

DIPLOMARBEIT

Titel der Diplomarbeit

The Molecular Biology of HIV Cloning and Characterization of SHIV-1157ipEL: A New Tool for Vaccine Studies

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

Verfasser:	Klemens Johannes Wassermann
Matrikel-Nummer:	0206718
Studienrichtung /Studienzweig	Biologie /Genetik Mikrobiologie
Betreuerin / Betreuer:	Prof. Dr. Thomas Decker
	Durchgeführt am Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute/Harvard Medical School (Prof. Dr. Ruth Ruprecht Laboratory) Boston, 2007/2008

Meiner Familie in Dankbarkeit gewidmet.

Abstract

The AIDS epidemic caused by the human immunodeficiency virus type 1 (HIV-1) continues to escalate but a protective vaccine remains elusive. With 33 million people infected world wide, 2.7 million people infected in 2007 (UNAIDS), and with a high mortality (2 million deaths in 2007), HIV is one of the most lethal pathogens. Thus, a vaccine and or better treatment are urgently needed.

To develop effective strategies, deep knowledge and detailed insights into HIV biology, its origin and interaction with the host immune system are crucial. In recent years, approaches to trigger CD8⁺ T-cell responses for eradication of HIV looked promising, but do not prevent primary infection. Thus, neutralizing antibodies (nAbs) are the preferential choice to effectively neutralize viral particles before establishing a systemic infection. To study nAbs, the simian-human immunodeficiency virus (SHIV)-macaque model has been shown to most likely mimic the natural way of infection in humans. The development of various chimeric SHIVs carrying HIV envelope sequences represents a great tool to screen and test vaccine efficacy. However, these SHIVs have to fulfill some important criteria to reflect the situation of HIV infection seen in humans.

The majority of primary infections in humans are with HIV-1 clade C, the predominant strain worldwide. Thus, new SHIV models should reflect this situation and carry envelopes from clade C. Ninety percent of all transmissions occur mucosally and among these, almost all involve R5 viruses. Current SHIV models lack at least one of these properties, if not all, and need to be replaced with mucosally transmissible R5 SHIVs.

In this work, we describe the construction of a SHIV which a) carries a clade C envelope, b) uses CCR5 as coreceptor, c) is mucosally transmissible and d) is neutralization sensitive to allow assessment of nAb efficiency upon vaccination. Through molecular cloning techniques, we could exchange the major envelope region of an already used SHIV with the early-form envelope of an HIV-1 isolated from a Zambian child. 293T cells were then transfected with the full-length genome of the new SHIV; virus was produced in these cells and tested for functionality in TZM-bl cells (modified HELA cells containing an infection-responsive reporter gene). A viral stock was grown and after neutralization susceptibility evaluation, we could show significant neutralization sensitivity of the new SHIV in contrast to a reference SHIV.

Contents

Abstract	5
1. Introduction	11
1.1. The Human Immunodeficiency Virus (HIV)	11
1.1.1. Discovery and Origin	11
1.1.2. Assembly and Genome Structure	12
1.1.2.1. Structure of the Viral Particle	12
1.1.2.2. Genome Organization	13
1.1.2.3. The Replicative Cycle	16
1.2. The Natural History of Infection	19
1.2.1. Infection and Progression to AIDS	19
1.2.2. The Immune Response to HIV	21
1.2.3. Immune Escape Mechanisms of HIV	23
1.2.4. The Neutralization and Neutralizing Antibodies	26
1.3. HIV Vaccine Development	28
1.3.1. Neutralizing Antibody Vaccines	29
1.3.2. T-Cell Stimulating Vaccines	29
1.3.3. The SHIV/Macaque Model	30
2. Aims	33
3. Materials	35
3.1. Vectors and Viral DNA	35
3.2. Bacteria	36
3.3. Cell Lines	36
3.4. Media	37
3.5. Chemicals, Enzymes and Kits	38
3.6. Antibodies and Monkey Sera	39

Contents

4. Methods	41
4.1. Molecular Cloning	41
4.2. Transformation	41
4.3. Glycerol Stocks	42
4.4. Plasmid Isolation	42
4.5. Freezing and Thawing of Cells	42
4.6. Preparation of Media and Maintaining of Cell Lines	43
4.7. Transfection and Production of Viral Particles	43
4.8. TZM-bl Assay	44
4.9. p27 Assay (ELISA)	45
4.10. Generation of Viral Stocks in PBMCs	46
4.11. Neutralization Assay	46
5. Results	47
5.1. Molecular Cloning of SHIV-1157ipEL	47
5.2. Testing the Functionality of the Clones	51
5.3. Measuring the TCID ₅₀	53
5.4. Neutralization	54
6. Discussion	55
7. Acknowledgments	57
8. Bibliography	59
Curriculum Vitae	65

Chapter 1

Introduction

1.1 The Human Immunodeficiency Virus (HIV)

In the 20th century, human immunodeficiency virus 1 and 2 (HIV-1, HIV-2) were introduced to the human population. HIV is a member of the *Retroviridae* and belongs to the family of lentiviruses and as such contains two positive-sense RNA strands that are reverse transcribed to double-stranded DNA in the host by the error-prone enzyme reverse transcriptase (see 1.1.2.3). Based on genetic variation of the surface envelope glycoprotein gp160, HIV-1 falls into three groups: M (Major/Main), N (Non-M, Non-O/New) and O (Outlier) – of which group M is the most common and is subdivided into clade A-H, J, K and many circulating recombinant forms (CRFs) and unique recombinants– of which clade B is mainly spread in Europe and the USA and clade C is primarily found in India, China and sub-Saharan Africa and causes the most infections. Following a study of UNAIDS, the global distribution of HIV-1 subtypes, based on sequence differences in the envelope, is dominated by clade C (56%), followed by clade A (25%), clade B (12%) and clade D, E and minor subtypes (each ~4%).

1.1.1 Discovery and Origin

In the 1980s, three independent groups (Francoise Barré-Sinoussi, Claude Chermann and Luc Montagnier [6], Robert Gallo and colleges [7] and the group of Jay A. Levy [8]) discovered a new retrovirus that was associated with an acquired immunodeficiency and was named human immunodeficiency virus (HIV) by an international committee in the year 1984 [9, 10].

Human immunodeficiency viruses HIV-1 and HIV-2, the causative agents of the acquired immunodeficiency syndrome (AIDS), were introduced to the human

population in Africa during the 20th century [11, 12]. Many species of non-human primates are naturally infected with closely related lentiviruses, but, curiously, AIDS is mostly not observed in the natural hosts. Phylogenetic analysis revealed that HIV-1 evolved from a simian immunodeficiency virus strain SIVcpz, which can be found in a subspecies of chimpanzee (*Pan troglodytes troglodytes*) and infected humans probably around the 1930^{ths}. The less pathogenic HIV-2 originated from SIVsm of sooty mangabeys (*Cercocebus atys*), having approximately 40-60% homology to HIV-1 [3]. These viruses were transferred to the human population via cross-species transmission, but only one SIVcpz ancestor gave rise to the global AIDS pandemic: HIV-1 group M with clades A to K. Group M, as well as group N arose from a geographically distinct chimpanzee population in Cameroon [13].

1.1.2 Assembly and Genome Structure

The viral genome, the production of viral proteins and the viral assembly are summarized in the following chapters. For a more detailed review of all HIV features and its life cycle, readers are referred to a review by B. Matija Peterlin and Didier Trono, [14].

1.1.2.1 Structure of the Viral Particle

On transmission electron microscopy (TEM) pictures, HIV appears as a 100 nm round spiked particle (Figure 1). The spikes are formed by trimers of the envelope glycoprotein units gp120 (surface glycoprotein; outer part) and gp41 (transmembrane part), which adhere by hydrophobic and hydrophilic interactions, advancing conformational changes leading to membrane fusion with the target cell. Gp120 is heavily glycosylated; different sugar residues take around 58% of its total mass [15, 16].

The outer shell of the virus is comprised by a double phospholipid bilayer that originates from the host cell after budding off of the virus and thereby includes several host membrane proteins, e.g., MHC molecules. Linked to the membrane are the p17

matrix proteins (MA); both the lipid bilayer and the MA are termed the outer core of the virus.

The cone-shaped inner core (capsid) consists of p24 capsid proteins (CA) and contains the two identical positive sense RNA molecules, stabilized by p7 nucleocapsid proteins (NC). The proteins reverse transcriptase (RT, p51), RNase H (p66) and integrase (IN, p32) bind to the NC proteins as well. Two copies of the tRNA^{Lys3}, needed for initiation of reverse transcription, and the accessory proteins Nef (negative effector) and Vif (viral infectivity factor) can also be found in the capsid. The viral proteins Tat (transcriptional transactivator), Rev (regulator of virion gene expression) and Vpu (viral protein u) cannot be found in the viral particle, but in the infected cell. The inactive viral protease (p10) can be found in between the matrix and the capsid and is important for maturation of the virion.

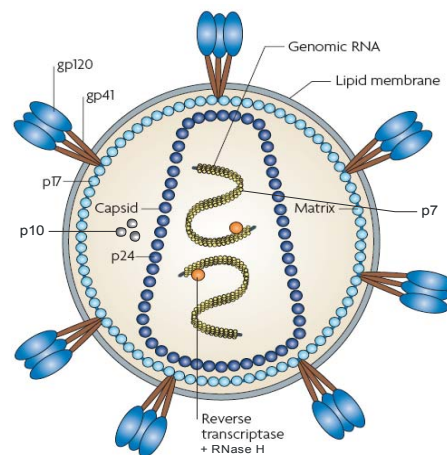


Fig. 1 | Schematic draw of an HIV particle

Modified from *The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus* by Karlsson Hedestam et al.

Nature Reviews Feb. 2008 [4]

1.1.2.2 Genome Organization

Integrated into the host chromosome, the 10 kb genome of HIV contains open reading frames for 16 proteins (Figure 2). The classical structural and enzymatic proteins derived from the genes *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) are required by all retroviruses. In addition, HIV encodes two regulatory proteins, Tat (transcription transactivator) and Rev (regulator of virion gene

expression), and the accessory proteins Nef (negative effector), Vif (viral infectivity factor), Vpr (viral protein r) and Vpu (viral protein u). The accessory proteins are not required for viral replication *in vitro* but enhance infectivity *in vivo*.

All viral proteins are synthesized by more than 30 messenger RNAs, which are all

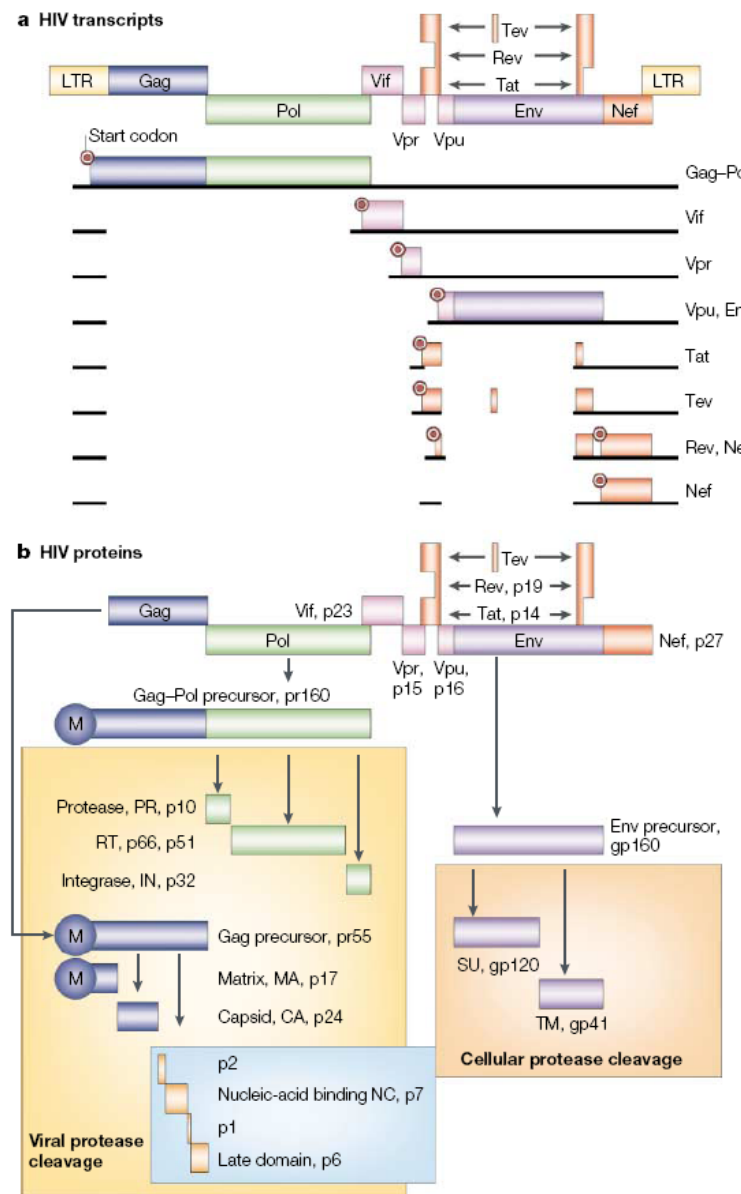


Fig. 2 | HIV genome, transcripts and proteins

a | The 10 kb genome of HIV contains ORFs for 16 proteins, 8 transcripts are shown in this figure. **b** | The synthesized precursor proteins get processed by viral (yellow box) and cellular (red box) proteases.

contains enhancer and promoter sequences with binding sites for several transcription

derived from the primary transcript but expressed and translated differently during the course of infection. The early transcripts for Tat, Rev and Nef are fully spliced, those that encode the late viral proteins, mainly structural and enzymatic components and factors that fine-tune infectivity, are singly spliced or unspliced. The transition between the early and the late phase of the viral gene expression is regulated by Rev by binding to the Rev response element (RRE) and transport of singly spliced or unspliced viral mRNA from the nucleus to the cytoplasm.

