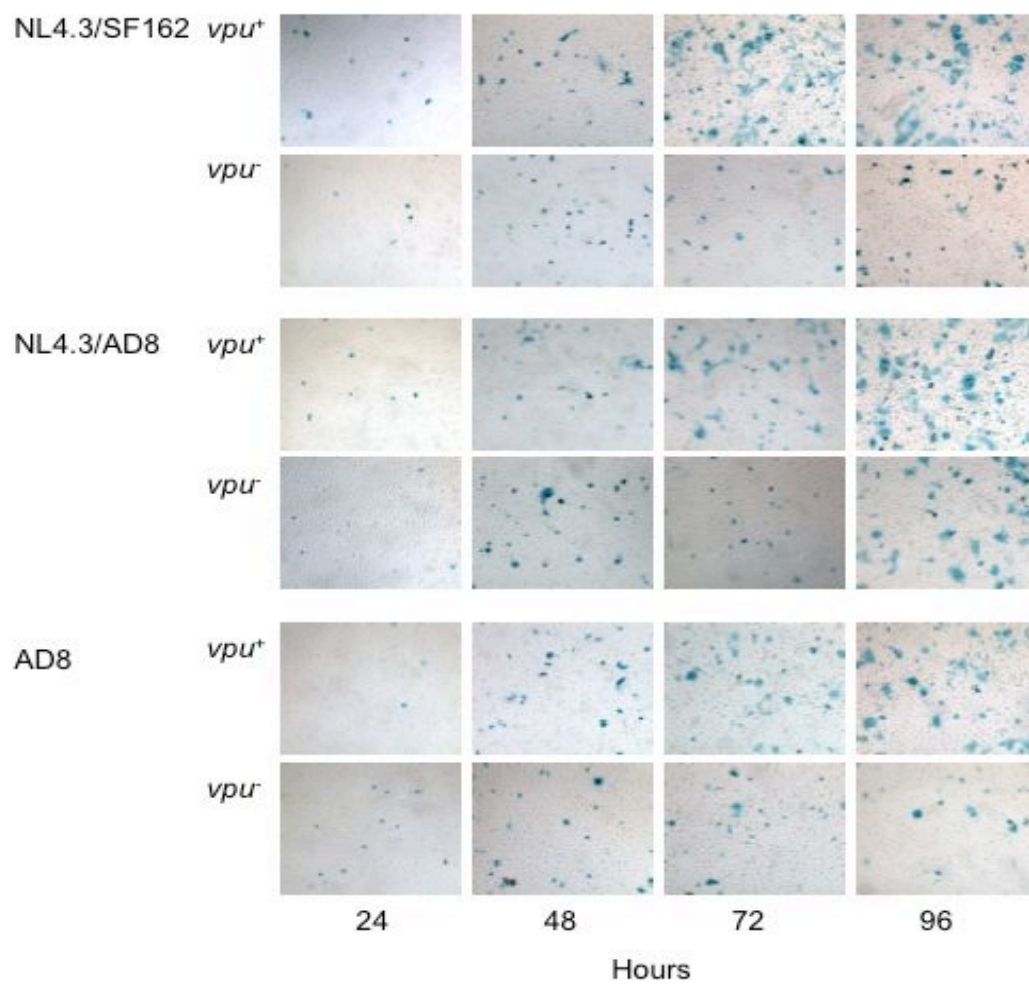
Fig. 3.4 Immunostaining of macrophages infected with *vpu⁺* and *vpu⁻* HIV-1. Macrophages were infected with *vpu⁺* and *vpu⁻* NL4.3/SF162, NL4.3/ AD8, and AD8. Infected cells were fixed and immunostained for p24 at 24-hour intervals. Blue-stained cells indicate infection. These micrographs show that *vpu⁺* viruses confer more efficient spread and formation of small syncytia. Magnification, x60.
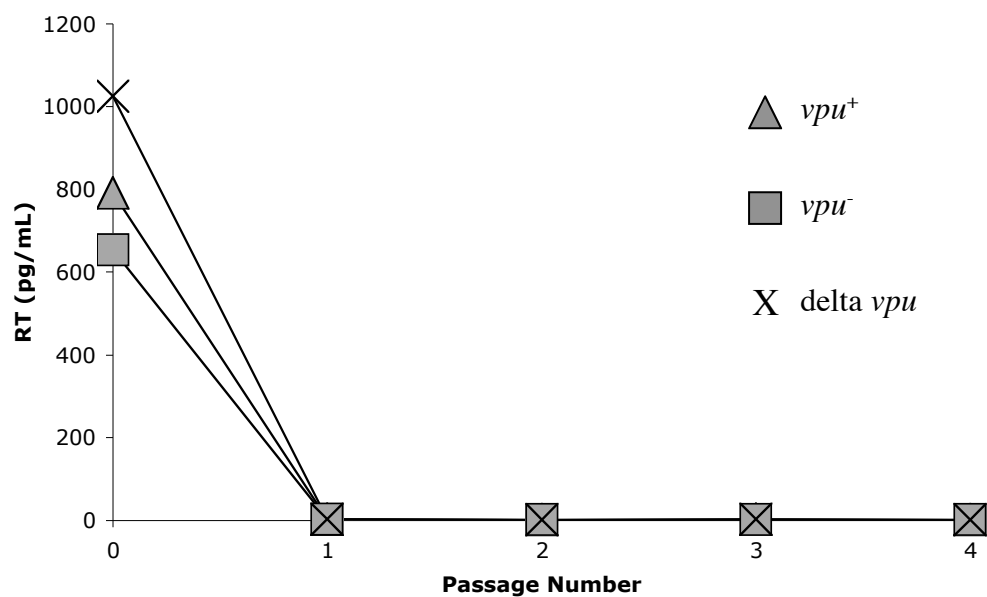
Fig. 3.5 Passage of chimeric viruses through macrophages. Macrophages were infected with a high dose of virus, and spinoculated. One week post-infection, supernatants were removed, and used to infect fresh macrophages. The RT of the infecting supernatant was quantified. Passage number 0 represents the first macrophage infection. Due to the low yield of virus particles after the initial infection, we were unable to force the SF162 envelope to become vpu-independent in macrophages. This graph is representative of multiple assays.

Short term virus release from infected macrophages

The infectivity assays described above followed virion release from macrophages during several rounds of replication over two weeks. It was possible that the vpu-independent phenotype reported for full-length AD8 [227] is more pronounced in early rounds of replication. I therefore examined whether the AD8 envelope conferred a vpu-independent phenotype when viral replication was limited to a single round. Macrophages were infected with high doses of virus, and treated with indinavir sulfate (IVS, a protease inhibitor) 3 hours after infection to prevent subsequent rounds of infection. Virus release was measured over 4 days by RT ELISA.

In the presence of 10 μM IVS, *vpu*+ viruses were released to higher levels than *vpu*- viruses (Fig. 3.6). I observed this phenotype for the SF162 and AD8 chimeras, as well as for full-length AD8.
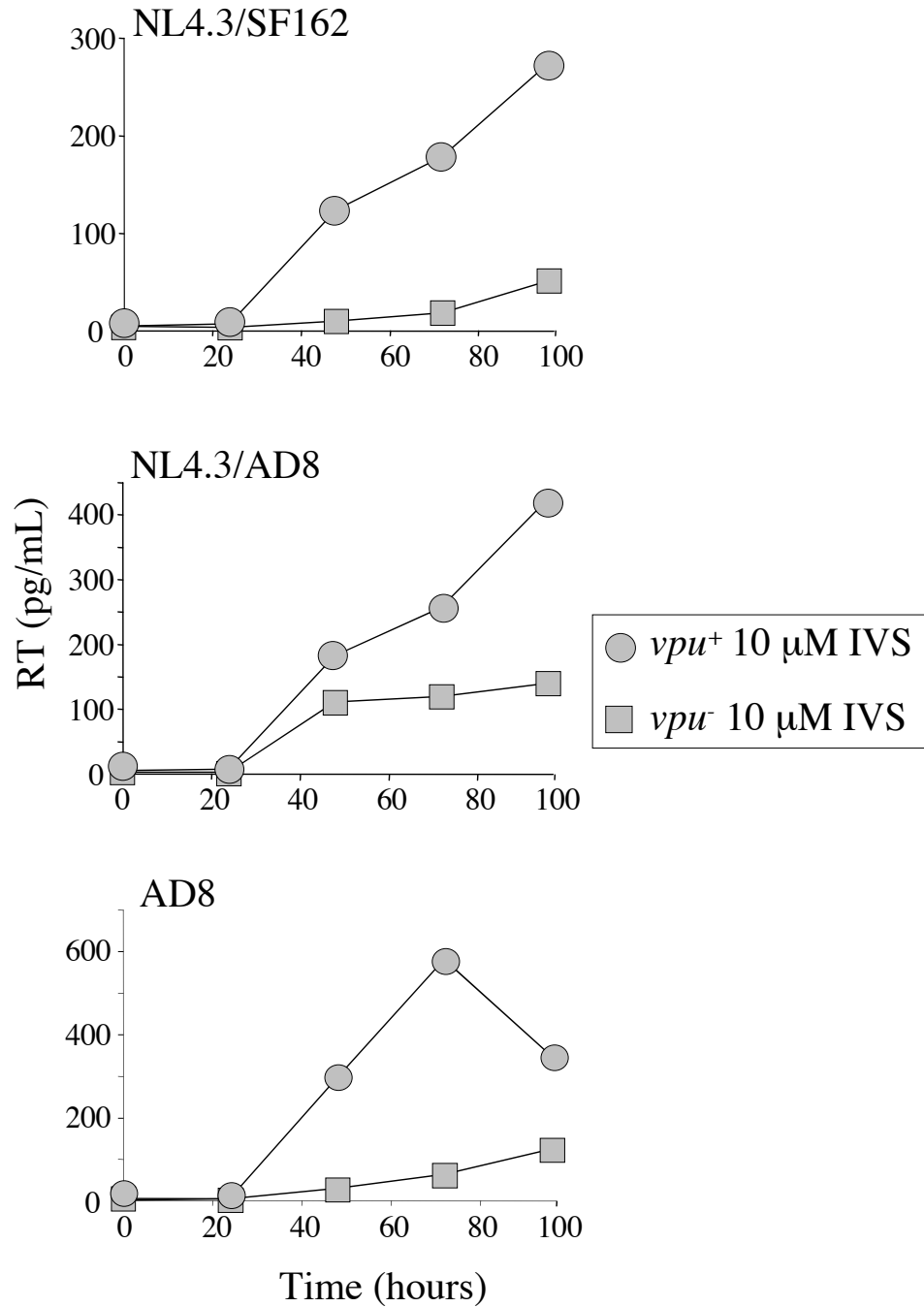
Fig. 3.6 Inhibition of viral spread by IVS. Macrophages were infected with a high dose of virus, spinoculated, treated with 10 μM IVS and incubated for 4 days. In all cases, *vpu⁺* viruses were released at higher levels than *vpu⁻* viruses. These graphs are representative of multiple assays.

The Role of Nef

The strategy used to create the chimeric clones used in these studies resulted in

chimeric *nef* genes (34 amino acids from AD8 or YU-2 and 162 amino acids from NL4.3;

Fig. 3.1). To determine whether nef influenced the phenotypes described above, I

introduced a premature stop codon in *nef* at the XhoI I site into the *vpu*⁺ and *vpu*⁻ chimeric

viral clones (Fig. 3.1b). Virus derived from these clones was then used to infect

macrophages, and virus released into the supernatant was measured over two weeks by

RT ELISA as described above.

NL4.3/SF162 and NL4.3/YU-2 chimeras carrying mutated *nef* genes

(*nef* ⁻) were released from macrophages at low levels similar to the *vpu*⁻ chimeras, while

the *vpu*⁺ *nef*⁺ counterparts replicated efficiently (Fig. 3.7a, 3.7b). Curiously, the *vpu*⁺ *nef*⁻

NL4.3/AD8 chimera varied in virus release depending on experiment. In one

experiment, the *vpu*⁺ *nef*⁻ NL4.3/AD8 chimera replicated as efficiently as the *vpu*⁺ *nef*⁺

chimera (Fig. 3.7c, right panel), while in a second experiment, virus release was low and

comparable to the *vpu*⁻ *nef*⁻ chimera (Fig. 3.7c, left panel).
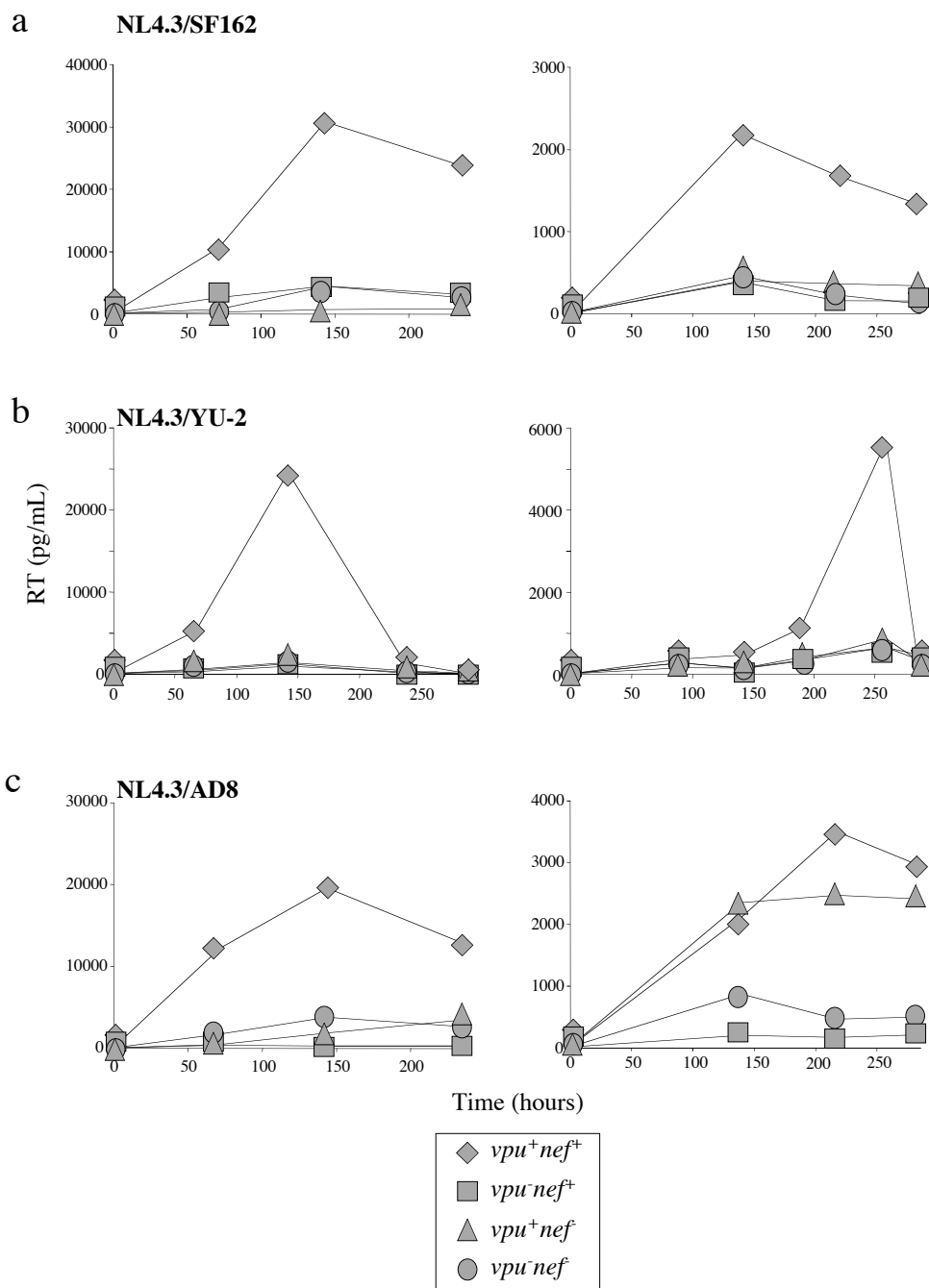
Fig. 3.7 Effect of *nef* on *vpu* phenotypes. Macrophages were spinoculated with equal amounts of *vpu*⁺/*vpu*⁻, *nef*⁺/*nef*⁻ viruses and incubated for 2 weeks. (a) NL4.3/SF162; (b) NL4.3/YU-2; (c) NL4.3/AD8. Results from two separate but representative experiments are shown in the left and right panels. For NL4.3/SF162 (a) and NL4.3/YU-2 (b), the presence of non-functional *vpu*, *nef* or both resulted in severely reduced virus release from macrophages. For NL4.3/AD8 (c), the presence of non-functional *vpu*, *nef*, or both resulted in reduced viral release for some experiments (left panel). However, virus release for *vpu*⁺ was observed even in the context of *nef*⁻ in other experiments (right panel). These graphs are representative of multiple assays.

Discussion

In this study, I determined that neither the AD8 nor the YU-2 envelope was able to compensate for the lack of functional vpu in terms of virus release from macrophages. Release of *vpu⁻* viruses from macrophages was consistently lower than their *vpu⁺* counterparts (Fig. 3.2, 3.3 and 3.7). However, release of *vpu⁻* AD8 was variable, reaching 60% of *vpu⁺* AD8 in one experiment. Short term virus release experiments showed that lower viral release by *vpu⁻* viruses is primarily due to a defect in release, but is compounded by reduced viral spread (Fig. 3.4 and 3.6). Nef does not compensate for the lack of functional vpu in HIV-1 replication in macrophages (Fig. 3.7).