Expression of viral genes is regulated by the long terminal repeat (LTR), which flanks the genome on the 5' and the 3' side. It

factors and a polyadenylation signal. The enhancer sequence binds the nuclear factor- κ B (NF- κ B), the nuclear factor of activated T cells (NFAT), as well as members of the ETS family of transcription factors [transcription factors containing the unique erythroblast transformation specific (ETS) DNA binding domain] [17] which ensures a high replication efficacy in activated T cells and differentiated macrophages. Further downstream, three SP1-binding sites, the TATA box, the initiator (Inr) and the transcription start are found. The most unusual feature of the LTR is the presence of a strong regulatory element located 3' to Inr. This RNA structure, which is known as the transactivation response (TAR) element, is found at the 5' end of all viral transcripts and binds Tat. In the absence of Tat, HIV transcription begins, but elongation is inefficient. Tat and its cellular co-factor, positive transcription elongation factor b (P-TEFb), cooperate to bind TAR with high affinity. The ability of Tat to recruit P-TEFb through an RNA sequence is unique among transcriptional activators, and it renders HIV replication particularly sensitive to inhibition of CDK9 (a component of P-TEFb). Although NF- κ B can partially substitute for Tat, as it can itself recruit P-TEFb, Tat leads to higher levels of gene expression and is essential for HIV replication in the host [14].

1.1.2.3 The Replicative Cycle

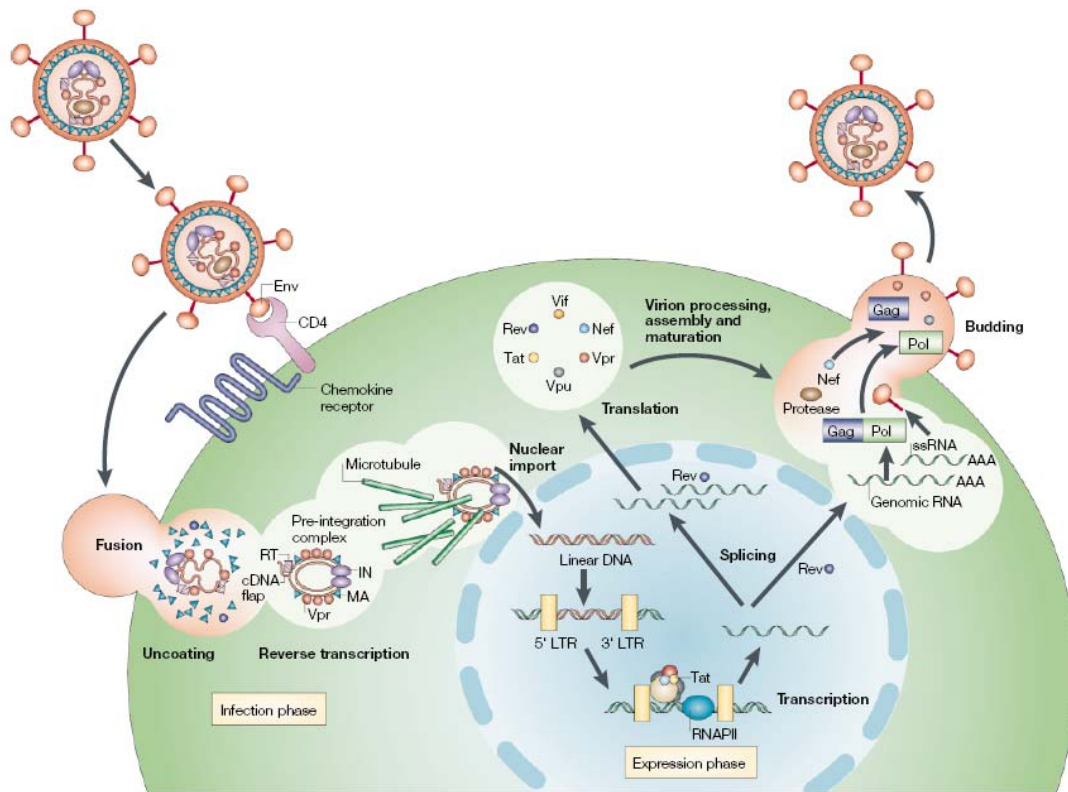


Figure 3 | The replicative cycle of HIV. The viral envelope protein binds CD4 first, leading to a conformational change and the binding of a co-receptor, either CCR5 or CXCR4, defining the tropism of the viral strain (R5 or X4). After fusion of the membranes, viral RNA gets reverse transcribed and transported through the microtubular network of the cell and the nuclear pores into the nucleus after the assembly of the so-called pre-integration complex. Viral DNA integrates and forms the pro-virus. After time-controlled transcription, splicing and translation, viral proteins get transported to lipid rafts in the cell membrane, where an immature virion assembles. After processing of Gag and Gag-Pol, the mature HIV virus buds off the cell membrane. [14]

HIV enters the body through exchange of body fluids and infects mainly T helper (T_H) cells, macrophages and to some extent, microglia cells and dendritic cells (DCs). Besides the use of CD4 as main receptor, the use of co-receptors after binding of CD4 and conformational change of envelope protein gp120, determines this target cell tropism. HIV R5 strains, detected in the early phase of infection, use CC-chemokine receptor 5 (CCR5) as their co-receptor and can, therefore, enter macrophages, DCs and T cells (mainly memory T cells), whereas X4 strains of HIV,

predominating with time, use CXCR4 as a co-receptor and can infect T cells (naïve T cells) only [18].

DCs are likely to play an important role in transporting the virus to lymphoid organs, as these cells can either be productively infected or capture the virus through the lectin-like receptor DC-SIGN (DC-specific ICAM3-grabbing non-integrin) and store the virus in an infectious form before presenting it to T cells [19, 20].

After binding of both, receptor (CD4) and co-receptor (CCR5/CXCR4), and further conformational changes in the envelope protein, HIV fuses with the host membrane [21], uncoats and reverse transcribes its two RNA molecules (with recombination events) into a double stranded DNA that is transported with the so-called pre-integration complex, a complex of integrase, matrix, viral protein *r* and viral DNA, into the nucleus. Because of the large number of nuclear-localization signals on the proteins of the pre-integration complex [22-24], a disintegration of the nuclear membrane, and therefore cell division, is not required for the nuclear import of the viral DNA.

Once in the nucleus, the viral DNA gets integrated via the help of integrase and several chromatin remodeling complexes [25] with preference for active genes. However, in resting lymphocytes, there are several barriers that preclude the completion of these early steps due to low energy levels, leading to accumulation of double-stranded viral genomes. Nevertheless, once the lymphocytes get activated, even partially, they become fully permissive for HIV to complete the replication cycle [26-28].

After integration, the now called pro-virus behaves like any other gene, with its promoter in the 5' LTR region and a poly-adenylation site in the 3' LTR, as described in 1.1.2.2. The viral genes get transcribed, spliced and translated in a time-dependent manner, controlled by Rev. After translation, viral structural and enzymatic proteins travel to the plasma membrane, where immature virions assemble in cholesterol-rich lipid rafts. Nef is important for this assembly and the viral protease p10 processes Gag and Gag-Pol, yielding to mature viral particles (Figure 3).

1.2 The Natural History of Infection

1.2.1 Infection and Progression to AIDS

After infection with HIV, either by sexual intercourse, blood-blood contact or breast feeding, the immune system reacts like it does for any other viral infection. First, infected cells get lysed by natural killer (NK) cells and cytotoxic T lymphocytes (CTL). Second, following this cell-mediated immune response, B cells produce immunoglobulin M (IgM) after a time course of two to three weeks, followed by a live long production of IgG.

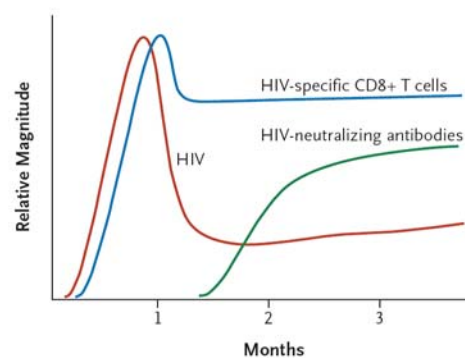


Fig. 4 | Immune responses to HIV infection,
Showing Plasma HIV levels, HIV-specific CD8+ T
cells, and HIV-neutralizing antibodies. [1]

In the first weeks after primary infection, an increase in viral RNA copies in the blood can be observed, which reaches its peak after 4-12 weeks post infection. Almost in parallel, the CD8⁺ T-cell population increases and patients show typical flu-like symptoms. Serum antibodies against viral proteins, mainly against p24, can be detected in Western blots, an observation called seroconversion, several weeks after virus exposure.

Following this viral peak, a significant drop of viral load in the blood is accompanied by a significant decrease of CD8⁺ cells; the immune system seemingly controls the infection. After this so-called acute phase of infection, the viral load in the blood may reach nearly undetectable levels or remain high, the so-called viral setpoint, which influences subsequent disease progression (Figure 4). This latent phase of infection is

deceiving. Although no replicating virus may be detected in the blood in some individuals, the virus is still replicating in two important latent reservoirs [29, 30]; the lymph nodes and, to some extent, the brain. Of note, during this phase of infection, only 0.005% of the lymphocytes are actively producing virus, but 0.5% and 0.05% of the lymphocytes contain non-integrated and integrated provirus, respectively [31, 32]. Besides this fact, almost all mucosally transmitted viruses use R5 and therefore primarily infect memory T cells with a half life of 22 weeks only [13]. Knowing this, it is clear how HIV is able to escape immune responses but maintain its replication and overwhelm the host with time.

During the course of infection (Figure 5), virus tropism can change as a result of mutations in the V3 loop of gp120 and the virus now uses also CXCR4 as a coreceptor to broaden its tropism to naïve T cells. This results in a sharp decline of CD4⁺ cells and renders the host susceptible to opportunistic infections and malignancies, leading to the acquired immunodeficiency syndrome, by definition at CD4⁺ T-cell counts in the blood below 200 cells/ μ l.

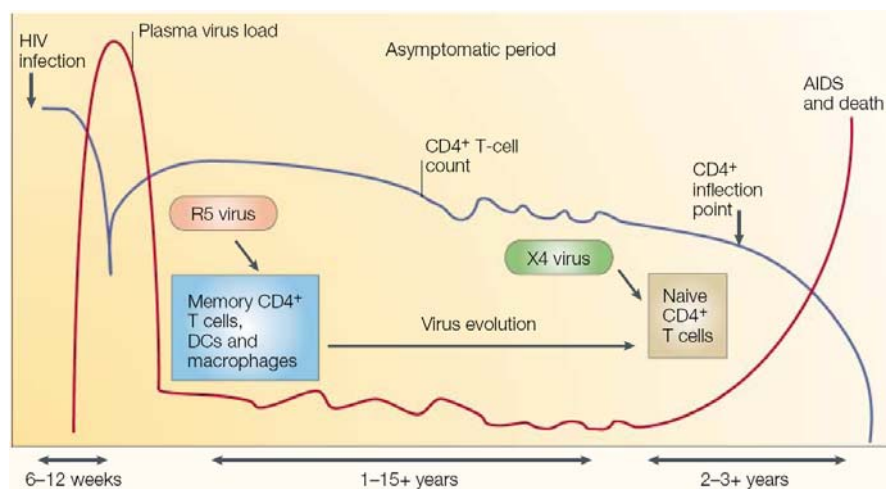


Fig. 5 | Schematic diagram of the course of HIV-1 infection. This diagram illustrates the relationship between HIV-1 virus load (red line) and CD4⁺ T-cell count (blue line) over time in a typical case of untreated HIV-1 infection. DCs, dendritic cells. [3]

The duration of the latent phase varies among patients and allows categorizing patients according to their disease progression profile: rapid progressors (RP), slow progressors (SP) and long-term non-progressors (LTNP), with the latter living more

than 10 years after infection without progression to AIDS. There is a variety of factors believed to play a role in the differing duration of the latent phase:

First, the viral load: the viral set point (see above) after acute infection predicts the outcome of the disease. The viral load of LTNP in the steady state is up to 20 times lower than those in RP [33, 34]. Second, the immune system: RPs show a lower diversity in the *env* region than quasi species of LTNPs, indicating a higher selection pressure by the immune system for the virus in LTNP [35, 36]. Third, the viral regulatory and accessory genes *vpr*, *vpu*, *vif*, *rev*, *nef* and *tat* can also play an important role. Their role in the replicative cycle and for the shielding against the immune system is explained in chapter 1.1.2.3 and 1.2.3. Fourth, chemokine receptors play a crucial part in determining the course of progression. Studies revealed that an extension in the V2 domain of gp120, responsible for the co-receptor usage, is only found in SP or LTNP. It is believed that this change in V2 keeps the virus from switching from CCR5 to CXCR4 and thereby prevents the naïve CD4⁺ cells to get infected [36-38]. Moreover it was found that in LTNP, CD8⁺ T cells secrete the natural ligands of CCR5, macrophage inflammatory protein 1 α (MIP1 α ; CCL3), macrophage inflammatory protein 1 β (MIP1 β ; CCL4) and RANTES (regulated on activation, normal T cell expressed and secreted; CCL5), to a higher extent. Fifth, the host genetic background: different HLA class I or MHCI, respectively, present different peptides of the virus to cytotoxic T lymphocytes and thus affect the quality of the immune response as some peptides are more conserved than others. HLA types associated with slow progression of infection are HLA-B27 and HLA-B57 [39]. Also homozygosity at the HLA class I loci is associated with rapid progression of infection. Last, a very prominent finding renders around 1% of the Caucasian population relatively resistant to HIV infections: the homozygous CCR5 Δ 32bp deletion, leading to a non-functional receptor protein [40].