Vpu's ability to enhance the release of virus particles is usually critical for HIV-1 replication in macrophages. Mutation of the *vpu* start codon occurs at low frequency during PBMC culture of HIV-1 isolates *in vitro* [69]. Thomas *et al.* described defects in the *vpu* genes present in several vpu-envelope sequences amplified from brain tissue, where macrophage lineage cells are the main targets for infection [255]. YU-2, a highly macrophage-tropic strain cloned directly from brain tissue of an AIDS patient, also carries a *vpu* start codon mutation [163]. Schubert *et al.* reported that the envelope of the HIV-1 AD8 isolate carried determinants that could compensate for the lack of vpu [227]. These observations led to the hypothesis that loss of vpu function *in vivo* may be compensated by adaptive mutation in envelope. Loss of vpu function could be advantageous in some environments, as it was reported to result in an increase in envelope synthesis [227] [245]. Here, I investigated whether the envelopes of AD8 and YU-2 could compensate for loss of vpu function for virus release and replication in

primary macrophage cultures. These results do not lend support to a role for envelope in compensating for a loss of vpu function in macrophages.

The data presented here appear to conflict with the study of Schubert *et al.*, which showed that AD8 viruses with and without a mutated *vpu* start codon replicate efficiently, and to equivalent levels in primary macrophages [227]. In the same study, Schubert *et al.* used a pseudovirion system to show that the AD8 envelope could enhance virion release from HeLa cells. Interestingly, several groups have also reported that the HIV-2$_{ROD}$ envelope enhanced virion release [2] [192] [31]. Therefore, it seems reasonable that some HIV-1 envelopes, e.g. AD8, may have evolved to perform the same virion-release function. It is unclear why I did not observe rescue of *vpu* start codon mutations for the AD8 envelope. Using non-chimeric AD8 infectious clones, *vpu*- AD8 did confer significant (although variable) levels of virion release in primary macrophages, but always less efficiently than *vpu*+ AD8. However, while Schubert *et al.* implicated the AD8 envelope as the determinant for vpu-independent virion release by using NL4.3/AD8 chimeras similar to those described here, I did not. In this study, I used primary macrophages prepared from blood monocytes by elutriation [107] [234] similar to those used by Schubert *et al.*. Both studies also monitored multiple rounds of viral replication in macrophages. I produced viruses from transfected 293T cells for macrophage infections, whereas Schubert *et al.* produced them in HeLa cells. HeLa cells carry the host-cell restriction overcome by vpu whereas 293T cells do not [190] [263] [3] [216]. *vpu*- viruses produced from HeLa cells may have altered envelope content and infectivity compared with virus particles from 293T cells. However, such a difference

would only affect the initial infection stage, and cannot explain the enhanced virion release observed here for *vpu*+ viruses compared with the equivalent virion release of Schubert *et al*. for *vpu*+ and *vpu*- viruses over multiple rounds of replication in macrophages. I attempted to produce virus stocks from HeLa cells, like Schubert *et al*. Unfortunately, the transfection efficiency was too low to produce enough virus to infect macrophages.

The apparent differences between my data and the data in the previous report by Schubert *et al*. led me to undertake several control experiments. First, I assessed infectivity: particle ratios of the VSV G+ virions that were produced from 293T cells to make certain that *vpu*- virions conferred similar levels of infectivity as *vpu*+ virions. I confirmed that virus production in 293T cells is not affected by vpu. Next, I evaluated virus release in a single replication cycle and in a spreading infection to confirm that rescue of virion release by the AD8 envelope did not occur early before being overwhelmed by cell:cell spread. Low virus release by AD8 *vpu*- viruses is not due to the lack of infected cells, but results partly from decreased spread. Therefore, increased virus release for AD8 *vpu*+ viruses is partly due to the spread to, and infection of, more macrophages. I also discounted an effect by nef, which, like vpu, downregulates CD4 [165]. These control experiments failed to alter my conclusion that the envelopes studied here did not affect *vpu*- virion release. Finally, I tested *vpu*+ and *vpu*- chimeric viruses constructed in two different ways resulting in either full-length SF162 *vpu* and chimeric *env*, or chimeric *vpu*. However, the different AD8 or YU2 chimeric constructs consistently failed to show that the loss of vpu function could be rescued by either AD8

or YU-2 envelope for macrophage replication.

In this study, I focused entirely on the effects of vpu on HIV-1 replication in primary macrophages and have avoided studying HeLa cells. HeLa cells are frequently used to examine the effects of vpu defects on viral replication [2] [227] [263]. However, while HeLa cells are valuable tools for studying events *in vitro*, it is unclear whether they are representative of any cell type targeted by HIV *in vivo*. A rapidly dividing culture of HeLa cells may not accurately model a culture of terminally differentiated macrophages. Neil et al. reported that vpu prevents the internalization of nascent virions by tetherin from the cell surface in HeLa cells conferring more efficient release of virions [190]. In contrast, newly budded virions from primary macrophages are predominantly found in intracellular vesicles even when HIV-1 carries *vpu* [199]. It is unclear whether tetherin or additional factors expressed in macrophages prevent virus release, and are overcome by vpu. These observations suggest that the mechanisms that lead to virion endocytosis in the absence of vpu may be significantly more potent in macrophages compared to HeLa cells. Therefore, the requirement for vpu for virus release in macrophages may be significantly more robust than the vpu requirement in HeLa cells. This possibility could explain the inefficient replication in macrophages by *vpu*⁻ AD8 observed here, if the putative vpu-independent AD8 envelope could not overcome the macrophage-imparted block on virion release.

I noted considerable variation from macrophage batch to batch, which presumably reflected macrophage donor variation. Some batches of macrophages failed to yield sufficient virus particles to provide meaningful results for my experiments.

Immunostaining *in situ* for p24 at the end of these assays showed that low levels of infected cells were the cause. Whether low infectivity was due to poor infection conferred by VSV G, or to post-entry restrictions is unclear. The fluctuating results seen with the NL4.3/AD8 *vpu⁺ nef* virus may also be due to variation between batches of macrophages.

In summary, using chimeric viruses based on NL4.3, I did not find evidence that the AD8 envelope can significantly rescue loss of vpu for macrophage replication. Moreover, the envelope from the YU-2 clone that carries a vpu start codon mutation, and is highly macrophage-tropic, also failed to confer macrophage replication. The variable replication in macrophages observed for *vpu⁻* AD8 suggests the presence of a viral determinant (presumably not envelope) that partially compensates for vpu loss. However, these results do not yet support the presence of fully vpu-independent HIV-1 variants that could preclude the development of vpu inhibitors for therapy.

Chapter IV: HIV-1 macrophage tropism varies substantially over the course of infection

Introduction

The Human Immunodeficiency Virus (HIV) is an enveloped virus with glycoprotein spikes inserted into its plasma membrane [274] [295] [81]. These glycoprotein spikes are encoded by the virus' *env* gene, and are synthesized as a polyprotein, gp160 [6]. gp160 is proteolytically digested by cellular furin/ furin-like proteases into gp120 and gp41 in the Golgi [116]. gp120 is found on the outside of the virus particle, while gp41 is inserted into the plasma membrane. gp120 and gp41 are weakly associated via a noncovalent bond on the virus particle [206] [122]. gp120 is responsible for interacting with CD4, the main host cell receptor, and either CCR5 or CXCR4, the coreceptor, triggering a series of conformational changes in gp41 that causes fusion of the virus and host cell membranes and subsequent infection [144] [182] [63] [48] [71] [73] [219].

Macrophages are an important reservoir of virus during infection, especially during the late stages of infection, when CD4^{+} T cell counts decline sharply [133]. Viruses that use CCR5 as a coreceptor are called R5-tropic; R5-tropic viruses are also frequently described as macrophage tropic. However, wide variation in R5-tropic viruses' ability to infect macrophages has been reported [68] [150] [239]. Non-macrophage-tropic R5 viruses are prevalent in immune tissue and blood, while macrophage tropic viruses are readily found in brain tissue, especially in HIV[+] patients diagnosed with dementia [203]

[111]. R5 viruses from late stages of disease have been reported to be more macrophage tropic [113] [162] [257].

Macrophages express low levels of CD4 and CCR5 [156]. R5 macrophage tropic viruses have an enhanced ability to use these low levels of CD4 [201] [203]. Determinants of R5 macrophage tropism have been mapped to residues in the CD4 binding site on gp120, and to residues likely to impact exposure of the CD4 binding site [78] [79] [202]. These structural changes may have consequences for neutralizing antibodies.

HIV-specific antibodies can be detected in infected individuals 4-8 weeks after infection. The antibody response against gp120 can be very strong: a variety of antibodies to different epitopes are produced to high levels [1] [5]. However, this response is not effective because the virus is able to quickly evolve to avoid binding of antibodies. Escape from these neutralizing antibodies may be conferred by mutations in the sequences encoding antibody epitopes. Escape can also be conferred by envelope changes that result in the shielding of epitopes, either by the variable loops or glycan groups [95] [169] [146] [273] [252]. Conserved gp120 epitopes that are critical for interactions with receptors may be protected in these ways. Therefore, neutralizing antibodies in immune tissues may act as modulators of virus tropism by selecting for variants that carry envelopes with critical receptor binding sites protected. When these sites are protected, viruses are less likely to use low levels of CD4 and CCR5, as on macrophages.

It has been reported that macrophage tropism correlates with increased resistance to inhibition by monoclonal antibodies that bind CD4 [202]. This suggests that these

viruses' gp120s have an increased affinity for CD4, allowing them to utilize the low levels of CD4 on macrophages. Macrophage tropic R5 viruses are frequently sensitive to b12, a human monoclonal antibody that targets a region proximal to the CD4 binding site on gp120 [202] [38]. This suggests that macrophage tropic envelopes may also have a more open conformation around their CD4 binding sites in gp120, allowing greater access to neutralizing antibodies like b12. Neutralizing antibodies that target the CD4 binding site may confer a strong selection for virus variants that have this site protected [164]. I hypothesize that the presence of neutralizing antibodies drives the development of viral variants that are unable to use low levels of CD4, as on macrophages.

Here, I evaluated whether macrophage tropism of patient envelopes varies early in infection before and after autologous and heterologous antibodies appear. Using HXBc2 chimeric viruses containing gp120 sequences cloned from patient PBMC at different time points during disease, I evaluated if these patient envelopes were able to use low levels of CD4 in cell lines and macrophages. I compared when macrophage-tropism appeared to when autologous and heterologous neutralizing antibodies appeared, and found that R5 macrophage tropism varied substantially in the patients studied here.

Results
Characteristics of patients and envelopes

HIV-1 HXBc2 clones containing gp120 sequences were provided by Marlen Aasa-Chapman (University College London). Four homosexual men (MM1, MM4, MM8 and MM23) with symptomatic primary HIV-1 infection were studied; characteristics of their disease have been published previously [1]. Briefly, patients developed influenza-

like symptoms between 12 and 35 days post high risk exposure incident, and

subsequently developed HIV-1 specific antibodies. All patients remained antiretroviral

therapy naïve throughout the study. Patients were removed from the study when they

started antiretroviral therapy.

PBMC and plasma samples were collected when patients presented with

influenza-like symptoms, then at approximately three-month intervals. Envelopes,

specifically the gp120 segment, were amplified by PCR from PBMC, and cloned into the

HXBc2 backbone via unique BstEII and MluI restriction enzyme sites by M. Aasa-

Chapman. This created full-length replication competent chimeras. Heteroduplex

mapping was used to determine if the cloned patient gp120s represented the bulk of the

viral population. The majority of the cloned envelopes were representative of the major

species circulating at the time of sampling [1]. Table 4.1 summarizes the characteristics

of the patients and envelopes used in this study.

Table 4.1 Summary of Patient Envelope Characteristics

Patient ID	Clone #	Isolation Day	Coreceptor Usage
MM1	1.2.1	28	R5
	1.2.3	28	R5
	1.5.58	84	R5
	1.12.1	833	R5
	1.12.4	833	R5
	1.12.7	833	R5
	1.12.8	833	R5
	1.20.B	2702	R5
	1.20.C	2702	R5
	1.20.E	2702	R5
	1.20.F	2702	R5
	1.20.I	2702	R5
	1.20.J	2702	R5
	1.20.K	2702	R5
MM4	4.1.33	17	R5
	4.1.34	17	R5
	4.10.1	493	R5
	4.10.3	493	R5
	4.10.4	493	R5
	4.10.7	493	R5
	4.12.1	844	R5
	4.12.4	844	R5
	4.12.6	844	R5
	4.12.7	844	R5
MM8	8.2.50	12	R5
	8.2.51	12	R5
	8.4.51	32	R5
	8.8.1	608	R5
	8.8.3	608	R5
	8.8.4	608	R5
	8.8.8	608	R5
	8.9.B	957	R5X4
	8.9.D	957	R5X4
	8.9.H	957	R5X4
	8.9.I	957	R5X4
	8.9.J	957	R5X4
MM23	23.2.D	15	R5
	23.2.E	15	R5
	23.2.F	15	R5
	23.2.H	15	R5
	23.8.12	316	R5
	23.8.18	316	R5
	23.12.3	316	R5
	23.12.7	316	R5

Autologous and heterologous neutralization by patient plasma

Serum samples from each patient had previously been evaluated for the presence of antibodies that neutralized envelopes derived from the same patient (autologous neutralization) [1]. Patient MM8 developed autologous neutralizing antibodies between 2 and 3 months post infection. The remaining patients did not develop autologous neutralizing antibodies until at least 4 months post-infection. Neutralizing antibody titers were between 1:10 and 1:80 [1]. Non-neutralizing HIV$^+$ antibodies were detected between 12 and 28 days post-infection [1].

The ability of these patient antibodies to neutralize the T-cell line adapted strain IIIB was also assessed (heterologous neutralization). Patient antibodies were typically unable to neutralize IIIB until 3 months or more after the onset of symptoms (Fig. 4.1b, 4.2b, and 4.4b). This neutralization also tended to be weak (titers of < 1:10, up to 1:20) [1]. Patient MM8 did not develop heterologous neutralizing antibodies during the study (Fig. 4.3b).

Several of the patient envelopes were found to be neutralization sensitive to b12. These were: 4.10.1, 4.10.3, 8.8.3, 8.8.8, 23.8.12, and 23.12.7 (M. Aasa-Chapman, personal communication).

Infection of CD4$^+$ CCR5$^+$ cell lines and macrophages by patient chimeras

I assessed the titers of these patient envelopes on a cell line that expressed low levels of CD4 (RC49 cells), as well as primary macrophages. I also used a cell line that expressed high levels of CD4 (TZM-bl cells) to determine the titers of the patient chimera

stocks. I compared the titers of these stocks on RC49 cells and macrophages to their titers on TZM-bl cells to determine if the patient envelopes were macrophage-tropic.

Most viruses had high titers on TZM-bl cells (1×10^3 to 1×10^4 ffu/mL) (Fig. 4.1a, 4.2a, 4.3a, and 4.4a, red bars). The majority of viruses were unable to infect RC49 cells or macrophages efficiently (Fig. 4.1a, 4.2a, 4.3a, and 4.4a, blue bars or black bars, respectively). For this study, macrophage-tropic viruses were defined as viruses that had a minimum titer of 1000 ffu/mL on macrophages (see Appendix A for details). By this definition, the following viruses are macrophage-tropic: Patient MM1 1.2.3, 1.20.B, 1.20.E, 1.20.I; Patient MM4 4.10.3, 4.10.7; Patient MM8 8.2.50, 8.8.3, 8.8.4, 8.9.B, 8.9.D8.9.H, 8.9.I, 8.9.J. Patient MM23 did not develop any macrophage-tropic envelopes. These macrophage-tropic envelopes did not cluster at any particular time point, but were found at different time points throughout infection.

Variation in macrophage tropism was seen between envelopes derived from the same PBMC sample. Three of the seven envelopes from PBMC collected on day 2702 from Patient MM1 were macrophage tropic (Fig. 4.1a). Two of the four envelopes derived from PBMC collected on day 493 from Patient MM4 were macrophage tropic (Fig. 4.2a). Two of the four envelopes derived from PBMC collected on day 608 from Patient MM8 were macrophage tropic. All five envelopes from day 957 were macrophage tropic (Fig. 4.3a).

Patient MM1

a)

b)

Fig. 4.1 Patient MM1 a)Titers on TZM-bl, RC49, and macrophages. TZM-bl cells, RC49 cells, and macrophages were infected, fixed and stained as described in the Materials and Methods. The titer for each virus was then calculated. Blue stars indicate macrophage-tropic viruses. b) heterologous neutralization of IIIB Neutralization assays were done by M. Aasa-Chapman at University College London. Neutralization was calculated by IC90.

Patient MM4

Fig. 4.2 Patient MM4 a) Titers on TZM-bl, RC49, and macrophages. TZM-bl cells, RC49 cells, and macrophages were infected, fixed and stained as described in the Materials and Methods. The titer for each virus was then calculated. Blue stars indicate macrophage-tropic viruses. b) heterologous neutralization of IIIB. Neutralization assays were done by M. Aasa-Chapman at University College London. Neutralization was calculated by IC90.

Patient MM8

a)

Fig. 4.3 Patient MM8 a)Titers on TZM-bl, RC49, and macrophages. TZM-bl cells, RC49 cells, and macrophages were infected, fixed and stained as described in the Materials and Methods. The titer for each virus was then calculated. Blue stars indicate macrophage-tropic viruses. b) heterologous neutralization of IIIB. Neutralization assays were done by M. Aasa-Chapman at University College London. Neutralization was calculated by IC90.

Patient MM23

a)
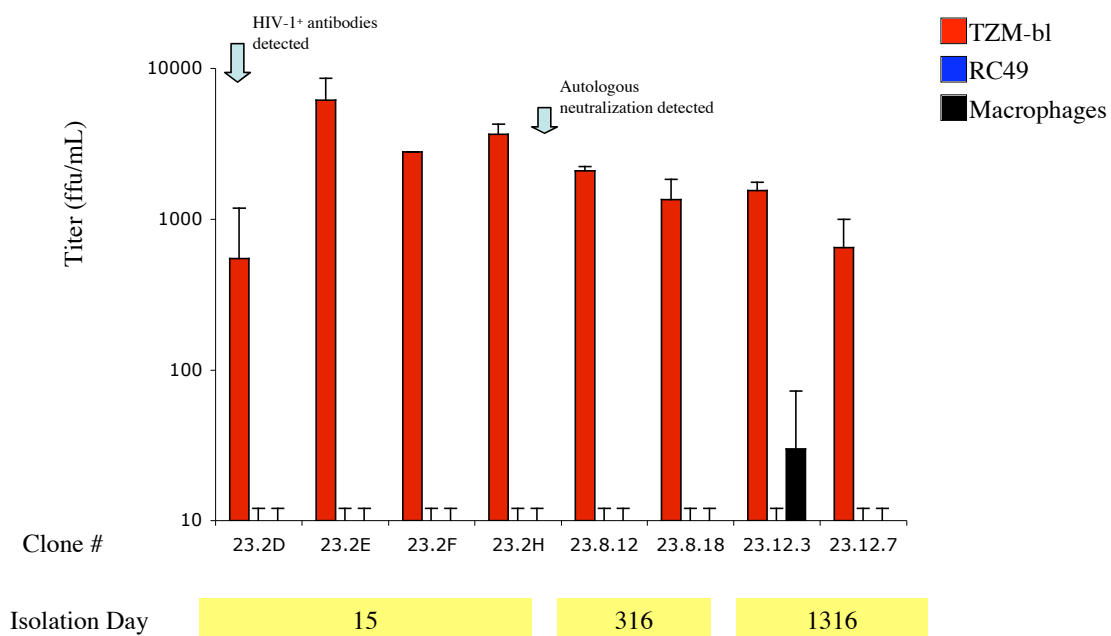
Fig. 4.4 Patient MM23 a)Titers on TZM-bl, RC49, and macrophages. TZM-bl cells, RC49 cells, and macrophages were infected, fixed and stained as described in the Materials and Methods. The titer for each virus was then calculated. Blue stars indicate macrophage-tropic viruses. b) heterologous neutralization of IIIB. Neutralization assays were done by M. Aasa-Chapman at University College London. Neutralization was calculated by IC90.

The effect of HXBc2 gp41 on macrophage tropism

In the chimeras used in this study, only the gp120 segment of envelope from patient PBMC was cloned into the HXBc2 backbone. It was possible that the HXBc2 gp41 could influence the phenotype conferred by patient gp120s, and their abilities to use low levels of CD4. I replaced the gp41 of a control virus, HXBc2/ YU-2 gp120, with the gp41 of YU-2, a macrophage-tropic virus. The infectivity of this envelope was compared with that of two other envelopes, B33 and LN85. These gp160s had previously been cloned into NL4.3, and their macrophage-tropism had been well-characterized. B33 is macrophage-tropic, while LN85 is not [201].

HXBc2/YU-2 gp120 and HXBc2/ YU-2 gp160 infected RC49 cells and macrophages with similar efficiencies (Fig. 4.5). NL4.3/B33 also infected RC49 cells and macrophages, while NL4.3/LN85 did not, as expected (Fig. 4.5).
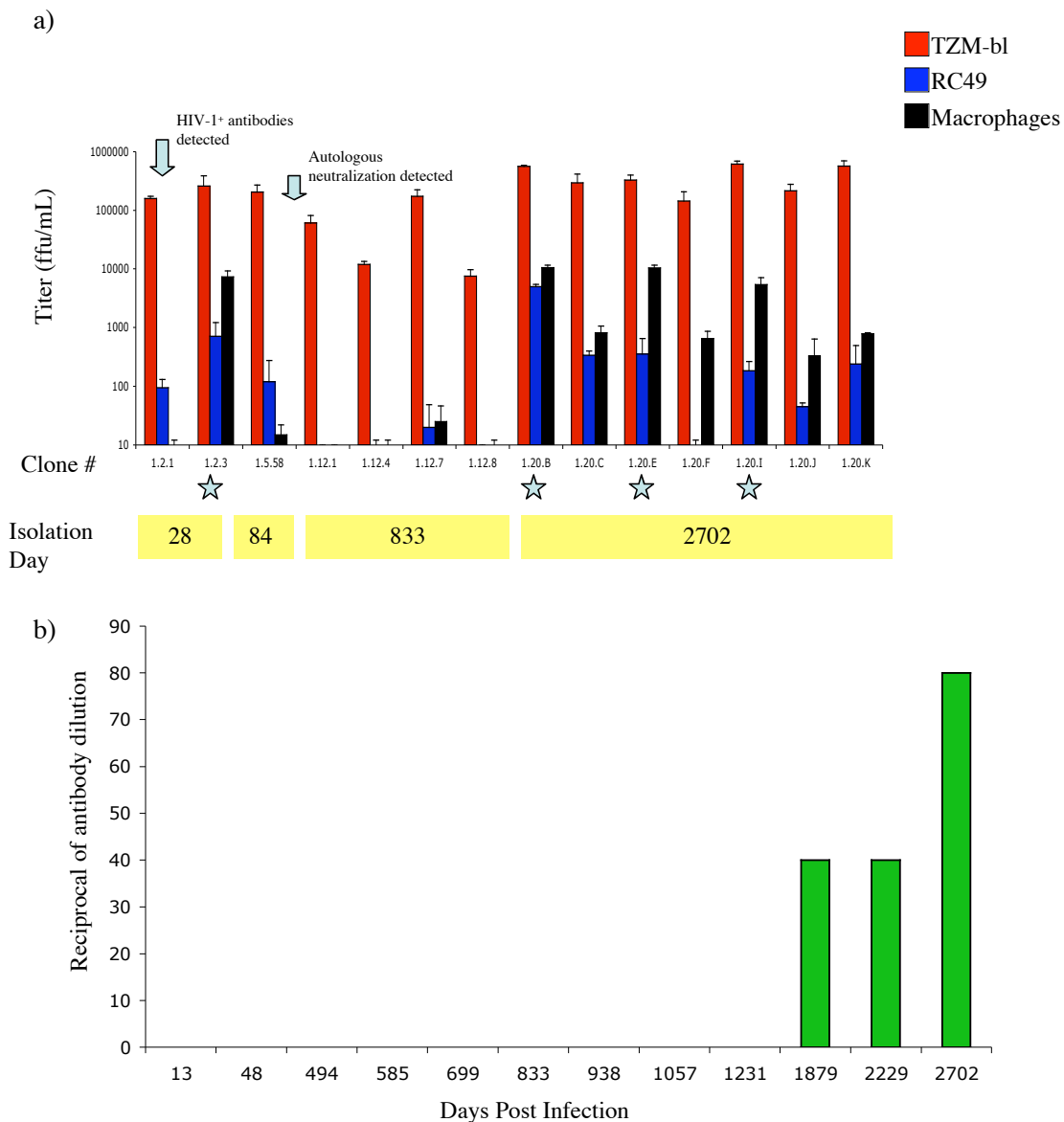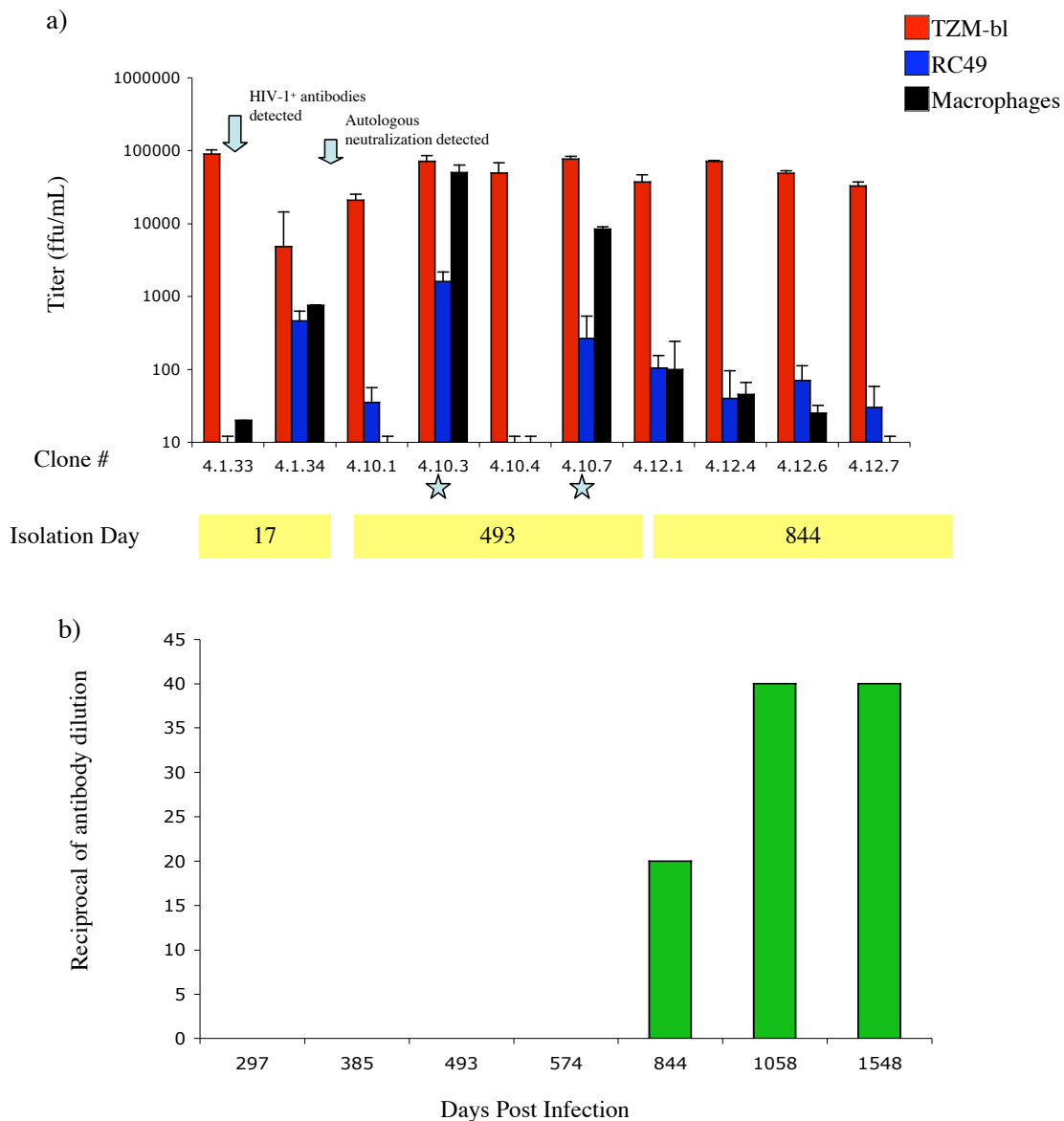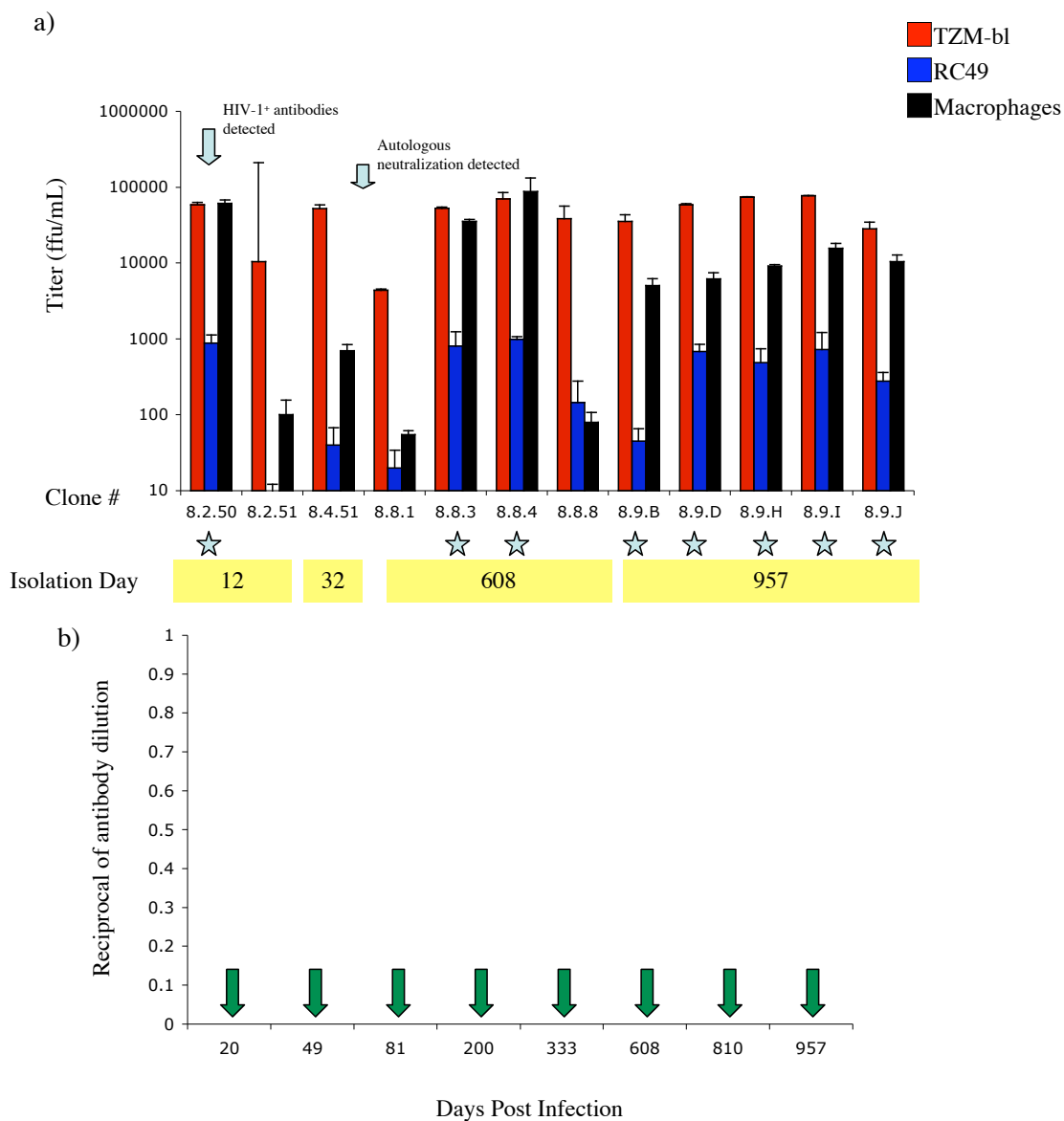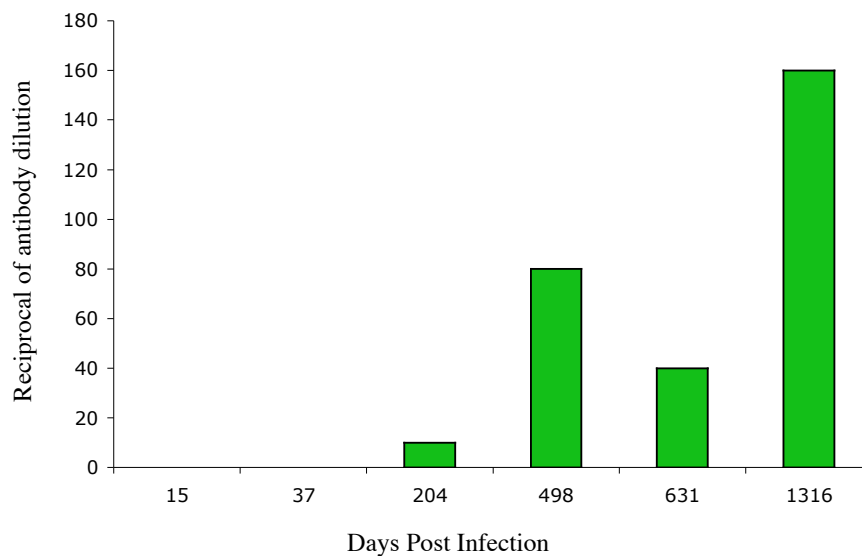
Controls
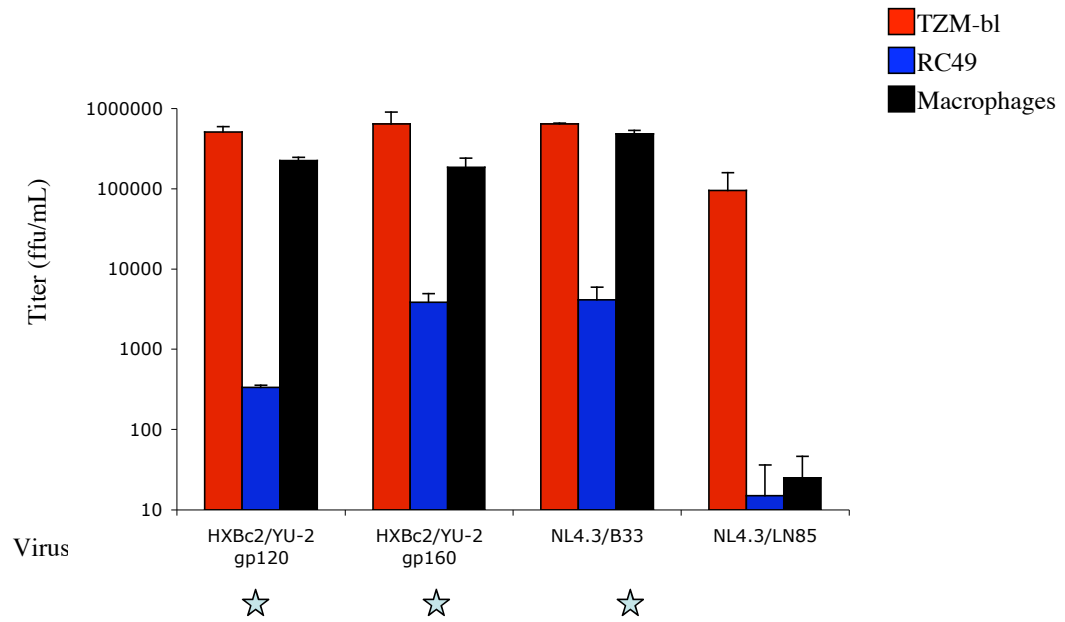
Fig. 4.5 Titers of control viruses on TZM-bl, RC49 and macrophages TZM-bl cells, RC49 cells, and macrophages were infected, fixed and stained as described in the Materials and Methods. The titer for each virus was then calculated. Blue stars indicate macrophage-tropic viruses.