1.2.2 The Immune Response to HIV

The first line of defense after infection is mediated by the cellular immune response. Natural killer (NK) cells lyse infected cells. CD4⁺ cells recognize viral peptides presented on dendritic cells via the MHC class II molecule, whereas CD8⁺ T cells (cytotoxic T lymphocytes, CTL) get primed for viral peptides presented on MHC

class I molecules and also lyse infected cells. $CD4^+$ T cells start producing and secreting cytokines: T_H1 (T helper cells type 1) mainly produce interleukin 2 (IL-2) and interferon gamma ($IFN-\gamma$), thereby activating NK cells and macrophages. T_H2 cells produce IL-4, IL-5, IL-6 and IL-10, which leads to activation of the humoral immune response. The cellular immune response can be nicely observed in the acute phase of infection, where the CTL response correlates with the decrease in viral load and the first drop in $CD4^+$ cell count but is not able to clear the virus because of reasons depicted earlier.

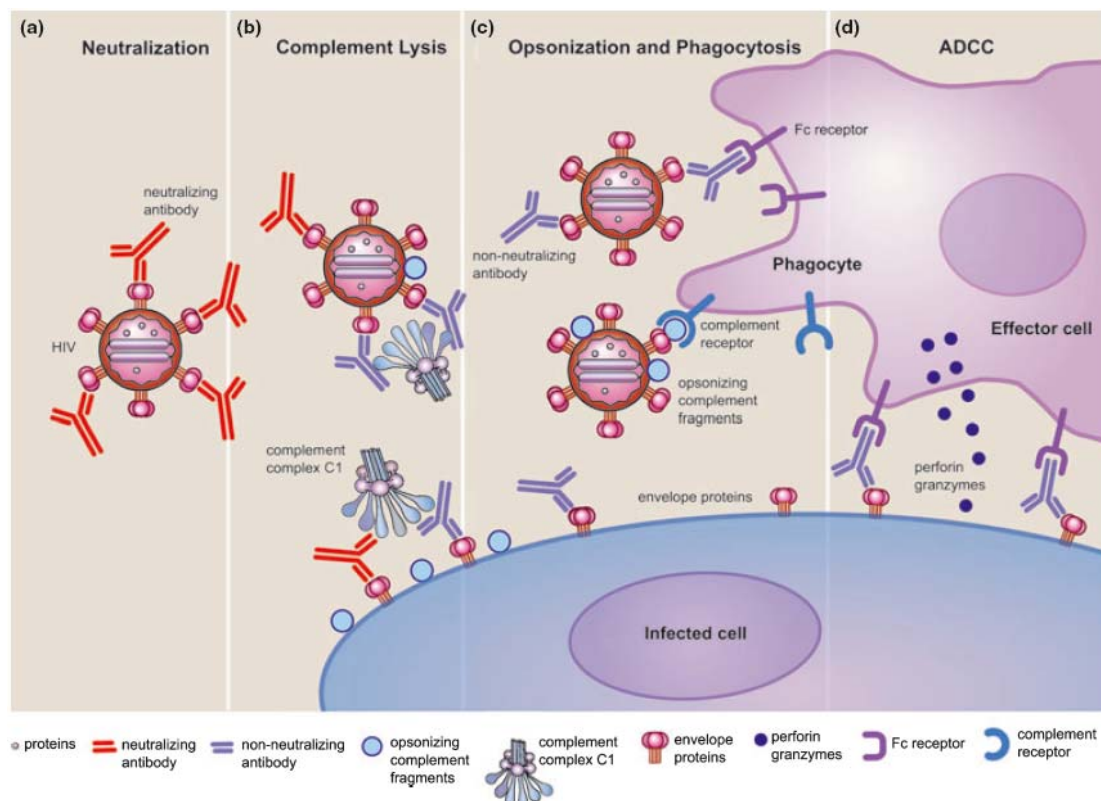


Fig. 6 | How antibodies combat HIV-1 a | Neutralization of free virus by antibodies, b | complement-mediated lysis of free virus and infected cells triggered by antibodies, c | opsonization of virus particles by antibodies and phagocytosis of virus particles via Fc- or complement-receptors, d | antibody-dependent cellular cytotoxicity (ADCC) against infected cells. Neutralizing antibodies (red), non-neutralizing (blue), Fc-receptors (violet), complement components (light-blue), complement-receptors (black). [2]

The second line of defense is represented by the humoral immune response: T_H2 cells produce IL-4, IL-5 and IL-6, which activates differentiation of B cells and antibody production in plasma cells. These antibodies are mainly directed against the viral envelope proteins gp120 and gp41 and the capsid protein p24, and play an important role in complement activation, opsonization and phagocytosis, antibody-dependent

cellular cytotoxicity (ADCC) and neutralization (Figure 6). Their neutralizing effects can be shown, but viral strategies make it difficult for the host to maintain good neutralizing antibody titers, which will be discussed in the following two chapters.

1.2.3 Immune Escape Mechanisms of HIV

Why and how a virus with only a 10 kb genome is able not only to hide from the immune system, but also to use it for its spread and against the host is fascinating. In this chapter, the manifold mechanisms with which HIV is able to hide and escape the immune mechanisms will be introduced.

One mechanism also used by many other viruses, the establishment of latent infection, is achieved by HIV through infecting resting T cells and integration of its proviral DNA into heterochromatin regions, which get expressed after activation of the cell. Additional targets that harbor latent viruses might include DCs, monocytes, astrocytes and seminal cells. HIV is also able to hide from immune responses by establishing a viral reservoir in the central nervous system, where cell-mediated immune response is reduced, by infecting microglia.

Another potent mechanism of HIV to escape CD8⁺ T cells is the mutation of prominent antigenic patterns of viral proteins, resulting in escape mutants. Selection and fitness gain or loss of such mutants by CTLs is probably one of the main features of a HIV infection. These escape mutants are products of virus replication and mutation rates and altered antigenic patterns of viral proteins thus preventing CTL-mediated killing of infected cells. The strong selective pressure and the resulting escape was shown by Koenig et al. [41], where a patient was treated with his own CTL clone, specific for a Nef epitope. Selection pressure and escape mutations resulted in the accumulation (30% of total virus) of a quasi-species that had deleted the presentation-relevant region in Nef. This pressure also explains the prevalence of humans with HLA-B27 and B57 to slow progression to AIDS (as explained in 1.2.1). Both select for epitopes in a well conserved and functionally important region of Gag p24; three distinct mutations were shown to be necessary for the virus to escape [42, 43].

Many viruses that establish long-term infections, such as members of the hepatitis virus family, typically alter MHC class I-mediated antigen presentation. Not

unexpectedly, HIV is no exception, as it decreases the expression of MHC class I molecules on the cell surface through Nef, a product of the early viral genes. Nef interferes with the migration and persistence of MHC class I-antigen complexes to and on the cell surface of infected cells. Interestingly, Nef has no enzymatic activity and carries out its function by protein-protein interactions only. There are two pathways for Nef dependent MHC class I down regulation. In the clathrin-dependent pathway, Nef connects the cytoplasmic tail of MHC class I with clathrin-coated pits, thereby triggering endocytosis. Nef is also able to down regulate CD4 in this manner, to ensure that released virions are fully infectious by making sure that gp120 is free to bind CD4 and chemokine receptors on recipient cells. In the clathrin-independent pathway, Nef mediates the internalization of MHC class I molecules into ARF6-specific early endosomal compartment [14] (Figure 7).

To avoid the trap of being lysed by NK cells, Nef is only specific for HLA-A and HLA-B, but not HLA-C and HLA-E, which still can bind inhibitory receptors on NK cells.

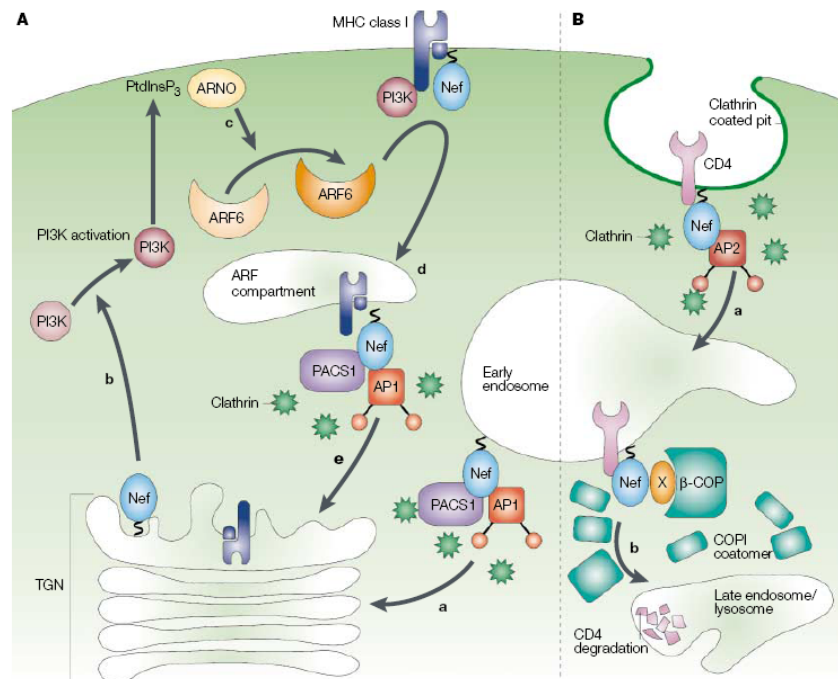


Figure 7 | Nef-induced downregulation of expression of MHC class I molecules and CD4.

A | Nef accelerates the endocytosis of MHC class I molecules through the phosphofurin acidic cluster sorting protein 1 (PACS1)/phosphatidylinositol 3-kinase (PI3K)-dependent activation of ADP ribosylation factor 6 (ARF6)-mediated endocytosis (a). Nef is probably targeted first to the trans-Golgi network (TGN), where it acquires the ability to activate PI3K (b). The formation of phosphatidylinositol-3,4,5-triphosphate (PtdInsP₃) ensues, which recruits the guanine exchange factor ARNO to the plasma membrane, where ARF6 becomes activated (c). Together with Nef, the latter mediates the internalization of MHC class I molecules from the plasma membrane to an ARF6-specific early endosomal compartment (d). From there, MHC class I molecules are retrieved to the TGN, where they remain trapped (e). To explain the target specificity of this process, an interaction between Nef and the cytoplasmic tails of HLA-A and HLA-B is probable, although it remains to be shown formally. **B** | The two steps of Nef-induced CD4 downmodulation. At the plasma membrane, Nef connects the cytoplasmic tail of CD4 with clathrin-coated pits through an interaction with adaptor protein 2 (AP2) and the vacuolar ATPase (v-ATPase), triggering rapid endocytosis of the CD4 receptor (a).

In the early endosome, Nef then interacts with the COPI coatome (a coat structure of early endosomes), which targets CD4 for lysosomal degradation (b). An as-yet unidentified co-factor (X) potentiates the interaction between Nef and the COPI coatome. Activation of PI3K and ARF6 is shown as a change in colour from light to dark.

But not only does HIV simply hide away from the host immune response, it also developed a way of striking back. *In vivo* experiments have shown that infection of lymphatic tissue with HIV is accompanied by enhanced apoptosis, which mainly affects bystander cells [44, 45]. This is a result of Nef-mediated up regulation of FAS

ligand (FASL) on the surface of infected cells. And as almost all HIV-specific T cells express FAS on their surface, they get killed by apoptosis before starting an efficient immune response. The mechanism how Nef up regulates FASL, however, is not yet known.

Although HIV infections trigger bystander cell apoptosis, Nef inhibits apoptosis in infected cells, at least until the cell has produced its load of new virions. Taken together, Nef inhibits the apoptotic signaling molecules apoptosis signal-regulating kinase 1 (ASK1), BCL-2 antagonist of cell death (BAD) and p53.

The last method of HIV to escape an immune system is to impair its response. CD4⁺ T cells are important for priming dendritic cells to initiate CD8⁺ T-cell responses, to maintain memory T cells and maturation of CD8⁺ T-cell function. All of these actions are impaired by the CD4⁺ cell specificity of HIV.

1.2.4 The Neutralization and Neutralizing Antibodies

Antibody (Ab)-mediated neutralization plays a key role in the fight against many pathogens and is defined as a measurable loss of infectivity due to direct and dose-dependent activity of Abs. Neutralizing antibodies (nAbs) will likely be required in the development of a vaccine to protect against HIV-1. In fact, classical HIV/AIDS vaccine approaches have failed to protect or have tended to elicit rather weak nAb responses. However, passively administered nAbs have shown to be protective in animal models [46-53] and therefore, a better understanding of nAb production and Ab-mediated neutralization is sufficient for further success in the HIV vaccine field.