Discussion

Here, I investigated the macrophage tropism of envelopes derived from patient PBMC from different time points during infection. For Patient MM1, macrophage tropic envelopes appeared at day 28 (1.2.3) and day 2702 (1.20.B, 1.20.E, 1.20.I) (Fig. 4.1a). For Patient MM4, macrophage tropic envelopes appeared at day 493 (4.10.3, 4.10.7) (Fig. 4.2a). For Patient MM8, macrophage tropic envelopes appeared at day 12 (8.2.50, day 608 (8.8.3, 8.8.4), and day 957 (8.9.B, 8.9.D, 8.9.H, 8.9.I, 8.9.J) (Fig. 4.3a). Patient MM23 had no macrophage tropic envelopes appear (Fig. 4.4a) Replacement of the gp41 in the control chimera HXBc2/ YU-2 gp120 did not affect macrophage-tropism (Fig. 4.5).

The viruses used in this project were gp120s amplified from patient PBMC, then cloned into an HIV-1 HXBc2 background. This approach results in chimeric envelopes that carry gp41 from HXBc2. It has been show recently that determinants in gp41 may impact the coreceptor tropism of gp120 [131]. It is therefore possible that the gp41 of HXBc2 impacts the tropism of gp120 sequences cloned into it. To ascertain whether HXBc2 gp41 affected the properties of a heterologous gp120, I removed the HXBc2 from HXBc2/ YU-2 gp120, and replaced it with that from YU-2 to create HXBc2/YU-2 gp160. I saw no difference in this virus' ability to infect TZM-bl cells, RC49 cells, or macrophages compared to HXBc2/YU-2 gp120 (Fig. 4.5). The HXBc2 molecular clone also has the unique feature of having a non-functional *vpu* gene, due to a mutated start codon (ATG to ACG). It is possible that this may also impact the properties of gp120s cloned into HXBc2. Both of these observations should be taken into account when interpreting results using this molecular clone.

Patients developed autologous neutralizing antibodies as early as 2 months post infection (Patient MM8), and as late as 4 months post infection (Patients MM1 and MM4, and MM23). Non-neutralizing HIV[+] antibodies appeared much earlier, between 12 and 28 days in infection. In two patients, MM1 and MM8, macrophage-tropic envelopes appeared after the initial antibody response, but before the appearance of autologous antibodies. In Patients MM1, MM4, and MM8, macrophage-tropic envelopes continued to appear after autologous neutralizing antibodies. These results suggest that the presence of neutralizing antibodies is not solely responsible for driving envelopes away from macrophage-tropism. It is likely that macrophage-tropism gives viruses an evolutionary advantage *in vivo*. One of these advantages may be that macrophages provide an excellent reservoir for HIV-1 infection.

It has been reported that enhanced macrophage-tropism can be conferred by changes in the CD4 binding site in gp120 [78] [79] [202]. These changes increase envelope's affinity for CD4, and consequently confer infection of cells expressing low levels of CD4. In addition, better exposure of the CD4 binding site may have a similar effect on gp120:CD4 affinity. R5 macrophage-tropic envelopes may be able to use different isoforms of CD4 or CCR5 on macrophages, or be more efficient at using attachment factors such as syndecans and C-type lectins [256]. Residues that enhance the fusogenicity of envelopes may also be responsible for macrophage-tropism. Sterjovki *et al*. reported that an asparagine at position 362 in the C3 region contributed to enhanced fusogenicity of R5 envelopes [246]. The loss of a N-linked glycosylation site at position 386 in the V4 loop has been found to enhance macrophage-tropism of envelopes [79].

The V3 loop, responsible for coreceptor usage, may also have a role in envelopes' ability to bind to low levels of CCR5. There are still other undefined determinants of macrophage-tropism in gp120. None of the envelopes described here carry the N283 motif, which has been reported to confer increased CD4 affinity and macrophage tropism [78] [79]. Two of the four envelopes from day 608 from Patient MM8 are macrophage-tropic. An alignment of these four sequences did not reveal consistent differences that could account for their distinct macrophage-tropism properties. It is likely that the macrophage-tropic envelopes presented here have still undefined determinants of macrophage-tropism, such as increased fusion ability.

In conclusion, the appearance of macrophage-tropic envelopes vary substantially over the course of infection in the patients studied here. These envelopes appeared before and after autologous neutralizing antibodies. This suggests that the presence of neutralizing antibodies does not necessarily force envelopes away from macrophage-tropism. Retaining macrophage-tropism, despite antibody pressure, must give HIV an evolutionary advantage. As macrophages provide a reservoir for infection, it would be valuable to examine how envelopes retain macrophage-tropism over time.

Chapter V: HIV-1 gp41 cytoplasmic domains of two envelopes carry determinants of virion infectivity

Introduction

The Human Immunodeficiency Virus (HIV) is an enveloped virus with glycoprotein spikes inserted into its plasma membrane [274] [295] [81]. These glycoprotein spikes are encoded by the virus' *env* gene, and are synthesized as a polyprotein, gp160 [6]. gp160 is proteolytically digested by cellular furin/ furin-like proteases into gp120 and gp41 in the Golgi [116]. gp120 is found on the outside of the virus particle, while gp41 is inserted into the plasma membrane. gp120 and gp41 are weakly associated via a noncovalent bond on the virus particle [206] [122]. gp120 is responsible for interacting with CD4, the main host cell receptor, and either CCR5 or CXCR4, the coreceptor, triggering a series of conformational changes in gp41 that causes fusion of the virus and host cell membranes and subsequent infection [144] [182] [63] [48] [71] [73] [90] [219]. These conformational changes in gp41 allow the fusion peptide in gp41 to insert into the target cell membrane. The 6-helix bundle brings the viral and cell membranes together, and the viral core is released into the cytoplasm of the cell [276] [96] [221] [70] [75] [76].

The cytoplasmic domain of gp41 contains several functional motifs responsible for assembly of envelope onto virus particles, and infectivity. Fig. 5.1 shows a schematic of gp41 in the virus plasma membrane, and Table 5.1 summarizes theses motifs. gp41 can be broken into 3 general domains: the ectodomain, the transmembrane domain, and the cytoplasmic domain. The ectodomain contains the fusion peptide and heptad repeats,

responsible for forming the 6-helix bundle needed to bring the viral and host cell membranes together for fusion and entry [42] [275]. It also carries determinants for interacting with gp120 [122]. The transmembrane domain anchors gp41 in the viral plasma membrane [267].

The cytoplasmic domain contains many functional motifs. The YSPL motif is an endocytosis signal. It recruits AP-2 in clathrin-coated pits for this purpose [32] [27] [21] [194]. The Kennedy/ Dimmock loop has no known function; however, it has been proposed to loop outside the viral plasma membrane and form a neutralizing antibody epitope [51] [43]. There are two palmitoylation sites at positions 764 and 837. These palmitoylation sites are responsible for envelope targeting to lipid rafts, which is an important step in virus assembly at the host cell membrane. These are relatively conserved cysteines; the 764 residue is more conserved. If these cysteines are mutated to amino acids with bulky hydrophobic side chains, infectivity is retained [213] [287]. The PRA1 binding site binds the cellular protein PRA1, a protein associated with Golgi membranes [85]. The YW motif binds TIP47, a cellular protein responsible for bringing gag and envelope together, and recruiting rab9, which is involved in vesicular trafficking [26] [166] [45]. The dileucine motif at the end of the cytoplasmic domain is responsible for binding AP-1 in clathrin-coated pits [286].
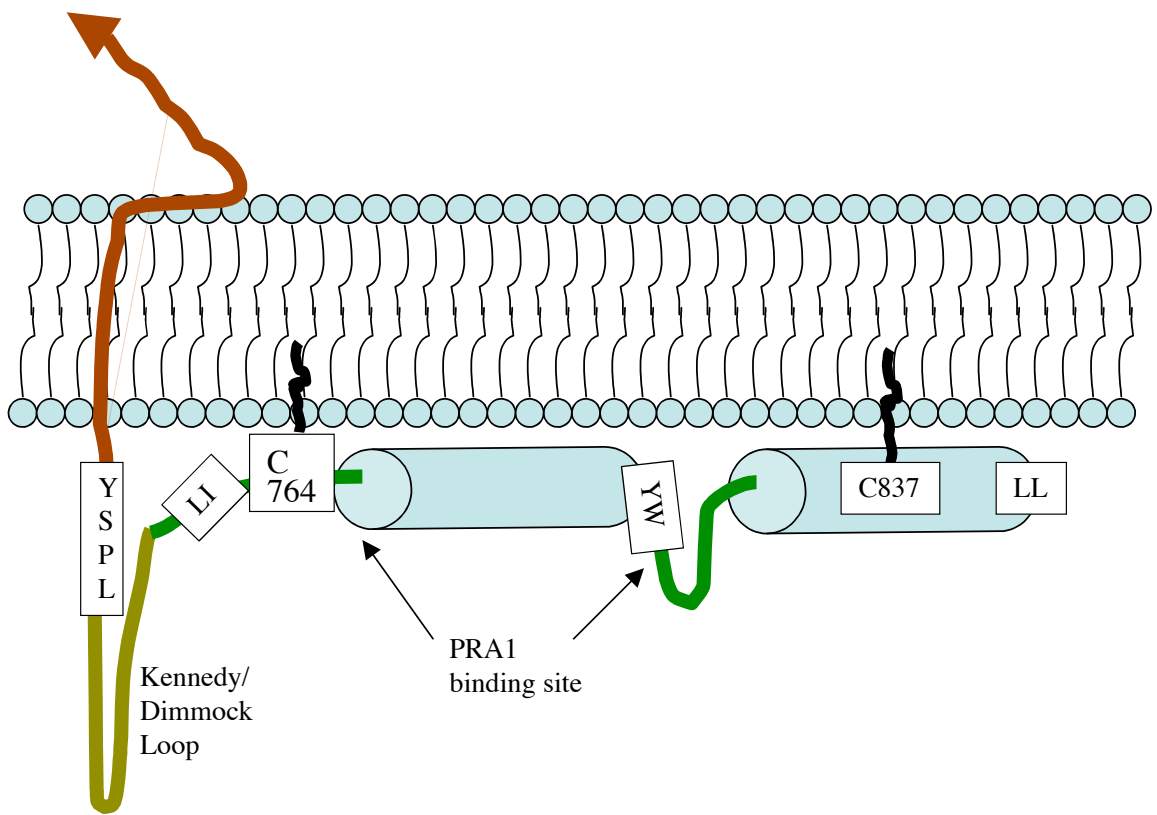
Fig. 5.1 Schematic of gp41 cytoplasmic domain, with locations of functional domains.

Table 5.1: Summary of functional motifs in the cytoplasmic domain of gp41

gp41 Cytoplasmic Domain Motif	Position	Function	Reference
YSPL	712-715	Endocytosis signal, recruits AP-2 in clathrin-coated pits	[32] [27] [21] [194]
Kennedy/Dimmock Loop	724-745	Unknown	[51] [43]
LI	755-756	Dileucine motif	[286]
C	764	Palmitoylation site	[213] [287]
LLP 2/3	770-796	Lentilytic peptides; interact with membranes	[186] [145]
PRA1 binding site	766-807	Interacts with PRA1, a protein associated with Golgi membranes	[85]
YW	802-803	TIP47 binding site	[26] [166] [45]
LLP-1	828-856	Lentilytic peptide; interacts with membranes	[186] [185]
F	837	Palmitoylation site	[213] [287]
LL	855-856	Dileucine motif	[286]

Our lab has previously reported on two envelopes derived from two separate tissues from the same patient. One envelope from brain, B33, confers high levels of infectivity, while the other envelope from lymph node, LN40, confers low levels of infectivity despite being fusogenic in cell:cell assays. When the entire gp41 of LN40 is replaced with that of B33, there is a restoration of infectivity [24]. I hypothesize that there are determinants in the gp41 of LN40 that cause its weak infectivity.

Here, the determinants of infectivity in gp41 were investigated using these two envelopes, B33 and LN40. Chimeric envelopes as well as point mutations were constructed. I found that no chimera or combination of point mutations completely restored infectivity; however changes in the first palmitoylation site at position 764, the Pr55^{gag} binding region at positions 787 and 788, combined with restoration of the dileucine motif at position 856 increased LN40 infectivity.

Results

Introduction to B33 and LN40 and construction of chimeras

B33 and LN40 are envelopes derived from two separate tissues from the same patient. B33 was derived from brain tissue; LN40 was derived from lymph node tissue [201]. B33 envelope confers high levels of infectivity to pseudovirions, while LN40 envelope confers low levels of infectivity (Fig. 5.2a). Both of these envelopes are fusogenic in cell:cell fusion assays (Fig. 5.2b).

a)

b)
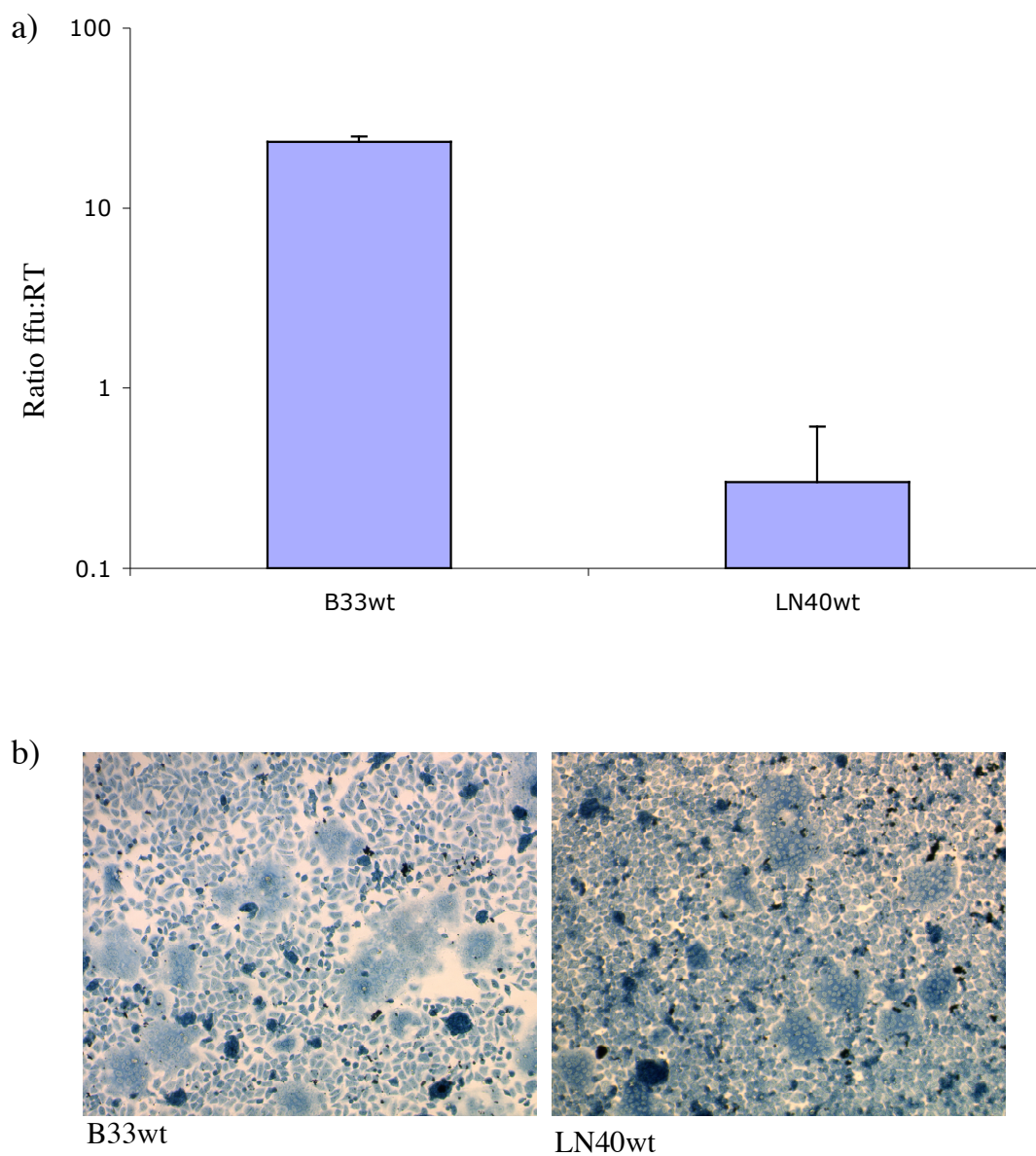
B33wt

LN40wt

Fig. 5.2 Characteristics of B33wt and LN40wt. a) Ratio of ffu: RT for B33 wt and LN40 wt. Virus stocks were titrated onto TZM-bl cells. Cells were fixed and stained 48 hours post-infection; stained cells were counted, and the ffu/mL value was calculated. The RT (in pg/mL) of the stocks was quantified by RT-ELISA. b) Cell:cell fusion assay. 293T cells were transfected with viral DNA; 24 hours later transfected cells were mixed with TZM-bl cells.Cells were fixed and stained when syncytia appeared. See text and Materials and Methods for experimental detail. B33wt-transfected cells form syncytia more quickly (4 hours) than LN40wt-transfected cells (6 hours).

There are fourteen amino acid differences between B33 and LN40 in gp41. These differences are highlighted in Fig. 5.3. Several are in, or near, functional domains. There are three differences within the transmembrane domain, one at the palmitoylation site (position 764), two at a potential Pr55^{gag} binding site (positions 787 and 788), two proximal to the TIP47 binding site (positions 687 and 690), and one in the dileucine motif at the end of LLP-1 (position 856). The remaining five differences are not in any defined functional domains.
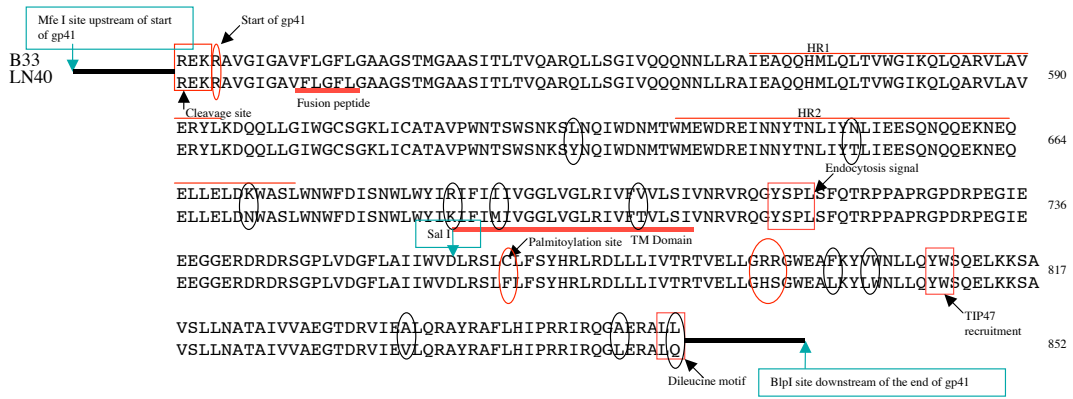
Fig. 5.3 Amino acid alignment of B33 and LN40 gp41 sequences. Important functional domains are highlighted in red. Differences between B33 and LN40 are circled. Unique enzyme sites used to construct chimeras are marked in green. Reference amino acid numbers are on the right.

Several chimeras of B33 and LN40 envelopes were constructed using unique MfeI, SalI, and BlpI sites (Fig. 5.4a). I constructed two chimeras using the MfeI site at the start of gp41, and the BlpI site at the end of gp41. One chimera has the gp120 of B33, and the complete gp41 of LN40; the other has the reverse (Fig. 5.4b). I replaced the RR in LN40/B33wt MfeI-BlpI at positions 787 and 788 with HS (Fig. 5.4b). This was to assess the effects of these potentially important gag binding sites on infectivity.

I constructed a second set of chimeras using the SalI site just upstream of the palmitoylation site at position 764. One chimera has the SalI-BlpI fragment of LN40 placed into B33; the other has the reverse (Fig. 5.4c). Again, I replaced the RR in LN40/B33 SalI-BlpI with HS (Fig. 5.4c).

The following results are all from using env^+ pseudovirions. B33, LN40, their chimeras, or their variant envelopes are all in the pSVIIIenv expression vector. These vectors are co-transfected with pNL4.3 env^- to create envelope$^+$ pseudovirons (see Materials and Methods for details).
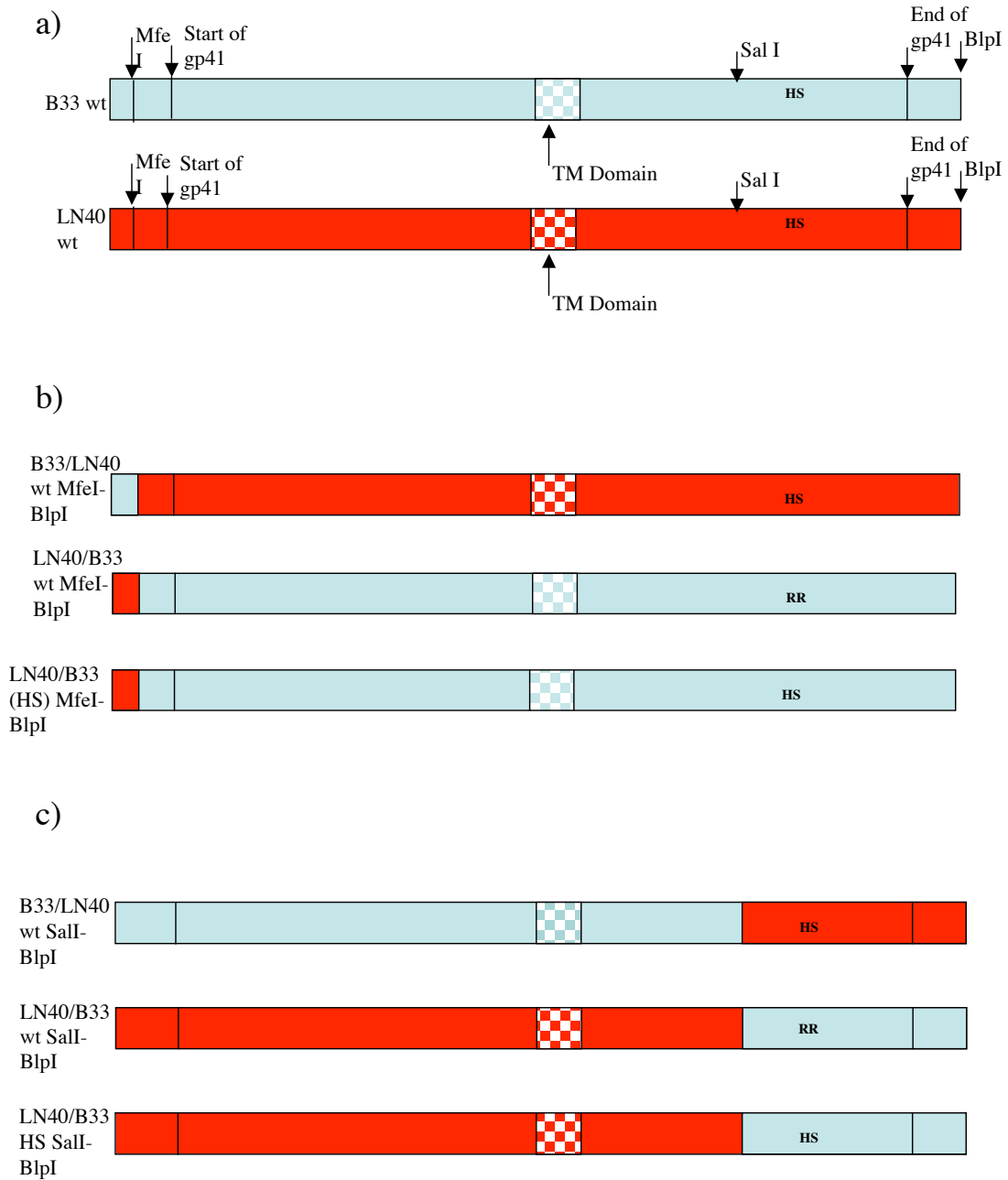
Fig. 5.4 Schematics of B33 and LN40 chimeras used in this study. a) B33 and LN40 wt, with unique restriction sites marked, b) B33/LN40 MfeI-BlpI chimeras, c) B33/LN40 SalI-BlpI chimeras

Mapping of determinants of infectivity in gp41 using MfeI-BlpI and SalI-BlpI chimeras

I first evaluated the infectivity of chimeric envelopes where the entire gp41 sequences of B33 or LN40 had been exchanged. For LN40/B33wt MfeI-BlpI, infectivity was restored, but was still about 10-fold lower than B33wt (Fig.5.5a). I also observed an approximately 3-fold reduction in infectivity in the B33/LN40wt MfeI-BlpI chimera, compared to B33wt (Fig. 5.5a)

Next, I evaluated chimeras in which the C-terminal end of the gp41 cytoplasmic domain (between the SalI and BlpI site) had been exchanged. A restoration of infectivity was observed for LN40/B33wt SalI-BlpI, although infectivity was about 6-fold lower than LN40/B33wt MfeI-BlpI, and about 16-fold lower than B33wt (Fig. 5.5b). There was also a reduction in infectivity in the B33/LN40wt SalI-BlpI chimera, compared to B33wt (Fig. 5.5b). This reduction was similar to the reduction seen for the B33/LN40wt MfeI-BlpI chimera. These results confirm that gp41 plays an important role in LN40 infectivity.

a)

b)
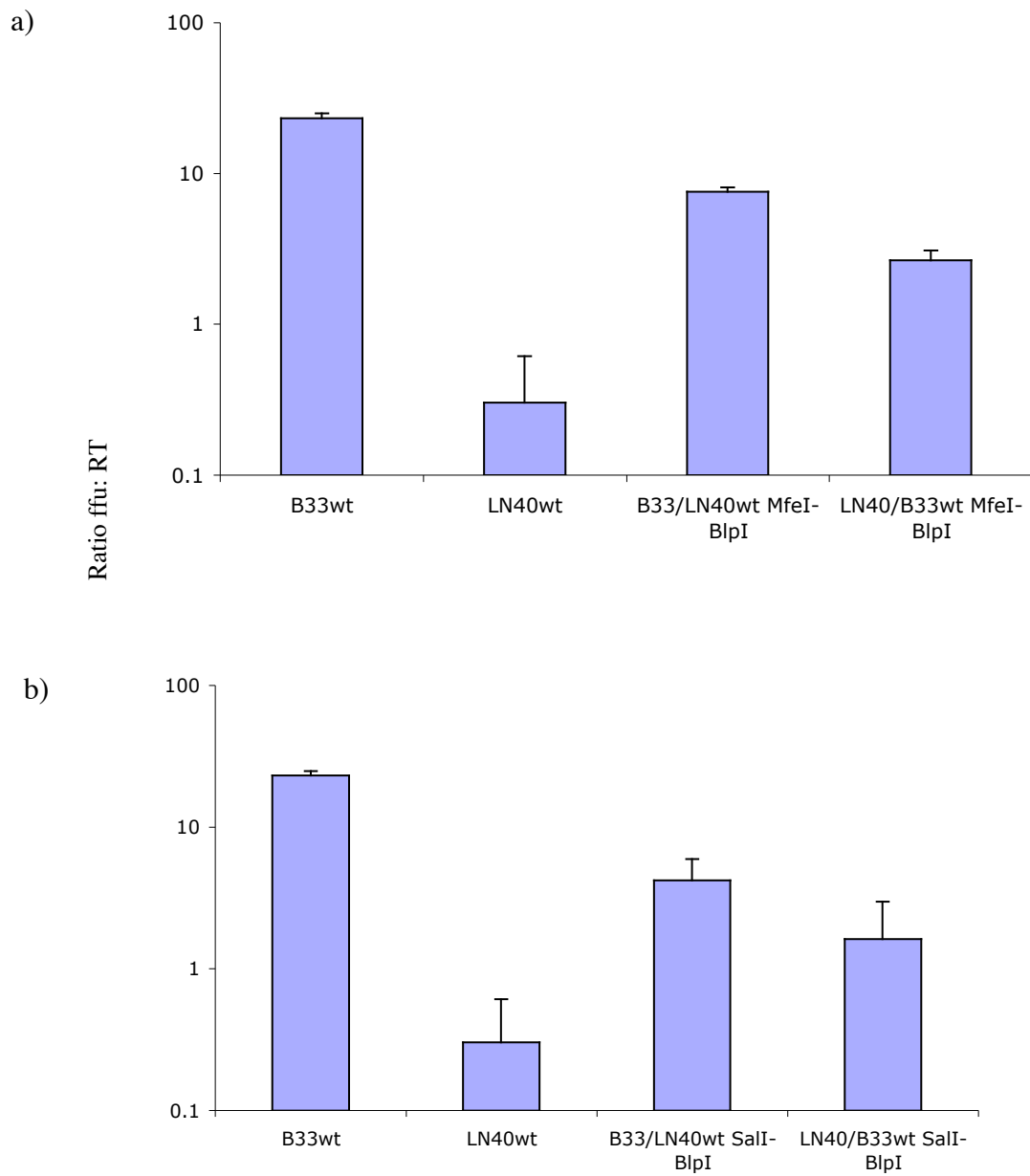
Fig. 5.5 Ratio of ffu:RT of B33wt, LN40wt, and B33/LN40 chimeras. a) MfeI-BlpI chimeras, b) SalI-BlpI chimeras. See Fig.5.2 and 5.4 legend for more detail.

The palmitoylation site at position 764

There is a conserved palmitoylation site at position 764 in gp41 that is responsible for targeting envelope to lipid rafts in the host cell plasma membrane [213][23][287]. The residue at this position is usually a cysteine, but can also be an amino acid with a bulky hydrophobic side chain for the virus to retain infectivity. In B33wt, position 764 is the conserved cysteine, whereas in LN40wt this residue is a phenylalanine. To determine if this palmitoylation site plays a role in LN40's reduced infectivity, the phenylalanine (F) in LN40 was substituted with a cysteine (C). We found that changing the F to a C did not increase the infectivity of LN40 (Fig. 5.6).

The gag binding site at positions 787 and 788

Two arginines in positions 787 and 788 in gp41 lie within a region of gp41 previously implicated in binding Pr55^{gag} [24][127][188]. B33wt has these two arginines. In LN40, these residues are substituted by a histidine and a serine at 787 and 788, respectively.

To determine if these residues were responsible for the difference in infectivity between LN40 and B33, several versions of LN40 and B33 with different mutations in these sites were constructed. These sites were also mutated in two of the chimeras made previously, LN40/B33wt MfeI-BlpI and LN40/B33wt SalI-BlpI (Fig. 5.4b and c).

I observed a slight reduction in infectivity when I substituted HS for RR in B33, compared to B33wt (Fig. 5.7a). The HS present in the two chimeras had some effect on infectivity. LN40/B33 HS MfeI-BlpI showed a 3-fold reduction in infectivity compared

to LN40/B33wt MfeI-BlpI (Fig. 5.7a). LN40/B33 HS SalI-BlpI showed a very slight

reduction compared to LN40/B33wt SalI-BlpI (Fig. 5.7a).

Specific mutations were made in the palmitoylation site at position 764 and in the

RR/HS residues at positions 787 and 788. For LN40, changing the F-HS to C-RR resulted

in a 4-fold increase in infectivity compared to LN40wt (Fig. 5.7b). However, other

combinations of residues, including LN40 F-RS, F-HR, C-RS, and C-HR had little

impact on infectivity (Fig. 5.7b). These results indicate that the HS is detrimental to

LN40 infectivity but has less effect on B33 infectivity.
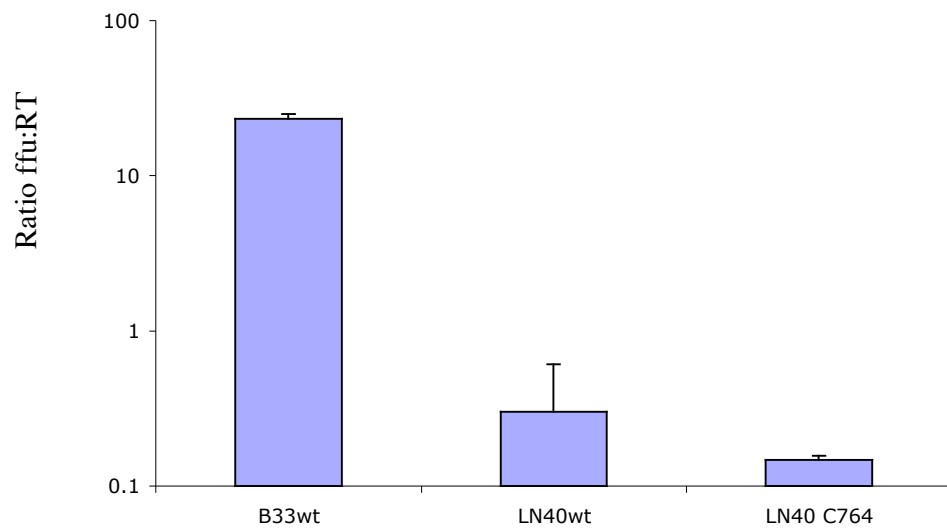
Fig. 5.6 Ratio of ffu:RT: The effect of the palmitoylation site at position 764. B33 wt, LN40 wt, and LN40 C. See Fig. 5.4 legend for more detail.