The targets of nAbs on HIV-1 are conserved motifs presented on functional envelope (Env) spikes (see Chapter 1.1.2.1). Relatively conserved regions of Env have been identified and contain elements crucial for a variety of functions *in vivo* (*i.e.* Env synthesis, folding, processing, virus incorporation, stability, receptor recognition and fusogenicity [54]). The most crucial challenge for vaccine-elicited nAb responses is to be high in titer and broad in recognition of different virus isolates, so that virus escape cannot occur by minor mutations.

There are some drawbacks in getting nAbs to work. Problematic issues are not only the fast change of the virus by mutations, the shielding of nAb epitopes by variable loops and glycans and the steric inhibition of Ab binding, but also the

immunodominance of non-neutralizing epitopes (formed by non-functional Env; *i.e.* mono-, dimers and gp41 stumps), which often overlap with epitopes for broadly nAbs. But these non-neutralizing Ab could play important roles in immunological pathways other than neutralization, *e.g.* for complement activation, opsonization, or most importantly, ADCC.

As HIV-1 only needs one [55] to five [56] spikes for infection, nAb clearly need to reach high titers to successfully neutralize virus *in vivo*.

With all these limiting factors being of concern, there are a few monoclonal Abs (mAbs) described to date that can potently neutralize a wide range of primary HIV-1 isolates. Briefly, neutralizing mAbs (nmAbs) against gp120 are b12 and 2G12, whose epitopes overlap with the CD4 binding site and a distinct cluster of glycans on the outer face of gp120, respectively. Neutralizing mAbs against gp41 are 2F5 and 4E10, which target epitopes within the membrane-proximal external region (MPER) of gp41 [54] (Figure 8). A drawback for the latter two was the finding that both cross-react with cardiolipin through a hydrophobic tip in the CD3 region and therefore are autoreactive [57].

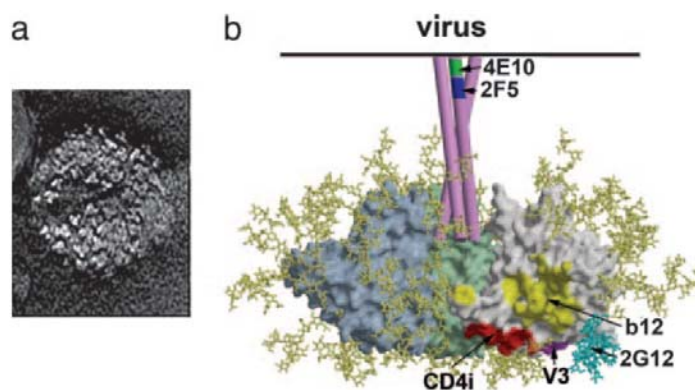


Figure 8 | The trimeric Env spike of HIV-1/SIV **a** | Electron micrographs of SIV particles showing trimeric Env spikes on the surface (63). This micrograph shows trimers on the surface of an SIV particle expressing high levels of Env. HIV-1 Env appears to be less stable than Env of SIV, and there is likely heterogeneity in the number of Env spikes per virion. **b** | Model of the Env spike based on the structure of core gp120 (11, 64), with three gp120 monomers shown in gray, pale green, and pale blue. gp41 is shown schematically as three pink tubes. Carbohydrate chains are shown in yellow, and the oligomannose cluster proposed to interact with mAb 2G12 is shown in cyan. The approximate locations of the epitopes for broadly neutralizing mAbs are indicated. [5]

There are many more mAbs showing weaker neutralization profiles (e.g. Z13e1, D5). Interested readers are referred to some reviews about neutralizing monoclonal antibodies in the HIV-1 vaccine field [51, 58-62].

All these mAbs have become vital tools not only for studying neutralization mechanisms, but also for characterizing Env vaccine candidates.

Neutralizing Antibodies, monoclonal or polyclonal, will play an important role in the fight against HIV-1 infections and in the development of a vaccine since only nAbs are able to prevent a primary infection.

1.3 HIV Vaccine Development

The problem with the natural immune response to HIV is that it is always chasing the persisting, variable virus. In these circumstances, the virus will always escape. Due to this fact and the high mortality, a prophylactic vaccine is desperately needed [63].

Since 1798, classical vaccination strategies have worked for a number of infectious diseases, but not for HIV infections. Thus far, no one has ever cleared an HIV-1 infection and because of the unique immunological properties of HIV-1, classical vaccine designs have failed.

In today's HIV vaccine research field, there are two main strategies: One is to investigate and develop vaccines inducing neutralizing antibodies, the other is to generate HIV-specific CD8⁺T cells.

The best vaccine-induced immune response would be the production of neutralizing antibodies, as; if present in high enough titers and with cross-clade neutralizing capacity; true sterilizing protection could be achieved.

As the search for adequate nAbs clearly became very difficult and there still might be the danger of cell-to-cell transmission, a vaccine-induced cellular immune response becomes important too. HIV-specific CD8⁺ T cells could then kill infected cells before release of new viral particles, but will not prevent infection.

1.3.1 Neutralizing Antibody Vaccines

When HIV was first identified and sequenced, many thought it would be straight forward to use the viral gp120 to create a vaccine. But because of the high variability and the irrelevance of monomers to mediate neutralization *in vivo*, these first attempts were thwarted and more than two dozen phase I clinical trials [64] and two phase III efficacy trials in the United States and Thailand showed no protection against infection.

The new idea then was to create live attenuated, *nef*-deleted viruses as vaccine candidates. Shortly after the initial hope that *nef*-deleted viruses may be used as vaccines, the group of Dr. Ruth Ruprecht showed that live attenuated viruses represent safety problems as monkeys infected with *nef*-deleted viruses developed disease [65, 66].

The field then concentrated on nmAb production and on screening of nmAb by establishing the SHIV/macaque model. Several SHIV challenge experiments also provided proof-of-concept that passively administered nAb can protect monkeys from infection [47-50, 52, 53, 67]. With this positive note and the fact that conserved regions do exist in the structure of HIV Env, a better understanding of the different forms of Env (unbound/bound and Env of early isolates vs. so-called late Env) will be sufficient for more successful SHIV trials and identification of more nAb or neutralization susceptible epitopes.

1.3.2 T-Cell Stimulating Vaccines

Induction of CD8⁺ T cells by vaccination with vectors encoding HIV genes (plasmid DNA, modified vaccinia virus Ankara (MVA), adenovirus carrying *gag*, *pol*, *nef* and *env*) might offer a chance for partial protection from HIV infection. CD8⁺ T cells cannot prevent infection of cells but they could abort an infection before it becomes established, or contain the virus at a significantly lower level. Encouraging data arose from the finding that some sex workers in Nairobi are regularly exposed but not infected by HIV. This resistance was found to derive from HIV-specific CD 8⁺ T cells in cervical mucosal lavage samples [68].

However, latently infected cells survive cytotoxic T-lymphocyte surveillance. When those latently infected cells become activated, they produce virions that could infect new cells before the initial cells die or are cleared. Thus, cytotoxic T lymphocytes help control the infection but do not clear the HIV reservoirs completely [1].

1.3.3 The SHIV/Macaque Model

SIV does not appear to cause AIDS in their natural African hosts (SIV_{cpz} in chimpanzees, SIV_{sm} in sooty mangabeys, SIV_{agm} in African green monkeys). Similar to humans, however, several species of Asian macaques develop AIDS when infected with a common non-pathogenic lentivirus of African sooty mangabeys (SIV_{sm} became SIV_{mac}).

SHIV constructs encode HIV-1 *env* in the genome of SIV_{mac} and rhesus macaques infected with such SHIVs show similar pathogenesis leading to AIDS as HIV-1-infected humans do.

SHIVs are unique in that they allow direct testing of HIV-1 Env-based vaccines and neutralizing antibodies isolated from HIV-1-infected individuals in an animal model and primate-tested active and passive immunization can directly enter clinical trial.

Other important advantages are the isolation of new HIV-1 Env neutralizing antibodies from infected primates and the possibility of envelope evolution studies in the primate model [69].

New SHIV constructs should therefore fulfill some criteria to be biologically relevant:

- A) As >50% of all individuals with HIV/AIDS harbor HIV-1 clade C infection, the development of anti-clade C vaccines should be a top priority. To date, however, most primate challenge studies use clade B SHIV strains.
- B) The majority of HIV-1 infections happen during sexual intercourse by mucosally transmissible R5 viruses.
- C) According to an intriguing study [70], recently transmitted HIV-1 clade C isolates surprisingly showed higher neutralization sensitivity. Compared to the predominant quasispecies from a donor, their sexual partner harbored more neutralization sensitive viruses. This finding suggests a bottleneck, which favors neutralization-sensitive quasispecies during or shortly after transmission.

New SHIV constructs therefore should carry HIV-1 clade C *env*, be mucosally transmissible, have R5 tropism and should be neutralization sensitive.

Chapter 2

Aims

As most SHIV constructs are clade B but >50% of all individuals harboring HIV/ADIS worldwide are infected with clade C, the group of Dr. Ruprecht developed a clade C SHIV from a biological isolate of an infected Zambian child [71]. This SHIV (SHIV-1157ipd3N4) was replication competent and more virulent because of an additional NF κ B site engineered in the viral LTR. But as this virus was re-isolated from a monkey 141 weeks post infection, this SHIV represents a late-stage, immune escape virus. A recent study [70] suggests that HIV clade C envelopes from late stage viruses show less neutralization sensitivity than do early biological isolates; a bottle neck during or shortly after sexual transmission seems to appear that favors neutralization-sensitive quasispecies. Therefore, the use of SHIVs containing envelope of late isolates will not only set the bar high for neutralizing antibody studies in rhesus monkeys but also won't mimic the natural course of infection due to their relatively neutralization resistance.

The aim of this project was to engineer a novel SHIV construct on the basis of SHIV-1157ipd3N4. By exchanging the major part of its envelope region with the corresponding region of an earlier viral isolate, the new SHIV will fulfill all criteria of biological relevance: mucosal transmissibility, early clade C envelope and R5 tropism. These criteria ensure this new SHIV to be a useful tool for vaccine efficacy studies in the macaque model.

Chapter 3

Materials

3.1 Vectors and Viral DNA

SHIV-1157ipd3N4 is a chimeric virus containing the SIVmac239 backbone with the HIV-1 clade C *envelope* of an isolate obtained from a Zambian infant (#1157i). The construct was serially passed through five rhesus macaques (p) by blood transfer at week 2 post infection and caused disease (d) in the first monkey (RPn-8) after 137 weeks. The viral DNA was re-isolated from RPn-8 at week 141, used for PCR and an additional NF κ B site was introduced in the 3' LTR of clone 3 to increase replication efficacy (3N4).

The parental virus of SHIV1157ipd3N4, SHIV-1157ip, was re-isolated after passage to RPn-8 before disease developed and represents the early-stage form.

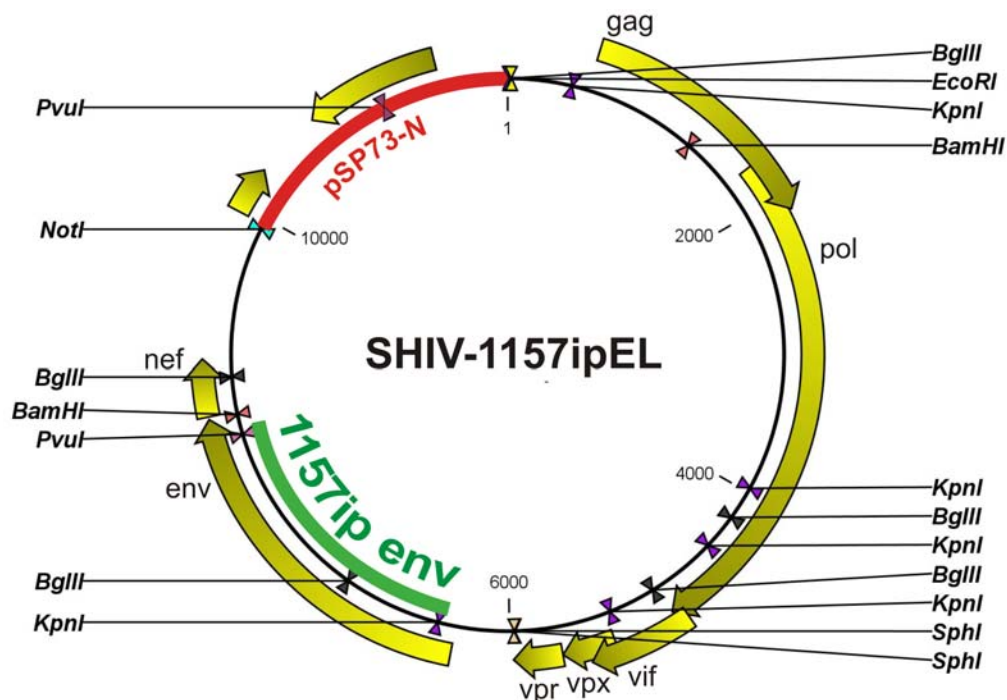


Figure 9 | Genetic map of a viral genome carrying plasmid comprising vector SP73-N (red). Important restriction sites as well as ORF (yellow) and the region of envelope exchange (green) are indicated.

SHIV-1157ipd3N4 whole genome and 3' half only (containing *env*) was provided as a plasmid containing an ampicillin-resistance carrying vector pSP73-N (Figure 9) by R.J. Song and Siddappa NB, as well as a plasmid containing the 3' half of SHIV-1157ip.

3.2 Bacteria

All bacterial work was done with Invitrogen MAX Efficiency Stbl2 Competent Cells. Genotype:

F^- *mcrA* $\Delta(mcrBC\text{-}hsdRMS\text{-}mrr)$ *recA1* *endA1lon* *gyrA96* *thi* *supE44* *relA1* $^-$ $\Delta(lac\text{-}proAB)$

3.3 Cell Lines

- **293T cells**

For the transfection of full-length viral DNA and production of viral particles, 293T cells were used. These cells derive from SV40 Large T antigen carrying human embryonic kidney cells, which allow the replication of episomal DNA.

- **TZM-bl cells**

TZM-bl cells derive from HELA cells and express CD4, CCR5 and CXCR4 as viral receptors. These cells were used in viral assays to quantify HIV infection since they carry a firefly *luciferase* and a *beta-galactosidase* gene under the control of the HIV promoter. Infection with HIV results in expression of both marker genes and can be detected by standard luciferase assays and/or staining of the cells.

TZM-bl cells were provided by the NIH AIDS Research & Reference Reagents Program.

3.4 Media

- **LB**

1%	Enzymatic digest of casein 10 g/L
0.5%	Yeast extract (low sodium) 5 g/L
0.5%	Sodium chloride 5 g/L
0.2%	Inert agents (necessary for tableting process) 2 g/L

Obtained from Sigma (LB Broth Tablets)

- **Carbenicillin**

Obtained from GIBCO (Carbenicillin, Disodium Salt)

- **LB Agar**

1%	Tryptone (10 g/L)
0.5%	Yeast extract (5 g/L)
1%	Sodium chloride (10 g/L)
1.5%	Agar (15 g/L)

Obtained from MP Biomedicals (LB-Agar)

- **SOC Medium**

2%	Tryptone (20 g/L)
0.5%	Yeast extract (5 g/L)
10 mM	Sodium chloride
2.5 mM	Potassium chloride
10 mM	Magnesium chloride
10 mM	Magnesium sulfate
20 mM	Glucose

Obtained from Invitrogen (S.O.C. Medium)

- **10% FCS DMEM**

88%	DMEM
10%	Fetal calf serum (FCS)

- 1% Glutamine
- 1% Penicillin/Streptomycin

- **20% DMEM**

- 78% DMEM
- 20% FCS
- 1% Glutamine
- 1% Penicillin/Streptomycin

- **RPMI**

- 78% RPMI minimal medium
- 20% FCS
- 1% Glutamine
- 1% Penicillin/Streptomycin

- **0.25% Trypsin, EDTA**

Obtained from Invitrogen

- **Minimal Medium**

Obtained from Invitrogen

- **Freeze Medium**

- 10% DMSO
- 90% FCS

3.5 Chemicals, Enzymes and Kits

- **Restriction enzymes**

All restriction enzymes, BSA (100x) and their buffers (5x) were obtained from New England Biolabs.

- **1% Agarose gel**
Gels were always run in 1% agarose in 1x TAE buffer with 4v% ethidium bromide.
- **10x TAE**
Obtained from commercial source
- **H₂O**
For all experiments, ultrapure H₂O (Sigma) was used.
- **Plasmid preparation kit**
To purify plasmids from bacterial cultures, Qiagens QIAprep Spin Miniprep Kit was used with spin columns for 5 ml and tip20 columns for 20 ml cultures
- **Gel elution kit**
To purify DNA from agarose gels, Qiagens QIAquick Gel Extraction Kit was used.
- **Ligase**
ROCHE Rapid DNA Ligation Kit was used for ligations
- **Lipofectamine LTX Reagent**
Obtained from Invitrogen

3.6 Antibodies and Monkey Sera

Serum from rhesus monkey RPn-8 was used as neutralizing antibody source for neutralization assays. Serum samples were collected 45, 65 and 100 weeks post infection and kept in aliquots on -80°C.

Chapter 4

Methods

4.1 Molecular Cloning

Plasmid map analysis and strategy development (genetic maps, open reading frame search and restriction site analysis as well as the identification of an in-frame cloning strategy) was done with CLC Free Workbench 4.0 (CLC bio; www.clcbio.com).

All restriction digests were done with enzymes and buffers from New England Biolabs in a heat block according to the manufacturer's instruction.

DNA and New England Biolabs 1 kb Marker for band size identification were run in general on a 1% agarose gel in 1x TAE buffer at 80-100V. Gel photos were taken with the Biorad Universal Hood II. Relevant bands were excised with sterile disposable scalpels and gel elution was done using the QIAquick Gel Extraction Kit.

The ligation reaction was performed with the ROCHE Rapid DNA Ligation Kit for 15 minutes at RT.

4.2 Transformation

Transformation of the MAX Efficiency Stbl2 E. coli strain was done according to the Invitrogen protocol with minor changes.

A stock of competent cells was thawed on wet ice together with empty polypropylene culture tubes used for subsequent steps.

With the use of the Bunsen burner, 50 µl of the cell suspension was aliquoted into the culture tubes in a sterile manner. For transformation of isolated and purified plasmid, 1 µl was used, for transformation of ligation mixes, 10 µl was used.

After adding the DNA and gentle tapping, the transformation mix was incubated 20 minutes on ice.

After a 30 seconds heat shock at 42°C in the water bath, the mixture was incubated for 2 minutes on ice, followed by the addition of 900 µl S.O.C. medium to each tube. To help the bacteria regenerate, the tubes were incubated 60-80 minutes at 30°C and 150 rpm.

The cultures were then plated out on LB Agar containing 50 µg/ml carbenicillin in a low concentration (100-110 µl of the transformation mix added to the plate) and in a high concentration (the left over ~900 µl of the transformation mix was centrifuged 10 minutes at 3000 rpm, most of the supernatant was discarded, the bacterial pellet resuspended in the leftover and plated out).

4.3 Glycerol Stocks

In a 1.5 ml tube, 0.5 ml autoclaved 30% glycerol in ddH₂O was added to 0.5 ml of the culture (in logarithmic growth phase). After gently inverting the tubes, the stocks were shock frozen using liquid nitrogen or dry ice/ethanol and stored at -80°C.

4.4 Plasmid Isolation

Plasmid isolation was done according to the Qiagen Protocol for Spin columns and tip-20 tubes (QIAprep Spin Miniprep/Midiprep Kit), respectively.

4.5 Freezing and Thawing of Cells

To freeze mammalian cells, 2x10⁶ cells were transferred to a cryo tube with 1 ml of freeze medium. The cells were frozen using a Nalgene cryofreezer and stored 24 hours at -80°C before transfer to a liquid nitrogen freezer for long time storage. Thawing of frozen cells was done by taking out the tube and putting it into a 37°C water bath immediately. The aliquot was then transferred to a 15 ml Falcon tube with 5 ml 10% FCS DMEM media. To wash the cells, the tube was gently mixed and then centrifuged 5 minutes at 1000 rpm.

The supernatant was aspirated and the pellet was gently resuspended in 5 ml 10% FCS DMEM, followed by transfer to a T75 culture flask already containing 10 ml 10% FCS DMEM.

4.6 Preparation of Media and Maintaining of Cell Lines

For preparation of media, the single components were warmed up in the 37°C water bath, mixed together into a 250 ml filter unit and sterilized through a 0.2 µm filter by applying vacuum. The flask was closed, labeled and stored at 4°C.

Before splitting the cells, the media and Trypsin-EDTA bottles were pre-warmed to 37°C in the water bath.

The splitting of cells was performed every 3-4 days, depending on the cell density and the media color in the culture flask. The supernatant was aspirated, 5 ml warmed (37°C) Trypsin-EDTA was added (all values related to T75 culture flasks) to the flask and incubated approximately 3 minutes at 37°C. When the cells detached, 5 ml warmed 10% FCS DMEM was added to rinse the flask. The whole cell suspension was mixed thoroughly; 1 ml was transferred to a 15 ml Falcon tube and centrifuged 5 min at 1000 rpm. After aspirating out the supernatant and gentle resuspending the pellet by tipping against the tube, 5 ml of media (10% FCS DMEM) was added. Further resuspending of the cells, transfer to the culture flask (already containing 10 ml fresh media), gentle swivel, marking the date on the flask and putting back the flask into the incubator (37°C, 5% CO₂) finished the procedure.

4.7 Transfection and Production of Viral Particles

Transfection of 293T cells was performed using the Lipofectamin LTX and Plus Reagent from Invitrogen. In a 12-well plate, 10⁴ cells/ml were plated per well one day before transfection to allow the 293T cells to adhere to the bottom.

For transfection, 200 µl minimal medium, 2 µg of DNA and 1 µl of Plus Reagent was added to Eppendorf tubes, mixed thoroughly and incubated 5 minutes at room

temperature. Five μl Lipofectamine was added to each sample, mixed thoroughly and incubated again 30 minutes. In the meantime, the media supernatant from the cells was discarded and 200 μl minimal medium was added to each well of cells and put back into the 37°C incubator.

After half an hour incubation of the transfection mixture, all ~210 μl were added to the wells (pos. control DNA was added each time in one well as transfection control) and incubated again for 3-4 hours at 37°C before adding ~600 μl DMEM (20% FCS) to each well, making the FCS concentration roughly 10% again. The plate was put back to 37°C and kept there for 24 hours and then transferred to the biocontainment suite, as viral particles were produced by these cells. To obtain virus, the supernatant from the transfected 293T cells was simply harvested for each clone, filtered through a 0.2 μm syringe filter unit and stored at -80°C.

4.8 The TZM-bl Assay

On day one, white 96-well luminometer-read out plates were seeded with 100 μl /well of TZM-bl cells at 5×10^4 cells/ml (diluted in 10% FCS DMEM), 9 wells in the bottom line were left empty for controls and the plate was incubated over night at 37°C.

On day two, viral dilutions (usually 1:5 serial dilution) were performed in a separate 96-well plate at an end volume of 100 μl in 10% FCS DMEM per well. To increase infectivity later on, 10 μl of a 400 $\mu\text{g}/\text{ml}$ DEAE dextran was added to each well of the virus dilution.

After carefully removing the 100 μl medium from each well of the TZM-bl plate, 110 μl from the virus dilution plate were transferred to each well.

	1	2	3	1	2	3	1	2	3	1	2	3
A	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5	1:5
B	1:25			1:25			1:25			1:25		
C	1:125			1:125			1:125			1:125		
D	1:625			1:625			1:625			1:625		
E	1:3025			1:3025			1:3025			1:3025		
F	1:15625			1:15625			1:15625			1:15625		
G	1:78125			1:78125			1:78125			1:78125		
H	pos. contr			Medium only			Cells only			Virus only		

Figure 10 | Pipetting scheme for the TZM-bl assay for 4 clones in a triplicate for each 96-well plate (red, turquoise, green, yellow) with viral dilution indicated. The bottom row wells are used for controls (grey).

The plate was incubated at 37°C, 5% CO₂ over night.

On day three, the medium was replaced.

On day four, 100 µl/well luciferase substrate (Bright Glo Luciferase, Promega) was added to each well and the plate was read in a Luminometer (PerkinElmer VICTOR Light 1420 Luminescence Counter).

4.9 The p27 Assay

Measuring the amount of SIV p27 (homologous to HIV p24) by a sandwich ELISA enables the quantification of the viral load in tissue culture samples. For this, 25 µl distribution buffer was added to each well of a micro-ELISA plate coated with anti-p27 murine mAb. Then, 100 µl SIV p27 standard dilution, cell-free samples of virus-infected PBMCs taken at different time points, or negative control (medium only) were added to the wells in triplicates. Following a 60 minute incubation time at 37°C and four washing steps, 100 µl of conjugate solution (peroxidase-conjugated mixture of monoclonal antibodies to SIV p27) was added to each well. The wells were then covered with a plate sealer and incubated again at 37°C for 60 minutes. After another

four washing steps 100 μ l Peroxidase Substrate was added to each well, incubated for 30 minutes at room temperature followed by adding 100 μ l of Stop Solution. Absorbance at 450 nm was then measured in a micro-ELISA plate reader.

The ELISA was done with all solutions and materials provided by Advanced BioScience Laboratories, Inc; SIV p27 Antigen Capture Assay.

4.10 Generation of Viral Stocks in rmPBMC

To grow viral stocks in PBMC derived from rhesus macaques (RM) (purified by Ficoll gradient centrifugation), one stock of RM PBMC was thawed, activated by IL-2 (20 μ g/ml) and transferred to a 12 well plate.

These cells then got infected with a molecular clone (filtered supernatant of transfected 293T cells) and TNF- α (10ng/ml) was added for optimal virus replication. Every two days, supernatant was collected and filtered through a 0.2 μ m syringe filter unit. For p27 measurement, 100 μ l were transferred to another plate; the rest was frozen as viral stock at -80°C.