a)

b)
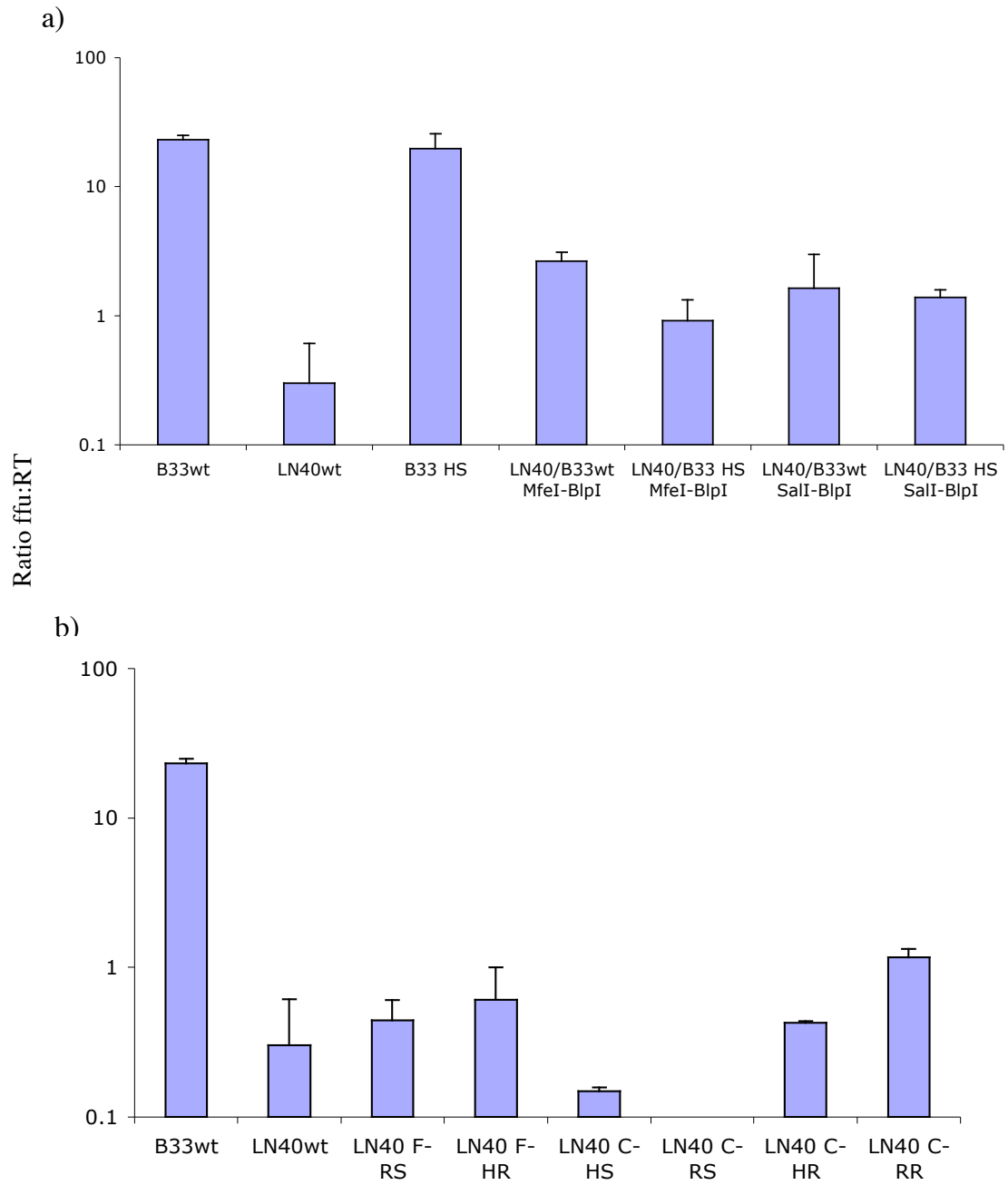
Fig. 5.7 Ratio of ffu:RT: The effects of the gag binding site at positions 787 and 788. a) The effects in chimeras. b) The effects as point mutations. See Fig. 5.4 legend for more detail.

TIP47 binding region at positions 793 and 796

TIP47 is a cellular protein that has been reported to connect gag and envelope [166]. TIP47 also recruits Rab9 GTPase, which directs endosome-to-Golgi vesicular transport [26][45]. The TIP47 binding motif at positions 802 and 803 is a tyrosine followed by a tryptophan (YW). This motif is preserved in LN40; however there are two changes just upstream of this site at 793 and 796, which may influence the function of the YW motif. In B33, these amino acids are a phenylalanine (F) and a valine (V), while in LN40, there are leucines (L) in both sites.

These sites were changed to FV in both LN40wt and LN40 C-RR. I found that changing these sites from two leucines to FV did not increase the infectivity of LN40 in either the wt or C-RR forms (Fig. 5.8).

Dileucine motif at position 855 and 856

There is a dileucine motif at the end of the cytoplasmic domain of gp41 that is important for recruiting AP-1 found in clathrin-coated pits [286]. This dileucine motif is not present in LN40wt, but is present in B33wt. I restored the dileucine motif in LN40wt, as well as in LN40 C-RR. Restoration of the dileucine motif in LN40wt increases infectivity slightly. However restoration of the dileucine motif in LN40 C-RR conferred a 24-fold increase in infectivity compared to LN40wt (Fig. 5.9).
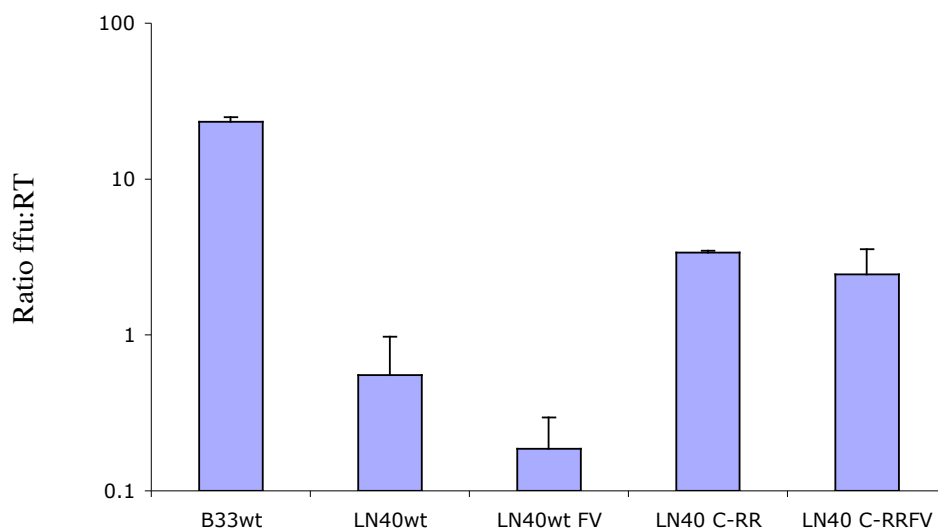
Fig. 5.8 Ratio of ffu:RT: The TIP47 binding region at positions 793 and 796. See Fig. 5.4 legend for more detail.
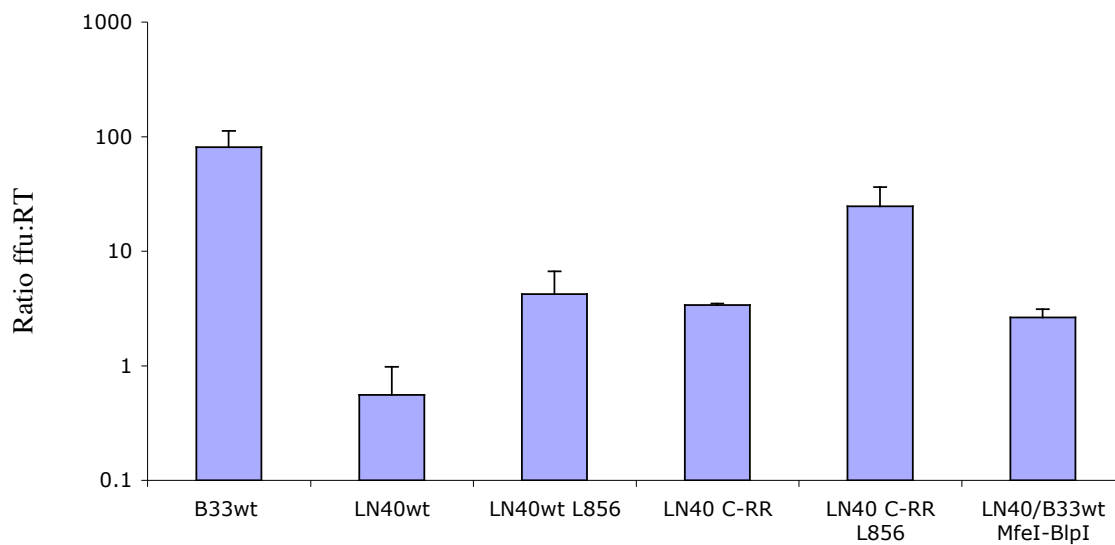
Fig. 5.9 Ratio of ffu:RT: The dileucine motif at position 856. See Fig. 5.4 legend for more detail.

Motifs between the MfeI and SalI sites

Above, I have described a number of changes made in LN40wt. All of these

changes were downstream from the Kennedy/ Dimmock loop, between the SalI and BlpI

sites in the C-terminal of gp41. Data from the LN40/B33 MfeI-BlpI chimera (carrying the

entire B33 gp41), and the LN40/B33wt SalI-BlpI chimera (carrying only the cytoplasmic

domain beyond the Kennedy/ Dimmock loop) indicate that there are possibly

determinants between the MfeI and SalI sites (Fig.5.7a). To explore the differences

between B33wt and LN40wt upstream of the SalI site, I substituted amino acids in LN40

for those in B33wt. These included a tyrosine-to-leucine change (position 625), a

threonine-to-asparagine change (position 649), an asparagine-to-lysine change (position

669), lysine-to-arginine and methionine-to-isoleucine changes (positions 687 and 690,

respectively), and a threonine to valine change (position 702). These substitutions were

all constructed separately, with the exception of the lysine-to-arginine and methionine-to-

isoleucine changes in the transmembrane domain. Because these were only two amino

acids apart, the substitutions were made together. All of these substitutions were made in

LN40wt and LN40 C-RRL856.

These substitutions had no effect on LN40 infectivity in either the LN40wt or

LN40 C-RRL856 backgrounds, and may even adversely affect infectivity (Fig. 5.10)

a)

b)
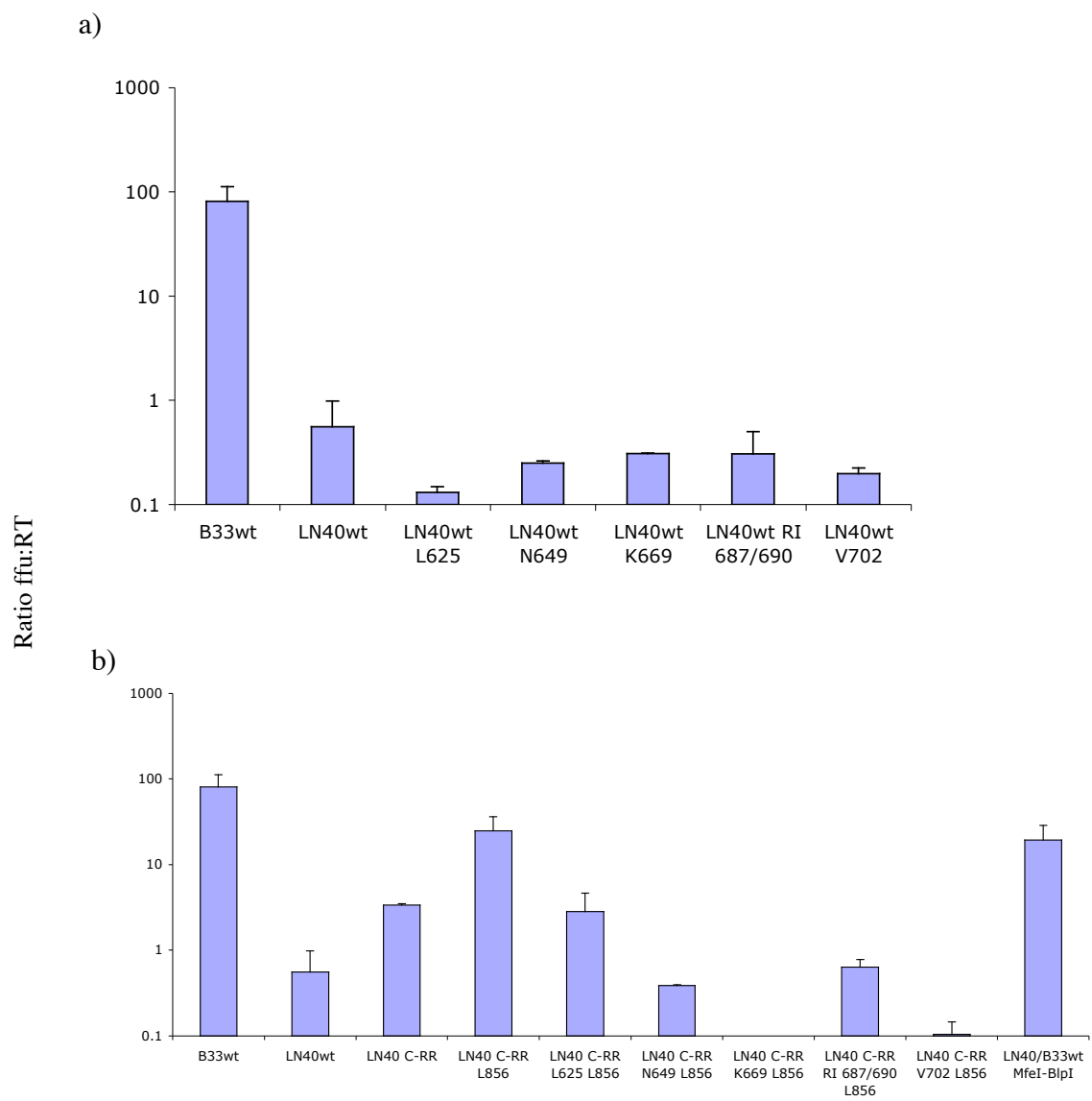
Fig.5.10 Ratio of ffu:RT: Motifs between the MfeI and SalI sites. a)Point mutations in LN40wt. b) Point mutations in LN40 C-RRL856, with LN40/B33wt MfeI-BlpI for comparison. See Fig. 5.4 legend for more detail.

Assembly of envelope onto virus particles

Previous data suggested that LN40wt virus particles had less envelope on them than B33wt virus particles as detected by Western blot [24]. I first examined properties of envelope[+] pseudovirions concentrated by centrifugation. Virus particles in envelope[+] pseudotype stocks were pelleted, and resuspended in DMEM. The titer (ffu/mL), RT activity (pg/mL, a measure of physical virus particles), and gp120 (ng/mL) content of the virus particles were estimated (see Materials and Methods). Using this method, I found that B33wt particles have about twice as much gp120 than LN40wt particles (Table 5.2).

I attempted to confirm this data, as well as the previous data, by evaluating envelope concentration of pelleted virus particles by Western blot using a monoclonal antibody to gp41, and was unable to do so. These Western blots were inconclusive, and showed similar amounts of envelope on both B33wt and LN40wt virus particles (Fig. 5.11a). When these envelopes were expressed alone without other viral proteins, envelope was still detected after filtration and centrifugation (Fig. 5.11b). There is "leak through" of envelope not on virions in our preparations. This leak through makes interpretation of the Western blots problematic. It also suggests that the data in Table 5.2 do not accurately represent envelope content on virions.

Table 5.2 Envelope content on B33wt and LN40wt virus particles

Virus:	Titer (ffu/mL)	RT (pg/mL)	gp120 (ng/mL)	Ratio gp120:RT	Percentage gp120:RT ratio (of B33wt)
B33wt	1.32×10^6	255858	46.1625	2×10^{-4}	100
LN40wt	2.6×10^4	253265	24.2525	9.6×10^{-5}	53
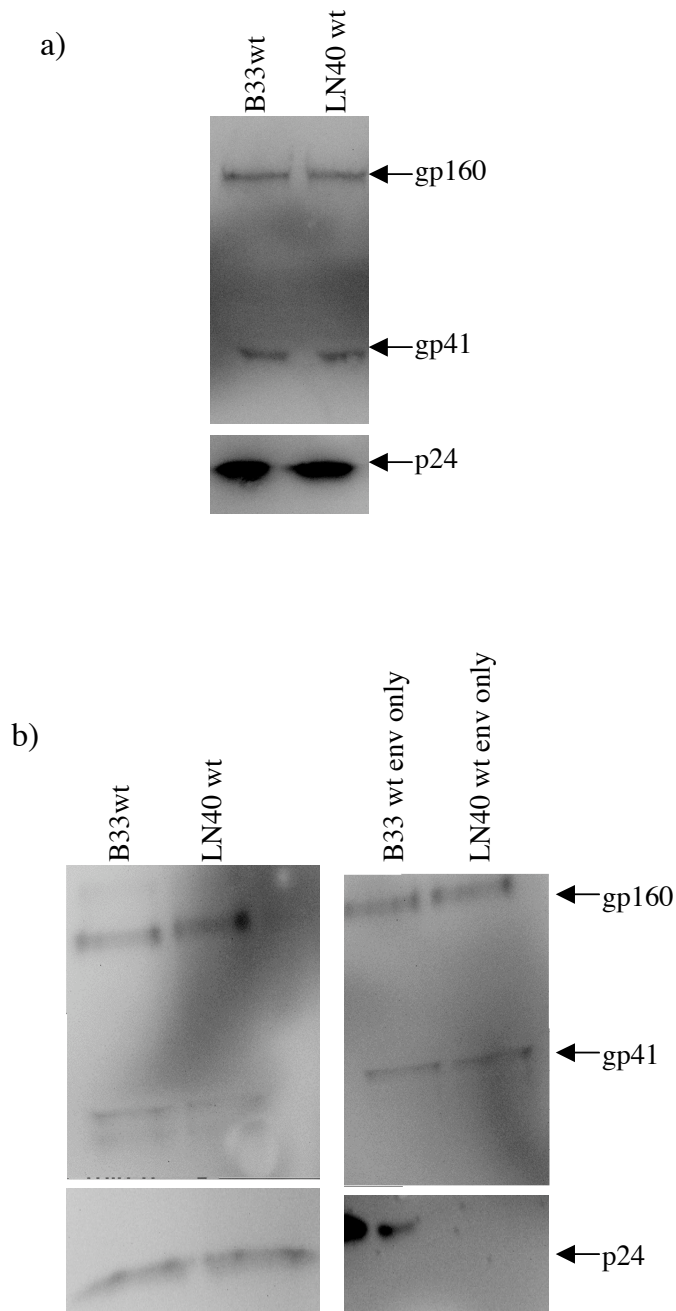
Fig. 5.11 Western blots for gp41 and p24. a) Representative Western blot of virus particles; b) Representative Western blot of virus particles and envelope only samples. Supernatants from transfections werespun at 3000rpm, filtered through .45um filters, then spun at 22,000rpm for 2 hours at 4^0C. Pellets were resuspended in 20uL Laemmli buffer, and loaded onto an SDS-PAGE gel. Blots were probed for gp41 and p24

Discussion

Here, I have identified determinants in the gp41 of LN40 envelope that are responsible for its decreased infectivity compared to B33. The LN40 chimera containing the entire gp41 of B33, LN40/B33wt MfeI-BlpI, has the greatest increase on LN40 infectivity (Fig. 5.5a). Several of these infectivity determinants are present in sites with known functions, including the palmitoylation site (position 764; Fig. 5.6), gag-binding sites (positions 787 and 788; Fig. 5.7), and the dileucine motif at the C-terminus (position 856; Fig. 5.9). The greatest effect on LN40 infectivity is seen when these determinants contribute together (Fig. 5.10b). Point mutations proximal to the TIP47 binding motif had no effect on LN40 infectivity (Fig. 5.8). Other point mutations between the MfeI and SalI sites also had no effect on LN40 infectivity (Fig. 5.10). Experiments to determine if LN40wt virus particles carried less envelope than B33wt particles were inconclusive due to problems with protein contamination of non-virion envelope following particle concentration (Fig. 5.11b).