4.11 The Neutralization Assay

TZM-bl based neutralization assays of SHIV-1157ipd3N4 and SHIV-1157ipEL were performed with autologous sera from the monkey of viral origin, RPn-8. The protocol was the same as for the TZM-bl assay with the only change that the virus concentration was constant and the serum was serially diluted.

The readout was done after addition of Luciferase substrate with the Luminometer PerkinElmer VICTOR Light 1420 Luminescence Counter.

Chapter 5

Results

5.1 Molecular Cloning of SHIV-1157ipEL

To avoid problems associated with working on a big construct, in this case 13kb, and to get rid of multiple restriction sites, a two-way strategy was devised (Figure 11): Briefly, the exchange of the *env* gene was done as the first step in the 3' half of the viral genome only. After screening for positive clones, the 3' half carrying the new envelope was fused in the second step with the 5' half of SHIV-1157ipd3N4, resulting in a full-length virus again (see **a.** and **b.**).

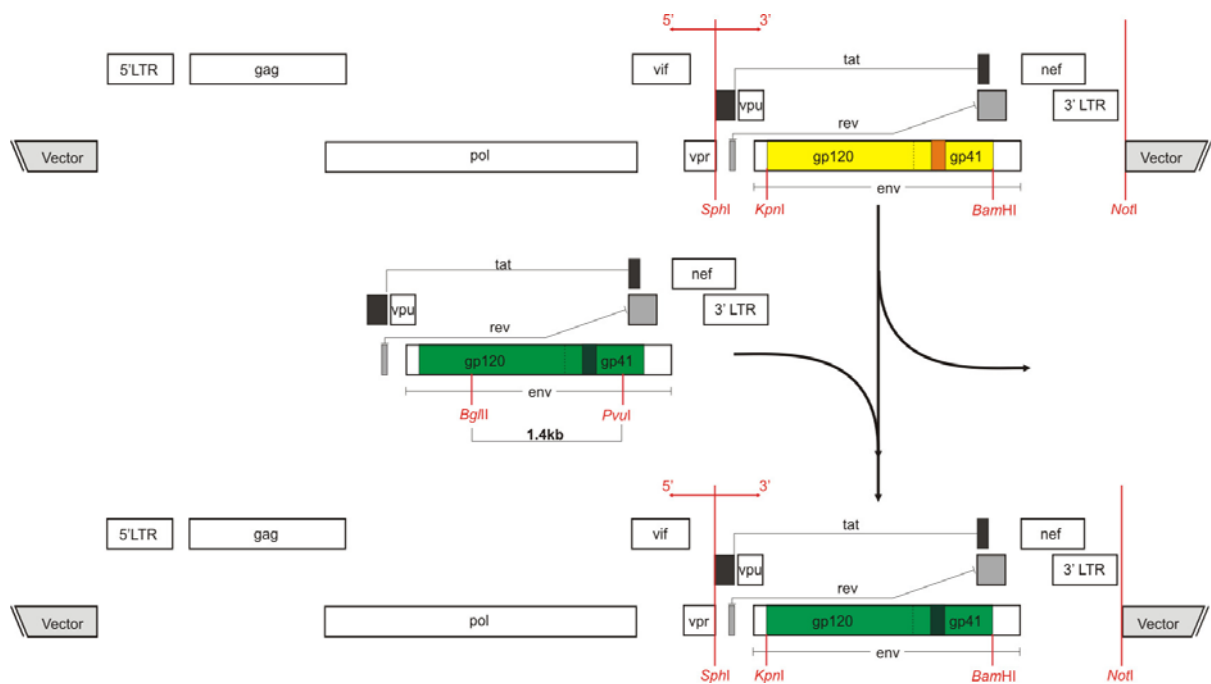


Figure 11 | Schematically diagram of the cloning strategy The pSP-73N plasmid (light grey) with the SHIV-1157ipd3N4 backbone and its *env* (yellow) which gets substituted by the early SHIV-1157ip *env* (green) are shown. The indicated restriction enzymes (red) provide the two way strategy, the substitution of *env* and the identification of positive clones as outlined in the text.

a. Substitution of the late envelope

To substitute the late *env* of SHIV-1157ipd3N4 with the early *env* of SHIV-1157ip, we identified *KpnI* and *BamHI* as two restriction sites allowing a substitution of the major part of the *env*, spanning most of gp120 as well as the entire gp41 extracellular domain and the transmembrane region (Figure 11). The resulting 2.1 kb fragment, representing the early *env* of SHIV-1157ip and the 4.2 kb fragment, representing the vector pSP73-N plus the Δenv SHIV-1157ipd3N4 3' were excised on an UV bench and gel eluted using a Qiagen kit (Figure 12).

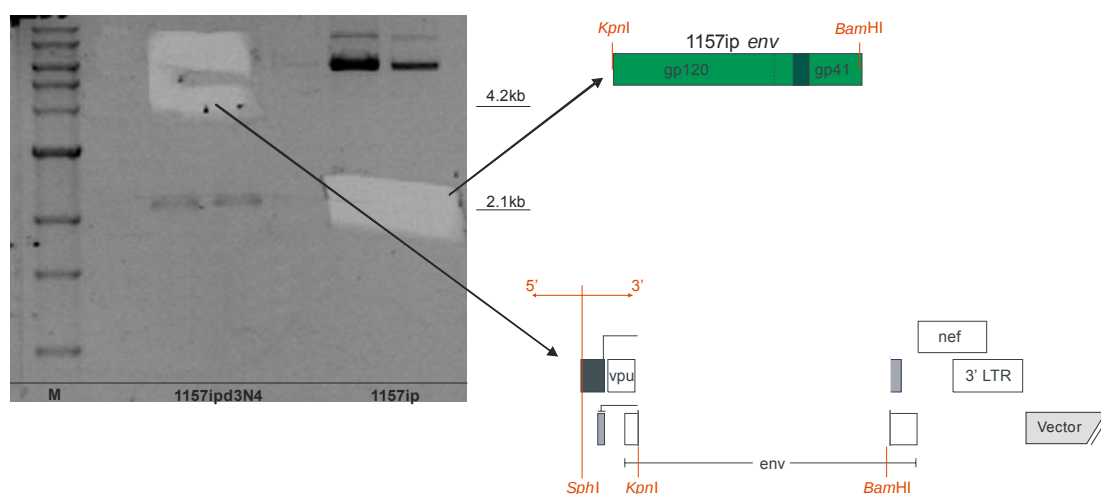


Figure 12 | Gel extraction scheme Picture of the cut gel after digestion of SHIV-1157ipd3N4 and SHIV-1157ip with the enzymes *KpnI* and *BamHI*. Cut out bands represent the 4.3kb fragment of the late 3' half backbone without the major part of *env* and the 2.1kb fragment of the early SHIV-1157ip *env*, respectively.

The purity, size and concentration of the extracted DNA was confirmed on a gel (data not shown), and equal amounts of SHIV-1157ip *env* and Δenv SHIV-1157ipd3N4 3' backbone were ligated and transformed into Sbl2 cells. All bacterial growth was done at 30°C and 150 rpm, as suggested for cloning of retroviral constructs to maximize the yield of positive clones.

Transformed cells were plated out, grown overnight and the next day, colonies were picked and inoculated in 5 ml LB/carbenicillin and cultured for ~16h at 30°C, 150 rpm.

Following the preparation of glycerol stocks of all ten clones and re-isolation of the plasmid DNA, a screening for positive clones was performed.

The identification of positive, early envelope-carrying clones was possible due to a unique *PvuI* restriction site in the sequence of the early *envelope*, resulting in a unique 1.4 kb fragment after double digestion with *PvuI* and *BglII* (Figure 11 and Figure 13). Figure 13 shows the result of the screen after this first step. Restriction with *PvuI* and *BglII* leads to the desired 1.4 kb fragment for all ten clones (see arrow), thus all screened clones are positive and represent the early envelope of SHIV-1157ip in the 3' half of the late SHIV-1157ipd4N4 backbone.



Figure 13 | Screening and identification of positive clones Plasmid DNA of 10 clones was digested with *BglII* and *PvuI*, resulting in a 1.4kb fragment, unique for positive clones.

b. Construction of full length virus containing the early envelope

To construct a full-length virus, clone 8 was picked for further ligation with the SHIV-1157ipd3N4 5' half. This was done with a 5' half comprising the pSP73-N vector provided by Siddappa N. B. (Figure 14 b) “p⁵”), as well as with a newly eluted 5' half (plus vector) of the original SHIV-1157ipd3N4 plasmid (Figure 14 b) “5' from 1157ipd3N4”). Clone 8 was digested with the restriction enzymes *SphI* and *NotI*, resulting in a 3.9 kb fragment, representing the 3' half without the pSP73-N vector, and ligated with the 9kb fragment comprising the entire 5' half of SHIV-1157ipd3N4 and the pSP73-N vector of either p⁵ or the newly excised 5' half of the original SHIV-1157ipd3N4 vector (Figure 14).

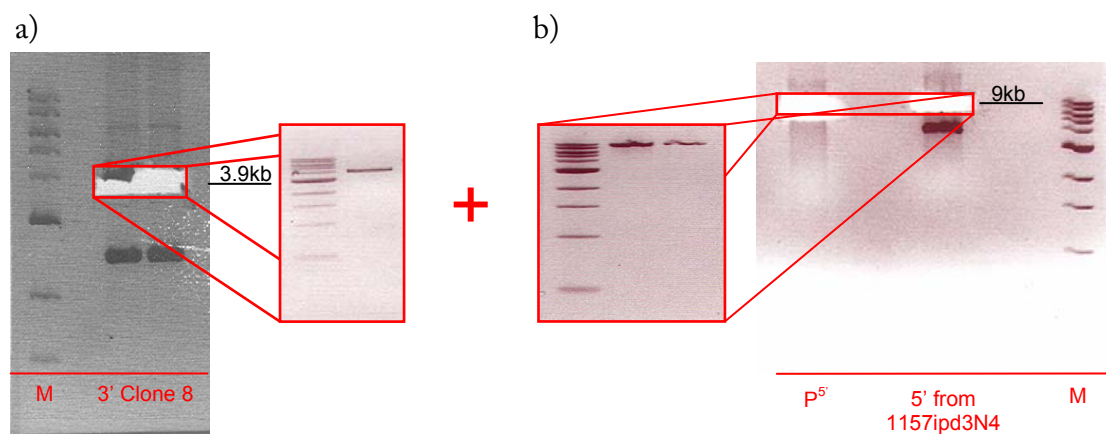


Figure 14 | Gel extraction and ligation scheme for full length clones Fusion of the 5' half of SHIV-1157ipd3N4 with the rearranged 3' half comprising the early *env*. **a** | Gel cut and gel elution control of the 3.9kb band representing the 3' half backbone only. **b** | Gel cut and gel elution control of the 9kb 5' half backbone plus pSP-73N vector derived from a provided plasmid (p^{5'}) and from the original SHIV-1157ipd3N4 plasmid

Stbl2 cells were transformed with the ligation mix, 12 colonies were picked from the plate and screened for positive clones and used for glycerol stocks and minipreps as previously described.

Seven out of 12 clones showed bands of different size, which sum up to the desired 13 kb, including the unique 1.4 kb band and thus were positive (Figure 15).

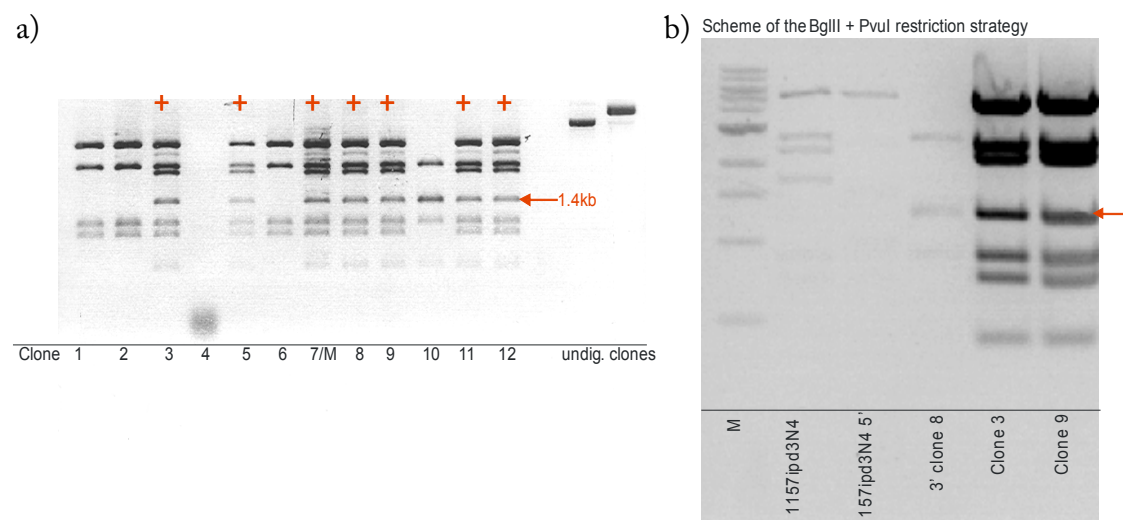


Figure 15 | Full length clone screen **a** | Screening of positive full length clones using *Bgl*II and *Pvu*II which resulted in a 1.4kb fragment, unique for positive clones (red arrow). Positive clones are indicated by a red plus symbol. **b** | Control gel – the 1.4kb band exclusively appears in the lane of the rearranged clones, not in the 5' alone or the original SHIV-1157ipd3N4. Also indicated the size shift from the rearranged 3' half (3' clone 8) to the full length clones (clone 3 and clone 9) of final ~13kb.

This virus, as it encodes an early *envelope* (1157ipE) in a late biological backbone (L), is named SHIV-1157ipEL.

5.2 Testing the Functionality of Clones

To identify functional, infective virus from large amounts of clones, the TZM-bl assay is the method of choice. TZM-bl cells are HeLa derived cells stably expressing CD4, CCR5 and CXCR4 as viral receptors and co-receptors, respectively.

They are also engineered to carry a luciferase gene under control of an LTR derived from virus and therefore, luciferase is expressed when viral Tat is present in the cell, meaning, if the virus is functional in infection, disassembly and synthesis, theoretically.

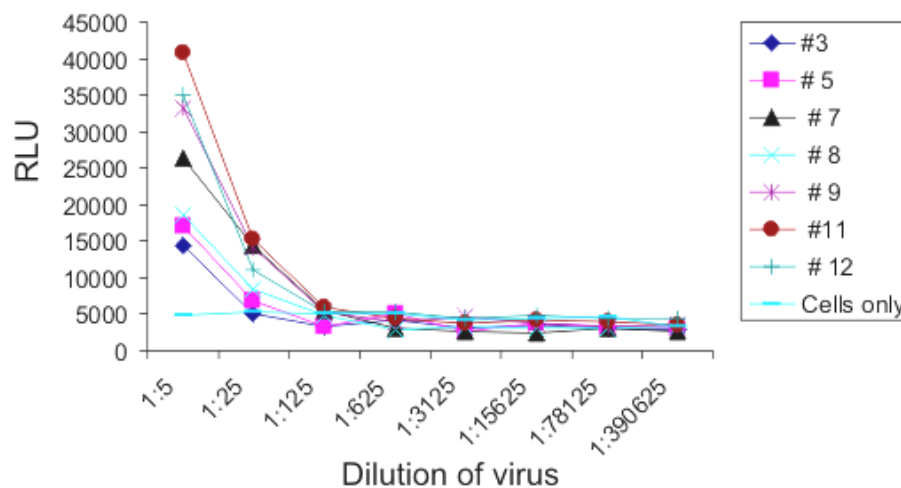


Figure 16 | Functionality of the clones in TZM-bl assay

TZM-bl cells were infected with different dilutions of supernatant from virus producing 293T cells. Functional virus infects TZM-bl cells and activates LTR-controlled luciferase. Luciferase activity is then measured with a luminometer and plotted against dilution. All 7 clones are positive; the variation in relative light units (RLU) likely is based on different viral production in 293T cells.

If the clones are positive in functionality, first, the viral particles produced from the 293T cells after transfection are infective. Second, if viral disassembly and transcription works, viral Tat is produced in the cells which then transactivates the

cellular LTR-controlled luciferase construct and luciferase activity can be measured after addition of luciferase substrate.

The result of the measurement can be presented as relative luciferase units (RLU) blotted against the dilution of the viral clones used (Figure 16).

In case of the seven full length clones of SHIV-1157ipEL (clone #3, #5, #7, #8, #9, #11, #12), we could show that all clones are positive, as luciferase was produced, which indicates that virus has infected TZM-bl cells and produced Tat. The range in RLU for the corresponding clones can result from different infectivity of the clones, but also, and more likely, from different concentration of virus as production of virus in 293T cells can vary from well to well (as the supernatant of the 293T cells is filtered and taken as undiluted virus sample). As all clones are positive and functional, we took clone #7 for further analysis and for setting up a viral stock in RM PBMC.

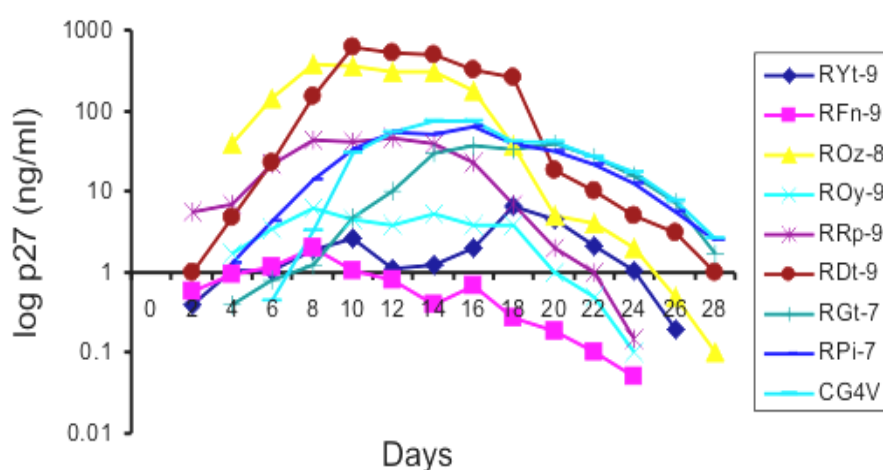


Figure 17 | Replication kinetics Nine different rhesus monkey peripheral blood mononuclear cells (rmPBMC) were infected with viral clone 7. Every two days samples were taken and viral p27 concentration was measured using an ELISA kit to evaluate replication kinetics and optimal supporting rmPBMC for growth of a viral stock.

To identify optimal growth support of the virus for viral stocks, SHIV-1157ipEL clone #7 was grown in RM PBMC from nine different macaques and daily samples were taken to measure the p27 concentration, which correlates with virus concentration, by a special p27 ELISA kit. As presented in Figure 17, the virus did grow in all nine RM PBMC, but with different kinetics as of genetic diversity. The PBMC from monkey RFn-9, for example, does not support replication very well,

whereas the virus replicates to a good extend in the PBMC of monkey ROz-8, RDt-9 and ROy-9.

The PBMC of the latter three monkeys were pooled for the growth of the viral stock.

5.3 Measuring the TCID₅₀

As for future experiments it is important to know the concentration of virus used. The concentration of virus can be depicted in two different ways: as multiplicity of infection (MOI), which gives a theoretically number of viral particles per cell or as tissue culture infective dose 50 (TCID₅₀). In our case, it was important to find out which sample of the virus grown in the pooled RM PBMC (see chapter 5.2) is the most infectious. Therefore TZM-bl cells were again infected with aliquots of samples from the viral stock frozen on different days after infection of the pooled RM PBMC and infectivity was measured according to the TZM-bl protocol.

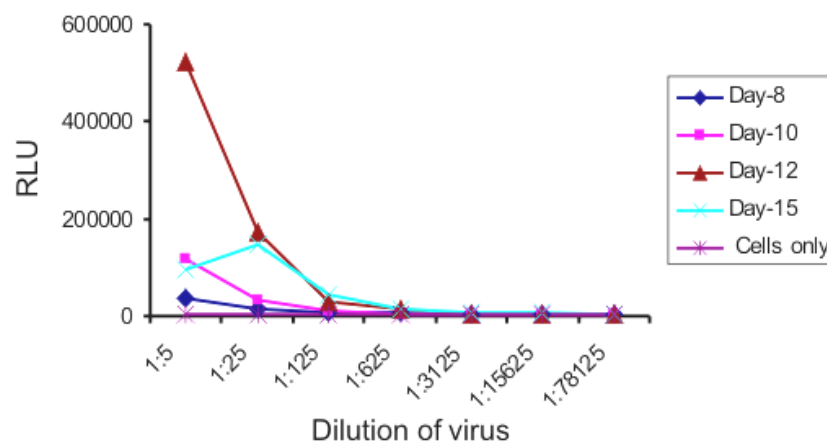


Figure 18 | TCID₅₀ of viral stock Different day samples (indicated on the right) of viral stocks were used to infect TZM-bl cells to measure the time point of highest viral load and tissue culture infective dose 50 (TCID₅₀), respectively.

After another luciferase activity read out shown in Figure 18, the viral load in the stock clearly reaches its maximum around day 12, making the viral stock from day 12 the most infectious.

5.4 Neutralization

To answer the question if we succeeded in cloning of a more neutralization sensitive envelope comprising SHIV, we set up a neutralization assay on TZM-bl cells with sera from monkey RPN-8. As SHIV-1157ip and SHIV-1157ipd3 were re-isolated from this monkey, the neutralization assay with these autologous sera from different time points of extraction gave us a great opportunity to compare the neutralization susceptibility of the early virus with the additional NF κ B sites to the late virus, also comprising additional NF κ B sites for better replication.

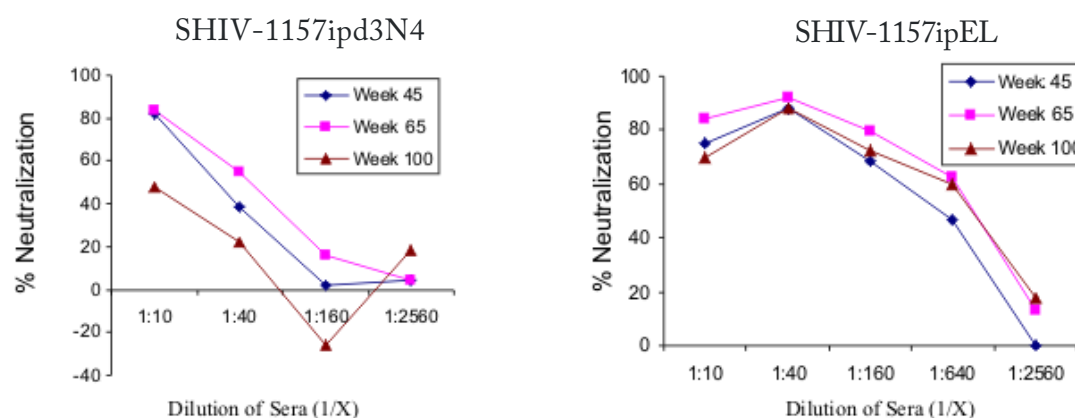


Figure 19 | TZM-bl neutralization assay with RPN8 rhesus monkey sera TZM-bl neutralization assay for comparison of the neutralization sensitivity of SHIV-1157ipd3N4 with the newly engineered virus SHIV-1157ipEL. Autologous sera from rhesus monkey RPN-8, extracted at different time points after infection, was added to TZM-bl cells in different dilutions (x-axis) before addition of virus. Percent neutralization, the reciprocal RLU, is given on the y-axis.

In comparison to the late virus SHIV-1157ipd3N4, the new virus SHIV-1157ipEL is neutralization sensitive for every serum sample (early serum week 45, mid-time serum week 65 and late serum week 100), indicated by percent neutralization, the reciprocal value of RLU (Figure 19). At serum dilution 1:40, 60-20% of SHIV-1157ipd3N4 gets neutralized with the different serum samples, respectively. For SHIV-1157ipEL, however, neutralization around 90% can be seen for all serum samples at the same concentration of 1:40. This data proves that we succeeded in our attempt to create a replication competent virus which presents the early envelope on its surface.

Chapter 6

Discussion

The overall goal of this thesis was to generate a R5 SHIV that encodes an early, neutralization sensitive HIV-1 clade C *env*. The group of Dr. Ruprecht recently published an engineered late stage clade C SHIV with an additional NFκB site in its LTR to render it more replication competent[71].

We used this SHIV construct as backbone and substituted the major part of its late envelope with the corresponding section of an early *env*.

The newly constructed molecular clone, SHIV-1157ipEL, has a number of relevant characteristics:

- 1) SHIV-1157ipEL encodes an HIV-1 clade C *env* from an early, neutralization sensitive isolate. According to an intriguing study, recently transmitted HIV-1 clade C isolates were surprisingly neutralization sensitive [70]. After mucosal transmission during sexual intercourse, recipients harbor more neutralization-sensitive viruses compared to the strains predominant in the donor, suggesting the occurrence of a bottleneck effect during or shortly after transmission. The use of late stage viruses in the SHIV/macaque model that have undergone multiple rounds of neutralizing antibody selection, followed by repeated escapes, therefore won't mimic the real situation of infection during sexual intercourse. Such viruses would set the bar unrealistically high for nAb challenge studies *in vivo*. To mimic sexual transmission in primates, SHIV constructs carrying *env* of recently transmitted, early HIV-1 isolates will be preferable over SHIV strains with *env* genes of late-stage viruses. The neutralization assay data in this thesis show significant higher neutralization susceptibility of SHIV-1157ipEL with autologous monkey sera compared to the neutralization sensitivity of the late stage virus SHIV-1157ipd3N4.
- 2) SHIV-1157ipEL is an R5-tropic virus (data not shown). As an R5-tropic virus, SHIV-1157ipEL does not induce the acute, severe pathogenicity

typically seen within 2 weeks after infection with other, often dual tropic R5/X4 SHIV constructs (e.g. SHIV89.6P), which does not reflect the biology of HIV-1 infection in humans, which is characterized by years of clinically stable, chronic infection before immune exhaustion sets in.

- 3) SHIV-1157ipEL contains an additional NFκB site in its LTR. An earlier work of this group showed a direct relation of the number of NFκB sites with LTR mediated gene expression [71]. This observation was used to engineer an additional NFκB site into the LTR of SHIV-1157ipd3N4 with the result of higher replication ability. As the SHIV-1157ipd3N4 backbone was used to incorporate the early *env*, SHIV-1157ipEL also includes an additional NFκB site, making this virus more replication competent too.