Data from the MfeI-BlpI and SalI-BlpI chimeras indicated that important infectivity determinants were in gp41. LN40/B33 chimeras showed an increase in infectivity, while B33/LN40 chimeras showed slight decreases in infectivity (Fig. 5.5). These results led me to investigate motifs in gp41, specifically in the cytoplasmic domain. The palmitoylation residue at position 764, usually a conserved cysteine, is a phenylalanine in LN40. Reportedly, this is still acceptable, as envelopes with amino acids with bulky hydrophobic side chains in this position also retain infectivity [23][213][287]. Changing the phenylalanine to a cysteine in LN40 unsurprisingly had little impact.

The residues at positions 787 and 788 in gp41 were previously reported to be important for binding Pr55^{gag} [213]. These residues are usually two arginines. In LN40, they are a histidine and a serine. Changing these residues to two arginines, along with restoration of the cysteine at the palmitoylation site, result in a modest increase in infectivity. Changing these two arginines to histidine and serine in B33 has no effect on infectivity. Replacing these residues with histidine and serine in the LN40/B33 chimeras also has little effect on infectivity. This suggests that the effect of these Pr55^{gag} binding residues is context dependent.

The dileucine motif at the C-terminus recruits AP-1 in clathrin-coated pits [286]. Restoration of this motif results in an increase in infectivity, especially in LN40 C-RR. The largest increase in infectivity is seen in the quadruple mutant, LN40 C-RR L856, when L856 is combined with C-RR. It is clear that a combination of these four residues make a major contribution to the low infectivity of LN40.

The simplest explanation for the differences in infectivity between LN40 and B33 is that LN40 envelope has an assembly defect as previously reported; however this could not be confirmed. If B33 and LN40 carry similar levels envelope on virions, and are both fusogenic, then the gp41 determinants identified here may conceivably contribute to an uncoating, post-fusion stage of entry.

When the LN40 gp41 was introduced in B33, there was only a marginal effect on infection, indicating that these gp41 determinants are context dependent. In addition, B33 gp41 failed to rescue LN40 infectivity to the level of B33, suggesting that determinants in gp120 also contribute. LN40 and B33 gp120s were reported to confer different levels of

infection in primary macrophages [201]. The capacity to use low CD4 may make B33

gp120 more efficient for fusion and overcome gp41 defects that either limit envelope

incorporation onto virions, or adversely affect entry events.

In conclusion, replacing the entire gp41 of LN40 with that of B33 results in an

increase in infectivity. Determinants within gp41 are part of the explanation for the

decreased infectivity of LN40; however, no combination of determinants completely

restores LN40 infectivity. It is likely that differences in gp120 between LN40 and B33

are the reasons for low LN40 infectivity. These differences may lie in the CD4 binding

site, V3 coreceptor binding regions, or in other undefined regions associated with the

ability to use low levels of CD4 for infection, like on macrophages. Mapping these

determinants in gp120 may help elucidate what regions are responsible for macrophage-

tropism.

General Discussion

In this thesis, I present data from three projects. These projects focused on three very different aspects of HIV-1 biology, but they ultimately had one common goal: the understanding of HIV-1 pathogenesis in macrophages. Infected macrophages play an important role during *in vivo* HIV infection; however some aspects of the relationship between HIV and macrophages are still undefined.

The first project I presented in this thesis explored the potential for vpu-independent envelopes to evolve *in vivo*. I looked at two envelopes, AD8 and YU-2, which had previously been reported to be vpu-independent [227] [163]. NL4.3/AD8 *vpu*⁻ and NL4.3/YU-2 *vpu*⁻ both were released to lower levels from macrophages than their *vpu*⁺ counterparts (Fig. 3.3). Full length AD8 *vpu*⁻ and full length YU-2 *vpu*⁻ were also released to lower levels from macrophages compared to their *vpu*⁺ counterparts (Fig. 3.2) These data show that neither the AD8 nor the YU-2 envelope were able to compensate for the lack of functional vpu, and are therefore not vpu-independent, contrary to these previous reports. In the discussion section of Chapter III, I went into detail about why my results did not agree with previously published reports. Here, I will discuss the implications of my results, speculate on their importance and propose future work for this project.

As a protein with ion channel characteristics important for its virus release function, vpu is an attractive target for ion channel blockers, such as amiloride derivatives [86]. Since HIV-1 can easily evolve to escape other drugs, such as protease

and reverse transcriptase inhibitors, it is possible that HIV-1 could evolve to escape

amiloride derivatives as well, if they become widely used. In this thesis, I present data

that show how absolutely essential vpu is to sustaining infection in macrophages.

Without functional vpu, viruses are unable to maintain high levels of virus output. It is

likely that treating patients with amiloride derivatives may vastly decrease the number of

macrophages infected, and the amount of virus infected macrophages are able to release.

Envelopes are not likely to evolve the ability to compensate for the lack of functional

vpu. Envelope is dependent upon functional vpu in two ways. One, envelope needs

functional vpu in order to eliminate CD4:envelope complexes from the endoplasmic

reticulum. Two, envelope, and the virus as a whole, require functional vpu for virus

particles to be released from host cell membranes. It would probably require a significant

number of changes in envelope to compensate for the lack of functional vpu, caused by

the presence of amiloride derivatives. This means that these drugs may be a valid

alternative for antiretroviral therapy. Future work may include passaging viruses through

macrophages in the presence of amiloride derivatives to check that the envelopes do not

evolve to compensate for the lack of functional vpu.

This project initially began with a very limited survey of *vpu* sequences derived

from a variety of patient tissues. The goal was to find patients with mutated *vpu* genes,

and study the envelopes associated with those mutated *vpu* genes. This sequence survey

was discontinued when the AD8 envelope was found not to be vpu-independent. I chose

to focus on the effects of non-functional *vpu* genes on HIV-1 infection of macrophages,

and try to resolve why the AD8 envelope was not vpu-independent. It may be useful to

resume this survey, since an exhaustive search for *vpu*-start codon mutations has never been done.

Two *vpu* start codon mutations from patient material have been reported, both from the brain tissue of infected patients [163] [255]. It may be especially useful to look specifically at brain tissue, or specific cell types in brain tissue using laser capture microscopy. If macrophages are the main route to the brain, *vpu* start codon mutations present there implies either a) a *vpu⁻* virus infected a macrophage, and that macrophage subsequently entered the brain, or b) that *vpu* start codon mutations can evolve in brain tissue *in vivo*, when sequestered from immune pressures. *Vpu⁻* viruses are known to express more envelope than *vpu⁺* viruses [227] [245]. The presence of more envelope may lead to more syncytia formation, and therefore more neuronal damage. *Vpu⁻* viruses' presence in the brain may lead to increased HIV-associated dementia.

The second project I presented in this thesis explored the relationship between the presence of neutralizing antibodies and macrophage-tropic envelopes over the course of infection. I assessed the macrophage-tropism of envelopes derived from different time points from four patients, knowing their neutralizing antibody profiles. All patients developed autologous neutralizing antibodies by 3 months post infection; however, only three of the four patients developed heterologous neutralizing antibodies (Patients MM1, 4, and 23; Figs. 4.1b, 4.2b, 4.3b, 4.4b). Patients MM1, MM4, and MM8 developed macrophage-tropic envelopes (Figs. 4.1a, 4.2a, 4.3a). Patient MM23 did not (Fig. 4.4a). Of the macrophage-tropic envelopes presented here, only two were also sensitive to the human monoclonal antibody b12. These data show that macrophage-tropism varies

substantially over the course of infection, and the presence of autologous neutralizing antibodies did not predict the absence of macrophage-tropic envelopes. Also, sensitivity to b12 neutralization did not correlate with macrophage-tropism, as has been previously reported with other envelopes derived from patient material [202].

HIV-1 is not unique in its ability to infect macrophages. The ability to infect, and replicate in, non-dividing cells is a characteristic of lentiviruses in general. HIV-1 has expanded its tropism to also infect CD4$^+$ T cells [266]. Infection of CD4$^+$ T cells is convenient and effective for HIV, yet HIV still retains the ability to infect macrophages. From an evolutionary point of view, retaining the ability to infect macrophages must give HIV an advantage. This advantage is likely linked to the fact that macrophages tolerate HIV-1 infection, exhibiting no cytopathic effects [266]. Macrophages provide a great reservoir for HIV-1, especially in later stages of infection, when the CD4$^+$ T cells have declined drastically [133]. In the patients presented in this thesis, macrophage-tropic envelopes appeared during the asymptomatic stages of infection, when CD4$^+$ T cell counts were still above 200 cells/µl. This suggests that the acquisition of macrophage-tropism occurs well before the onset of CD4$^+$ T cell decline, and is retained over time.

The patients examined here were discharged from the study when they commenced HAART. Because of this, there is no data from these patients from the symptomatic stages of infection. It would be interesting to examine envelopes from these patients in the late stages to see if their macrophage-tropism is retained, or if the number of macrophage-tropic envelopes present increases. It would also be interesting to see of the commencement of HAART affects the macrophage-tropism of these patients'

envelopes. There is evidence as well that suggests the compartmentalization of macrophage-tropic envelopes (macrophage-tropic envelopes are more prevalent in brain than in blood) is less obvious after long-term antiretroviral therapy (D. Gabuzda, P. Clapham, personal communications). These patients would be good to examine to see if this breakdown of compartmentalization does exist.

The patients presented here all developed strong autologous neutralizing antibody responses. However, they did not develop strong heterologous neutralizing antibody responses. These patients' antibody responses were very specific to the envelopes that evolved during their infections, but their responses were not broadly neutralizing. This is worrisome, since a vast majority of vaccine strategies developed against HIV depend on creating a broadly neutralizing antibody response. This may not be possible, and more work should be done to develop vaccines that utilize the cell-mediated response as well as the antibody response.

In two patients, macrophage-tropic envelopes appeared before the detection of autologous neutralizing antibodies, and these patients retained this macrophage-tropism. The third patient had macrophage-tropic envelopes after the appearance of autologous neutralizing antibodies. If macrophage-tropic envelopes are more sensitive to neutralizing antibodies, these envelopes should disappear after the onset of the antibody response. This is not the case in the patients presented here. Therefore, the antibody response does not drive viruses away from macrophage-tropism. The driving force behind macrophage-tropism is more likely the slow decline of alternative target cells as disease progresses.

Macrophage-tropic envelopes tend to be sensitive to the human monoclonal antibody b12. The b12 antibody is unique. It has an unusual structure for an antibody. It is an IgG antibody with an extended hypervariable loop that can fit into gp120, unlike other IgG antibodies [211, 217]. b12 binds to an area proximal to the CD4 binding site on gp120. The CD4 binding site on gp120 is tucked up inside the trimer before contact with CD4 [152]. Upon binding, the CD4 binding site becomes exposed, and a series of conformational changes occur in gp120 and gp41 that result in the fusion of the viral and host cell membranes. When the CD4 binding site is tucked away in the native gp120 conformation, the virus typically needs high levels of CD4 in order to infect cells. However, some envelopes have a more exposed CD4 binding site, which is thought to help them use lower levels of CD4, like on macrophages. Macrophage-tropic envelopes probably have a more exposed CD4 binding site than non-macrophage-tropic envelopes. Since b12 binds a region proximal to the CD4 binding site, envelopes that are macrophage-tropic tend to be more sensitive to b12 neutralization because their CD4 binding site is more exposed.

Of the macrophage-tropic envelopes presented in this thesis, only two are also sensitive to b12 neutralization (4.10.3 and 8.8.3). This suggests that these two envelopes have more exposed CD4 biding sites, but that the other macrophage-tropic envelopes do not. The CD4 binding site is an important determinant of macrophage-tropism, but it is not the only determinant. The V3 loop, which carries determinants for coreceptor usage, and the V4 loop, which has an N-linked glycosylation site important for macrophage infection, also provide determinants of macrophage-tropism [125] [132] [208][79]. There

are likely many other determinants of macrophage-tropism, in gp120, and possibly gp41, that need to be mapped.

The envelopes from these four patients may provide an excellent way to map these additional determinants. Chimeric gp120 sequences could be created using envelopes from the same point in which one or more envelopes are macrophage-tropic, and one or more envelopes are not macrophage-tropic. Sections of gp120 could be swapped back and forth between these envelopes to find what sections of gp120 can make a non-macrophage-tropic envelope able to infect macrophages.

Other work may include using anti-CD4 antibodies to elucidate the structure of these macrophage-tropic envelopes. These envelopes have not been tested for their sensitivity to anti-CD4 antibodies, which may be useful. The structures of the b12 sensitive macrophage-tropic envelopes versus the b12 resistant macrophage-tropic envelopes are possibly different. This work may suggest what changes have occurred in these envelopes' CD4 binding sites.

The third project I presented in this thesis explored possible determinants in gp41 responsible for infectivity. Two envelopes, B33 and LN40, are from two separate tissues from one patient; they have very different characteristics of infectivity on cells expressing high levels of CD4 (Fig. 5.2a). When the gp41 of LN40 is replaced with that of B33, there is a restoration in infectivity (Fig. 5.5a). Changes in the palmitoylation site (at position 765), $Pr55^{gag}$ binding sites (at positions 787 and 788), and the dileucine motif at the C-terminal (at position 852) increase the infectivity of LN40, but these changes do not completely restore infectivity (Fig 5.9). Virus particle isolation and Western blots to

determine the amount of envelope on LN40wt virus particles were inconclusive due to non-virion particle contamination (Fig. 5.11; Appendix B).

While the combination of determinants described here increase the infectivity of LN40 slightly, it is unknown if these determinants are universal. The amino acids in these determinants in LN40 could be placed in well-characterized molecular clones, such as NL4.3 and AD8, to assess whether they decrease infectivity. It is possible that these amino acids are context dependent, in that they only decrease the infectivity of the LN40 envelope.

This project began with the observation that replacing the gp41 of LN40 with that of B33 restored infectivity to LN40 envelope. gp41 has many attractive domain that may impact infectivity. Therefore, I began trying to map these determinants of infectivity. As I created new chimeras and point mutations, I found that no combination of determinants completely restored infectivity to LN40, even when its entire gp41 is replaced. There are likely additional determinants in gp120 that impact LN40's infectivity.

These determinants may be in LN40 envelope's inability to use low levels of CD4. LN40 is not macrophage-tropic, while B33 is macrophage-tropic. The B33 gp120 may have a more exposed CD4 binding site than LN40 gp120. LN40 is resistant to b12 neutralization, which is consistent with previous reports that non-macrophage-tropic envelopes are more resistant to b12 neutralization because their CD4 binding sites are hidden [202].

As was mentioned above, there are still undefined determinants of macrophage-tropism outside of the CD4 binding site. The B33 and LN40 envelopes provide an

opportunity to map these determinants because they are closely related. Creating chimeras using gp120 sequences from B33 and LN40 may be a way of mapping these determinants.

A key question in this project is how much envelope is present on B33 and Ln40 pseudotyped virus particles. Previous work in my lab indicated that there was less envelope on LN40 particles compared to B33 particles [24]. This provided a fairly simple explanation: LN40 gp41 had an assembly defect responsible for this envelope's decreased infectivity. Unfortunately, I was unable to repeat this previous result. There seems to be non-virion protein contamination in my preparations, which makes interpreting the amount of envelope on LN40 virus particles difficult. Using Optiprep gradients may provide the best solution for eliminating this non-virion contamination. I tried using Optiprep gradients, but there was not enough antigen in the gradient to detect on the Western Blot. Increasing the amount of antigen loaded onto the gradient before centrifugation would solve this issue. The immunoprecipitation protocol used to obtain virus particles from the steps in the gradient may also need to be optimized to extract the maximum amount of antigen. However, radioactively labeling the antigen in the gradient would allow me to skip the immunoprecipitation protocol, and would be more direct. Once the virus particle isolation protocol has been optimized, the LN40 chimeric and point mutation constructs can also be assessed for the amount of envelope assembled onto particles. It may be interesting to find if one of the constructs improves the assembly of envelope onto LN40 virus particles.

Since the experiments for this project were done using pseudotyped virus particles, is also possible that the differences in infectivity between B33 and LN40 may be due to problems in LN40's ability to NL4.3 gag. LN40 may be able to bind to its own gag better. Unfortunately, it would be extremely difficult to derive the gag that came from the same provirus as LN40. However, gag sequences from the same autopsy lymph node tissue could be amplified, and LN40 envelope could be pseudotyped onto these gag sequences. Another option is immunoprecipitation and Western blotting of LN40 pseudotyped onto NL4.3 particles could clarify if this envelope properly binds to NL4.3 gag.