Taken together, this new SHIV construct fulfils all criteria for biological relevance: Clade C envelope, neutralization susceptibility, replication ability, R5 tropism and mucosal transmissibility. SHIV-1157ipEL may therefore represent a practical tool to test the efficacy of vaccine candidates targeting Env of the world's most prevalent clade, HIV-1 clade C, in a relevant primate model and will most closely mimic the sexual transmission in humans.

Currently, four rhesus macaques are infected with this SHIV for viral adaptation to the rhesus monkey model, and future experiments will concern evaluating virus behavior *in vivo* and monitoring disease progression of the monkeys. Other experiments will include investigating envelope evolutionary patterns during adaptation and low-dose multiple titration to identify the exact dose required for systemic infection. If the virus successfully replicates in these monkeys and causes AIDS, this SHIV construct represents a good tool for vaccine efficacy studies targeting envelope antigens, especially for passive immunization, due to its neutralization sensitivity.

Chapter 7

Acknowledgments

First, I want to thank Prof. Dr. Ruth Ruprecht for the great chance and opportunity to work at her laboratory at the Dana-Farber Cancer Institute. Thanks to my mentor, Dr. Siddappa Nagadenahalli, for his support, guidance and supervision. Thanks to their great support, I was the first student to be allowed to enter the BL 2+ “Biocontainment Suite” of the Dana-Farber Cancer Institute in company with my mentor, to work with live-virus.

I also want to thank all the colleagues in the Ruprecht Laboratory for their great companionship, especially Agnès-Laurence Chenine, Michael Humbert and Victor Kramer for their never-ending support in and out the lab.

Last but not least, I am very thankful for Susan Sharp’s efforts and help with all the bureaucracy.

Chapter 8

Bibliography

1. Johnston, M.I. and A.S. Fauci, *An HIV vaccine--evolving concepts*. N Engl J Med, 2007. **356**(20): p. 2073-81.
2. Huber, M. and A. Trkola, *Humoral immunity to HIV-1: neutralization and beyond*. J Intern Med, 2007. **262**(1): p. 5-25.
3. Rowland-Jones, S.L., *Timeline: AIDS pathogenesis: what have two decades of HIV research taught us?* Nat Rev Immunol, 2003. **3**(4): p. 343-8.
4. Karlsson Hedestam, G.B., et al., *The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus*. Nat Rev Microbiol, 2008. **6**(2): p. 143-55.
5. Burton, D.R., R.L. Stanfield, and I.A. Wilson, *Antibody vs. HIV in a clash of evolutionary titans*. Proc Natl Acad Sci U S A, 2005. **102**(42): p. 14943-8.
6. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)*. Science, 1983. **220**(4599): p. 868-71.
7. Gallo, R.C., et al., *Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS*. Science, 1984. **224**(4648): p. 500-3.
8. Levy, J.A., et al., *Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS*. Science, 1984. **225**(4664): p. 840-2.
9. Coffin, J., et al., *Human immunodeficiency viruses*. Science, 1986. **232**(4751): p. 697.
10. Rabson, A.B. and M.A. Martin, *Molecular organization of the AIDS retrovirus*. Cell, 1985. **40**(3): p. 477-80.
11. Heeney, J.L., A.G. Dalgleish, and R.A. Weiss, *Origins of HIV and the evolution of resistance to AIDS*. Science, 2006. **313**(5786): p. 462-6.
12. Arien, K.K., G. Vanham, and E.J. Arts, *Is HIV-1 evolving to a less virulent form in humans?* Nat Rev Microbiol, 2007. **5**(2): p. 141-51.

13. Keele, B.F., et al., *Chimpanzee reservoirs of pandemic and nonpandemic HIV-1*. Science, 2006. **313**(5786): p. 523-6.
14. Peterlin, B.M. and D. Trono, *Hide, shield and strike back: how HIV-infected cells avoid immune eradication*. Nat Rev Immunol, 2003. **3**(2): p. 97-107.
15. Leonard, C.K., et al., *Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells*. J Biol Chem, 1990. **265**(18): p. 10373-82.
16. Mizuochi, T., et al., *Diversity of oligosaccharide structures on the envelope glycoprotein gp 120 of human immunodeficiency virus 1 from the lymphoblastoid cell line H9. Presence of complex-type oligosaccharides with bisecting N-acetylglucosamine residues*. J Biol Chem, 1990. **265**(15): p. 8519-24.
17. Jones, K.A. and B.M. Peterlin, *Control of RNA initiation and elongation at the HIV-1 promoter*. Annu Rev Biochem, 1994. **63**: p. 717-43.
18. Doms, R.W. and D. Trono, *The plasma membrane as a combat zone in the HIV battlefield*. Genes Dev, 2000. **14**(21): p. 2677-88.
19. Geijtenbeek, T.B., et al., *DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells*. Cell, 2000. **100**(5): p. 587-97.
20. Kwon, D.S., et al., *DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection*. Immunity, 2002. **16**(1): p. 135-44.
21. Liu, J., et al., *Molecular architecture of native HIV-1 gp120 trimers*. Nature, 2008.
22. Gallay, P., et al., *HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway*. Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9825-30.
23. Bukrinsky, M.I., et al., *A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells*. Nature, 1993. **365**(6447): p. 666-9.
24. Heinzinger, N.K., et al., *The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells*. Proc Natl Acad Sci U S A, 1994. **91**(15): p. 7311-5.
25. Miller, M.D., C.M. Farnet, and F.D. Bushman, *Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition*. J Virol, 1997. **71**(7): p. 5382-90.

26. Stevenson, M., et al., *Molecular basis of cell cycle dependent HIV-1 replication. Implications for control of virus burden.* Adv Exp Med Biol, 1995. **374**: p. 33-45.
27. Unutmaz, D., et al., *Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes.* J Exp Med, 1999. **189**(11): p. 1735-46.
28. Ducrey-Rundquist, O., M. Guyader, and D. Trono, *Modalities of interleukin-7-induced human immunodeficiency virus permissiveness in quiescent T lymphocytes.* J Virol, 2002. **76**(18): p. 9103-11.
29. Piatak, M., Jr., et al., *High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR.* Science, 1993. **259**(5102): p. 1749-54.
30. Pantaleo, G., et al., *HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease.* Nature, 1993. **362**(6418): p. 355-8.
31. Chun, T.W., et al., *Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection.* Nature, 1997. **387**(6629): p. 183-8.
32. Wain-Hobson, S., *Down or out in blood and lymph?* Nature, 1997. **387**(6629): p. 123-4.
33. Candotti, D., et al., *Status of long-term asymptomatic HIV-1 infection correlates with viral load but not with virus replication properties and cell tropism. French ALT Study Group.* J Med Virol, 1999. **58**(3): p. 256-63.
34. Pantaleo, G., et al., *Studies in subjects with long-term nonprogressive human immunodeficiency virus infection.* N Engl J Med, 1995. **332**(4): p. 209-16.
35. Deacon, N.J., et al., *Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients.* Science, 1995. **270**(5238): p. 988-91.
36. Mikhail, M., B. Wang, and N.K. Saxena, *Mechanisms involved in non-progressive HIV disease.* AIDS Rev, 2003. **5**(4): p. 230-44.
37. Masciotra, S., et al., *Temporal relationship between V1V2 variation, macrophage replication, and coreceptor adaptation during HIV-1 disease progression.* Aids, 2002. **16**(14): p. 1887-98.
38. Wang, B., et al., *HIV-1 strains from a cohort of American subjects reveal the presence of a V2 region extension unique to slow progressors and non-progressors.* Aids, 2000. **14**(3): p. 213-23.
39. Kaslow, R.A., et al., *Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection.* Nat Med, 1996. **2**(4): p. 405-11.

40. Agrawal, L., et al., *Role for CCR5Delta32 protein in resistance to R5, R5X4, and X4 human immunodeficiency virus type 1 in primary CD4+ cells.* J Virol, 2004. **78**(5): p. 2277-87.
41. Koenig, S., et al., *Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression.* Nat Med, 1995. **1**(4): p. 330-6.
42. Goulder, P.J., et al., *Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS.* Nat Med, 1997. **3**(2): p. 212-7.
43. Kelleher, A.D., et al., *Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses.* J Exp Med, 2001. **193**(3): p. 375-86.
44. Finkel, T.H., et al., *Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes.* Nat Med, 1995. **1**(2): p. 129-34.
45. Mueller, Y.M., et al., *Increased CD95/Fas-induced apoptosis of HIV-specific CD8(+) T cells.* Immunity, 2001. **15**(6): p. 871-82.
46. Mascola, J.R., *Defining the protective antibody response for HIV-1.* Curr Mol Med, 2003. **3**(3): p. 209-16.
47. Parren, P.W., et al., *Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro.* J Virol, 2001. **75**(17): p. 8340-7.
48. Baba, T.W., et al., *Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection.* Nat Med, 2000. **6**(2): p. 200-6.
49. Ferrantelli, F., et al., *Time dependence of protective post-exposure prophylaxis with human monoclonal antibodies against pathogenic SHIV challenge in newborn macaques.* Virology, 2007. **358**(1): p. 69-78.
50. Ferrantelli, F., et al., *Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies.* J Infect Dis, 2004. **189**(12): p. 2167-73.
51. Mc Cann, C.M., R.J. Song, and R.M. Ruprecht, *Antibodies: can they protect against HIV infection?* Curr Drug Targets Infect Disord, 2005. **5**(2): p. 95-111.

52. Ruprecht, R.M., et al., *Antibody protection: passive immunization of neonates against oral AIDS virus challenge*. Vaccine, 2003. **21**(24): p. 3370-3.
53. Safrit, J.T., et al., *Immunoprophylaxis to prevent mother-to-child transmission of HIV-1*. J Acquir Immune Defic Syndr, 2004. **35**(2): p. 169-77.
54. Zwick, M.B. and D.R. Burton, *HIV-1 neutralization: mechanisms and relevance to vaccine design*. Curr HIV Res, 2007. **5**(6): p. 608-24.
55. Yang, X., et al., *Stoichiometry of antibody neutralization of human immunodeficiency virus type 1*. J Virol, 2005. **79**(6): p. 3500-8.
56. Herrera, C., et al., *Dominant-negative effect of hetero-oligomerization on the function of the human immunodeficiency virus type 1 envelope glycoprotein complex*. Virology, 2006. **351**(1): p. 121-32.
57. Haynes, B.F., et al., *Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies*. Science, 2005. **308**(5730): p. 1906-8.
58. Pantophlet, R. and D.R. Burton, *GP120: target for neutralizing HIV-1 antibodies*. Annu Rev Immunol, 2006. **24**: p. 739-69.
59. Phogat, S. and R. Wyatt, *Rational modifications of HIV-1 envelope glycoproteins for immunogen design*. Curr Pharm Des, 2007. **13**(2): p. 213-27.
60. Srivastava, I.K., J.B. Ulmer, and S.W. Barnett, *Neutralizing antibody responses to HIV: role in protective immunity and challenges for vaccine design*. Expert Rev Vaccines, 2004. **3**(4 Suppl): p. S33-52.
61. Zolla-Pazner, S., *Identifying epitopes of HIV-1 that induce protective antibodies*. Nat Rev Immunol, 2004. **4**(3): p. 199-210.
62. Zwick, M.B., *The membrane-proximal external region of HIV-1 gp41: a vaccine target worth exploring*. Aids, 2005. **19**(16): p. 1725-37.
63. McMichael, A.J., *HIV vaccines*. Annu Rev Immunol, 2006. **24**: p. 227-55.
64. Spearman, P., *Current progress in the development of HIV vaccines*. Curr Pharm Des, 2006. **12**(9): p. 1147-67.
65. Ruprecht, R.M., T.W. Baba, and V. Liska, *Attenuated HIV Vaccine: Caveats*. Science, 1996. **271**(5257): p. 1790b-1791b.
66. Baba, T.W., et al., *Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques*. Nat Med, 1999. **5**(2): p. 194-203.

67. Mascola, J.R., et al., *Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies.* Nat Med, 2000. **6**(2): p. 207-10.
68. Kaul, R., et al., *HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi.* J Immunol, 2000. **164**(3): p. 1602-11.
69. Vlasak, J. and R.M. Ruprecht, *AIDS vaccine development and challenge viruses: getting real.* Aids, 2006. **20**(17): p. 2135-40.
70. Derdeyn, C.A., et al., *Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission.* Science, 2004. **303**(5666): p. 2019-22.
71. Song, R.J., et al., *Molecularly cloned SHIV-1157ipd3N4: a highly replication-competent, mucosally transmissible R5 simian-human immunodeficiency virus encoding HIV clade C Env.* J Virol, 2006. **80**(17): p. 8729-38.

Curriculum Vitae

Personal Data

Name	Klemens Johannes Wassermann
Date of Birth	May 5, 1984
Place of Birth	Vienna
Nationality	Austria

Education

1990–1994	Primary school, Primary school Theodor-Körner-Gasse and Prießnitzgasse
1994–2002	Secondary school Bundesrealgymnasiums Franklinstr. 26, with focus on natural science
Mai 2002	Matura (High School Diploma)
2002–2004	Study of Biology at the University of Vienna
2004	First Diploma Exam
2004–present	Study of Microbiology/Genetics at the University of Vienna with focus on Molecular Immunology
August 2007 – April 2008	Diploma Thesis in the field of HIV Vaccine Research at Dana Faber Cancer Institute/Harvard Medical School , Boston, MA