The B33 and LN40 envelopes provide an interesting snapshot of envelopes at the end stages of AIDS. Both envelopes were derived from tissues at autopsy of an HIV$^+$ patient that died of complications of AIDS [201]. B33 was derived from brain tissue, and is very macrophage-tropic. This suggests that at some point, this virus infected a macrophage in the blood, and was carried to the brain. LN40, from the lymph node tissue, is not macrophage-tropic. This envelope was restricted to T cells, and the blood. These two envelopes provide a unique case of macrophage-tropism becoming useful at one point in infection, and therefore retained. Developing macrophage-tropism expanded the repertoire of cells the viruses in this patient could infect.

HIV has impacted the entire population of the planet Earth. This virus has gained a fantastic foothold into humans. It has become uniquely adapted to its host, exploiting a necessary cell type of the immune system: macrophages. Why and how HIV infects macrophages are complex questions.

HIV uses macrophages as a reservoir over the course of infection. This allows HIV to persist, without interference from the rest of the immune system. If we can find a way to prevent HIV infection of macrophages, we may have a chance at beating AIDS. Macrophages may be some of the first cells infected. If this initial event could be prevented, the person may not become infected at all. However, this requires understanding the initial events of HIV infection of macrophages, including the characteristics of viruses that successfully infect macrophages, and what could happen if treatments targeting genes important for macrophage infection are used. The key to preventing and treating disease is understanding it at its most basic level. The basic understanding of HIV infection of macrophages is still needed.

In this thesis, I attempted to contribute to this understanding. There is still a great deal to be explored, but I hope the work I presented here provides some insight. Thank you, and goodnight.

Appendix A: Primary data for Chapter IV

The following Appendix contains primary data from one set of infections for Patients MM1, MM4, MM8, MM23, along with control viruses. The following tables have the titer (in ffu/mL) from TZM-bl, RC49 and macrophage infections, in duplicate, the average titers from these infections, and the standard deviation.

These tables also have the percent infectivity for each virus on TZM-bl cells, RC49 cells, and macrophages. The percent infectivity was calculated by dividing the average titer on the cell type of interest by the average titer on TZM-bl cells, and multiplying by 100.

Appendix A: Chapter 4 Primary Data

Patient MM1

Clone:	Isolation Time Point	TZM-bl					RC49					Macrophages				
		FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl
1.2.1	28	170000	150000	160000	14142.1356	100	70	120	95	35.3553391	0.059375	0	10	5	7.07106781	0.003125
1.2.3	28	170000	350000	260000	127279.221	100	350	1070	710	509.116882	0.27307692	8700	5900	7300	1979.89899	2.80769231
1.5.58	84	160000	250000	205000	63639.6103	100	10	230	120	155.563492	0.05853659	20	10	15	7.07106781	0.00731707
1.12.1	833	46000	76000	61000	21213.2034	100	10	10	10	0	0.01639344	10	10	10	7.07106781	0.01639344
1.12.4	833	13000	11000	12000	1414.21356	100	0	10	5	7.07106781	0.04166667	10	0	5	7.07106781	0.04166667
1.12.7	833	140000	210000	175000	49497.4747	100	0	40	20	28.2842712	0.01142857	40	10	25	21.2132034	0.01428571
1.12.8	833	9000	6000	7500	2121.32034	100	10	10	10	0	0.13333333	10	0	5	7.07106781	0.06666667
1.20.B	2702	540000	580000	560000	28284.2712	100	5300	4600	4950	494.974747	0.88392857	11200	9700	10450	1060.66017	1.86607143
1.20.C	2702	210000	380000	295000	120208.153	100	290	380	335	63.6396103	0.11355932	640	990	815	247.487373	0.27627119
1.20.E	2702	380000	280000	330000	70710.6781	100	150	560	355	289.91378	0.10757576	11200	9400	10300	1272.79221	3.12121212
1.20.F	2702	190000	100000	145000	63639.6103	100	10	0	5	7.07106781	0.00344828	800	500	650	212.132034	0.44827586
1.20.I	2702	670000	550000	610000	84852.8137	100	130	240	185	77.7817459	0.03032787	6600	4100	5350	1767.76695	0.87704918
1.20.J	2702	260000	170000	215000	63639.6103	100	40	50	45	7.07106781	0.02093023	110	550	330	311.126984	0.15348837
1.20.K	2702	480000	660000	570000	127279.221	100	60	420	240	254.558441	0.04210526	810	750	780	42.4264069	0.13684211

Patient MM4

Clone:	Isolation Time Point	TZM-bl					RC49					Macrophages				
		FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl
4.1.33	17	81000	99000	90000	12727.9221	100	0	10	5	7.07106781	0.0055556	20	20	20	0	0.02222222
4.1.34	17	4200	5500	4850	919.238816	100	340	580	460	169.705627	9.48453608	740	760	750	14.1421356	15.4639175
4.10.1	493	18000	24000	21000	4242.64069	100	20	50	35	21.2132034	0.16666667	0	10	5	7.07106781	0.02380952
4.10.3	493	82000	61000	71500	14849.2424	100	2000	1200	1600	565.685425	2.23776224	60000	40000	50000	14142.1356	69.9306699
4.10.4	493	63000	36000	49500	19091.8831	100	10	0	5	7.07106781	0.01010101	10	0	5	7.07106781	0.01010101
4.10.7	493	72000	82000	77000	7071.06781	100	70	460	265	275.771645	0.34415584	8900	7900	8400	707.106781	10.9090909
4.12.1	844	44000	30000	37000	9899.49494	100	70	140	105	49.4974747	0.28378378	200	0	100	141.421356	0.27027027
4.12.4	844	70000	73000	71500	2121.32034	100	0	80	40	56.5685425	0.05594406	30	60	45	21.2132034	0.06293706
4.12.6	844	52000	46000	49000	4242.64069	100	100	40	70	42.4264069	0.14285714	30	20	25	7.07106781	0.05102041
4.12.7	844	30000	36000	33000	4242.64069	100	10	50	30	28.2842712	0.09090909	10	0	5	7.07106781	0.01515152

Patient MM8

Clone:	Isolation Time Point	TZM-bl					RC49					Macrophages				
		FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl
8.2.50	12	62000	55000	58500	4949.74747	100	690	1060	875	261.629509	1.4957265	57000	66000	61500	6363.96103	105.128205
8.2.51	12	9000	12000	10500	2121.32034	100	10	0	5	7.07106781	0.04761905	60	140	100	56.5685425	0.95238095
8.4.51	32	48000	57000	52500	6363.96103	100	60	20	40	28.2842712	0.07619048	600	800	700	141.421356	1.33333333
8.8.1	608	4500	4200	4350	212.132034	100	10	30	20	14.1421356	0.45977011	50	60	55	7.07106781	1.26436782
8.8.3	608	54000	52000	53000	1414.21356	100	490	1120	805	445.477272	1.51886792	37000	34000	35500	2121.32034	66.9811321
8.8.4	608	81000	60000	70500	14849.2424	100	920	1050	985	91.9238816	1.39716312	57000	119000	88000	43840.6204	124.822695
8.8.8	608	26000	51000	38500	17677.6695	100	50	240	145	134.350288	0.37662338	100	60	80	28.2842712	0.20779221
8.9.B	957	30000	41000	35500	7778.17459	100	30	60	45	21.2132034	0.12676056	4200	5900	5050	1202.08153	14.2253521
8.9.D	957	60000	58000	59000	1414.21356	100	570	800	685	162.63456	1.16101695	7100	5200	6150	1343.50288	0.08813559
8.9.H	957	75000	74000	74500	707.106781	100	300	670	485	261.629509	0.65100671	8900	9400	9150	353.553391	12.2818792
8.9.I	957	77000	78000	77500	707.106781	100	380	1080	730	494.974747	0.94193548	13800	17400	15600	2545.58441	20.1290323
8.9.J	957	33000	24000	28500	6363.96103	100	220	340	280	84.8528137	0.98245614	12100	8700	10400	2404.16306	36.4912281

Patient MM23

Clone:	Isolation Time Point	TZM-bl					RC49					Macrophages				
		FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl
23.2D	15	1000	100	550	636.396103	100	10	0	5	7.07106781	0.90909091	0	10	5	7.07106781	0.90909091
23.2E	15	4400	7900	6150	2474.87373	100	10	0	5	7.07106781	0.08130081	0	10	5	7.07106781	0.08130081
23.2F	15	2800	2800	2800	0	100	10	0	5	7.07106781	0.17857143	0	10	5	7.07106781	0.17857143
23.2H	15	3200	4100	3650	636.396103	100	10	0	5	7.07106781	0.1369863	0	10	5	7.07106781	0.1369863
23.8.12	316	2200	2000	2100	141.421356	100	0	10	5	7.07106781	0.23809524	0	10	5	7.07106781	0.23809524
23.8.18	316	1700	1000	1350	494.974747	100	10	0	5	7.07106781	0.37037037	0	10	5	7.07106781	0.37037037
23.12.3	1316	1400	1700	1550	212.132034	100	0	10	5	7.07106781	0.32258065	0	60	30	42.4264069	1.93548387
23.12.7	1316	400	900	650	353.553391	100	10	0	5	7.07106781	0.76923077	0	10	5	7.07106781	0.76923077

Controls

Clone:	MOOT	TZM-bl					RC49					Macrophages				
		FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl	FFU #1	FFU #2	Average	Standard Deviation	Percentage of TZM-bl
HXBc2/YU-2 gp120		450000	570000	510000	84852.8137	100	320	350	335	21.2132034	0.06568627	240000	205000	222500	24748.7373	43.627451
HXBc2/YU-2 gp160		820000	460000	640000	254558.441	100	3100	4600	3850	1060.66017	0.6015625	146000	224000	185000	55154.3289	28.90625
NL4.3/B33		650000	630000	640000	14142.1356	100	2900	5400	4150	1767.76695	0.6484375	436000	516000	476000	56568.5425	74.375
NL4.3/LN85		140000	50000	95000	63639.6103	100	30	0	15	21.2132034	0.01578947	40	10	25	21.2132034	0.02631579

Appendix B: Western blots for Chapter V

The following Appendix contains pictures of Western blots done to determine the amount of envelope on B33wt and LN40wt pseudovirus particles. When I was unable to repeat the data previously published [24], I made several changes to the original ultracentrifugation protocol (see Materials and Methods). The following blots are representative of the different changes I made in the attempt to repeat the original data, as well as eliminate the protein leak through problem mentioned in Chapter V (Fig. 5.11). Descriptions of the changes I made to the protocol are detailed in the figure legends. Each picture here, and in Fig. 5.11, represents many attempts.

a)

B33 wt

LN40 wt

LN40 wt env only

← gp160

← gp41

← p24

b)

B33 wt

LN40 wt

LN40 wt env only

← gp160

← gp41

← p24
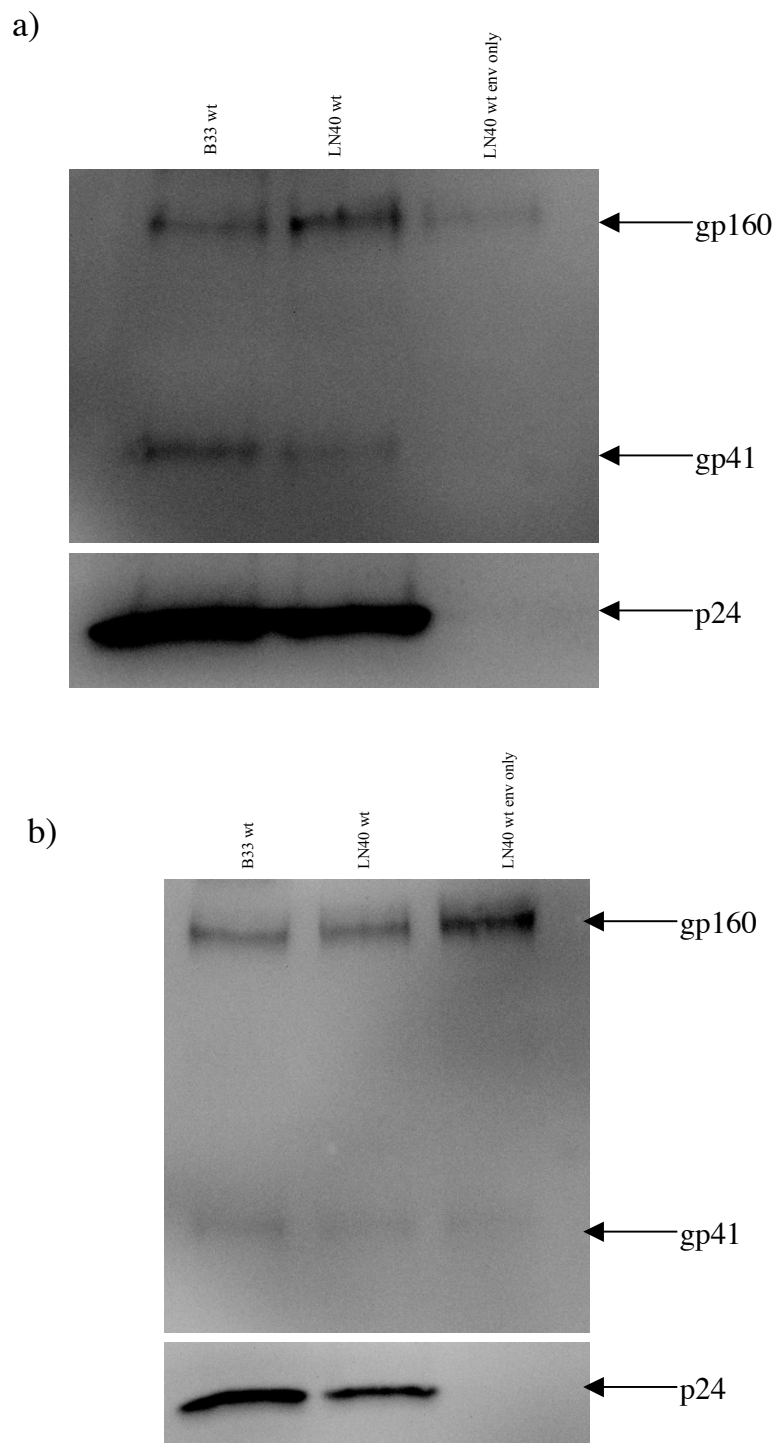
Fig. B1 Representative Western blots. Protocol changes: supernatants spun at 5000rpm (instead of 3000rpm), filtered through .22um filter (instead of .45um filter), then layered onto 2.5% sucrose cushion. Samples were ultracentrifugated at 22000 rpm for 2 hours at 4ºC. . On some occasions, the protein leak-through was lessened (a), other times not at all (b).
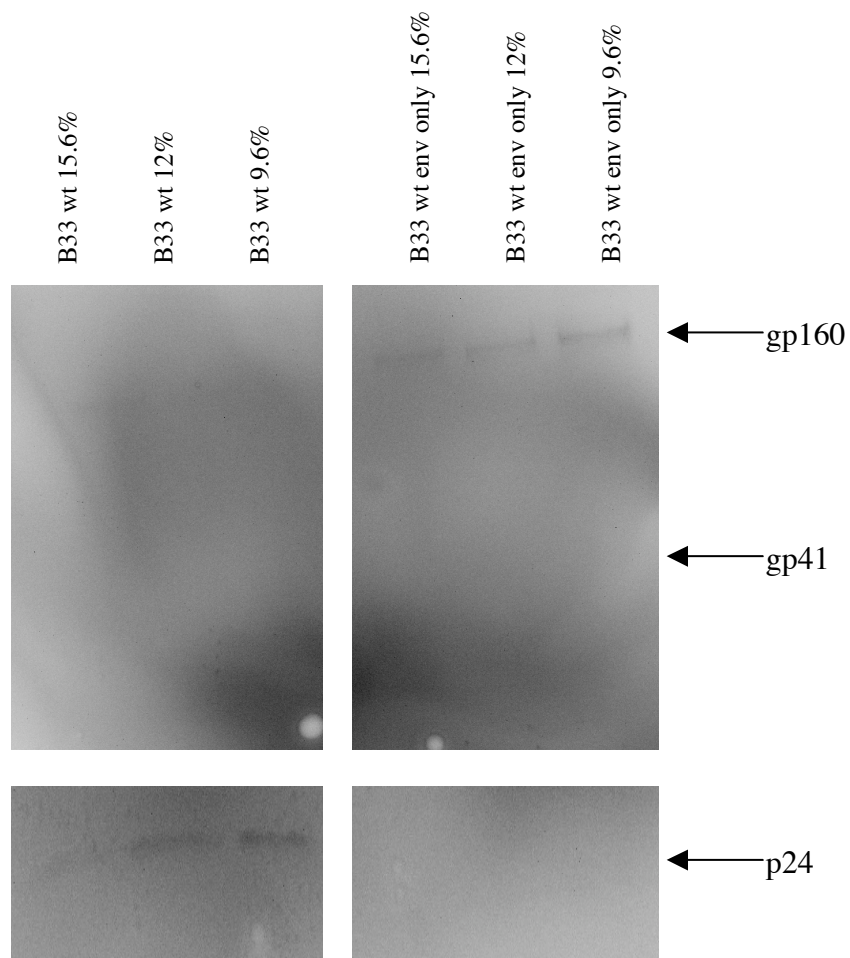
Fig. B2 use of Optiprep. My first attempts at using an Optiprep gradient (11 steps, in 1.2% increments, from 6% to 18%; Dettenhofer & Yu, *J.Virol*, 1999) were unsuccessful (not shown). Next, I tried using Optiprep cushoins. Supernatants were spun at 3000rpm, then layered onto Optiprep cushions as indicated. Pellets were resuspended, then run on SDS-PAGE gel, then blotted. These optiprep cushoins do not solve the problem of protein leak-through. They also decrease the amount of antigen, making interpreting the blots difficult.

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