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Proviral Cloning of Primate Immunodeficiency Viruses Encoding the HIV Subtype C Envelope

MASTER'S THESIS

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List of Abbreviations

%	Percent	**min**	minutes
(m)RNA	(messenger) Ribonucleic acid	**ml**	milliliter
(n)Ab	(neutralizing) antibodies	**Nef**	Negative factor
°C	Degree Celsius	**ng**	Nanogram
µg	microgram	**NK**	Natural killer cell
µl	microliter	**nm**	nanometer
α	Alpha	**NRM IgG**	naive rhesus monkey IgG
AIDS	Acquired immunodeficiency syndrome	**ORF**	Open reading frame
APC	Antigen presenting cell	**PAMPs**	Pathogen associated molecular patterns
APOPEC3G	apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G	**PBMC**	Peripheral Blood Mononuclear Cells
AZT	Azidothymidine	**PBS**	Phosphate buffered saline
bp	base pair	**PCR**	Polymerase Chain Reaction
C1-C5	conserved regions	**PHA**	Phytohaemagglutinin
C'ADE	Complement mediated antibody-dependent enhancement	**pmol**	picomol
CD4	Cluster of differentiation 4	***pol***	encodes polymerase
cDNA	complementary DNA	**PRR**	Pattern recognition receptors
CRF	circulating recombinant forms	**R5**	CCR5 tropic
DC	Dendritic cells	**R5X4**	dual tropic (CXCR4 and CCR5 tropic)
DFCI	Dana Farber Cancer Institute	**Rev**	Regulator of expression of virion proteins
DNA	Deoxyribonucleic acid	**RLU**	Relative Fluorescence Units
E.Coli	*Escherichia coli*	**RM**	Rhesus monkey
EB	Elution Buffer	**rpm**	rounds per minute
ELISA	Enzyme-linked immunosorbent assay	**RT**	Room temperature
env	encodes envelope	**S.O.C.**	Super-optimal broth with catabolite repression
FBS	Fetal bovine serum	**SAMHD1**	SAM domain and HD domain-containing protein 1
FDU	Fast Digest Unit	**SHIV**	Simian-human Immunodeficiency Virus
gag	Encodes group specific antigen	**SIV**	Simian Immunodeficiency Virus
GFP	Green fluorescent protein	**SIV_{cpz}**	SIV from chimpanzee
GM	Growth Media	**SIV_{mac}**	SIV from macaque
gp120	Envelope glycoprotein 120	**SIV_{smm}**	SIV from sooty mangabey
gp41	Envelope glycoprotein 41	**Tat**	Trans-activator of transcription

HIC	heat inactivated complement	**TCID50**	Tissue Culture Infectious Dose 50
HIV	Human Immunodeficiency Virus	**TNF**	Tumor necrosis factor
IFN	Interferon	**TRIM5α**	Tripartite motif-containing protein 5 α
Ig	Immunoglobulin	**URF**	Unique recombinant forms
IL	Interleukin	**UV**	Ultraviolet
IMC	Infectious molecular clone	**V**	Volt
K	Kappa	**V1-V5**	Variable loops
kb	kilobase	**Vif**	Viral infectivity factor
LB	Luria-Bertani	**Vpr**	Viral protein R
LTR	Long terminal repeat	**Vpu**	Viral protein U
LTNP	Long term non progressor	**Vpx**	Viral protein X
LucR	*Renilla reniformis* luciferase	**X4**	CXCR4 topic

1.Introduction

1.1. Discovery of HIV

Acquired Immune Deficiency Syndrome (AIDS) was first discovered as a new disease in 1981, when an unusual high number of young homosexual men died of rare opportunistic infections and malignances (CDC 1981). Subsequently, the retrovirus, now termed Human Immunodeficiency Virus type 1 (HIV-1), was identified as causative agent (BARRE-SINOUSSI et al. 1983; GALLO et al. 1984; LEVY et al. 1984). Now, more than 30 years after its discovery in humans, HIV-1 has become one of the most devastating infectious diseases that have emerged in recent history. To date, approximately 34 million people worldwide live with HIV and about 25 million people have died of AIDS. In 2011, an estimated 1,7 million people died because of HIV/AIDS (www.who.int). The greatest HIV-related morbidity and mortality is experienced by developing countries, in which young adults, especially in Sub-Saharan Africa, display the highest prevalence rate.

Antiretroviral treatment has reduced the number of AIDS related deaths, however access to such therapy is not universal and the promise of curative treatments and effective vaccines are elusive. Hence, HIV-1 infection will most likely continue to pose a public health treat for many years to come (SHARP and HAHN 2011).

1.2. Human Immunodeficiency Virus (HIV)

HIV is a member of the lentivirus genus of the *Retroviridae* family. Infections with lentiviruses, which originate from the Latin word lentus meaning slow, typically show a chronic course of disease, with a long period of clinical latency and persistent viral replication.

HIV isolates can be grouped into two types, HIV-1 and HIV-2. Diversity, mainly in the envelope genes, helps to distinguish them. The two groups differ at least by 30% (KORBER et al. 2001).

HIV-1 can be divided into four distinct lineages, termed groups M, N, O and P. Group M was the first discovered and represents the pandemic form of HIV-1. Within the course

of the HIV endemic in humans, the sequences of the different HIV-1 groups have diversified, giving rise to nine group M subtypes (or Clades) designated by the letters A-D, F-H, J and K. Between the subtypes, a 15-20% difference is found. To date, subtype C is the most prevalent worldwide, found especially in Sub-Saharan Africa. Recombination between strains/subtypes occurs frequently, especially between HIV-1 group M subtypes. They are designated as circulating recombinant forms (CRF) when found in more than three epidemiologically unlinked individuals, or otherwise as unique recombinant forms (URF) (ROBERTSON et al. 2000).

HIV-2 is less pathogenic than HIV-1 and largely restricted to West Africa, to date eight lineages have been identified termed HIV-2 clade A-H (FANALES-BELASIO et al. 2010; HEMELAAR 2012).

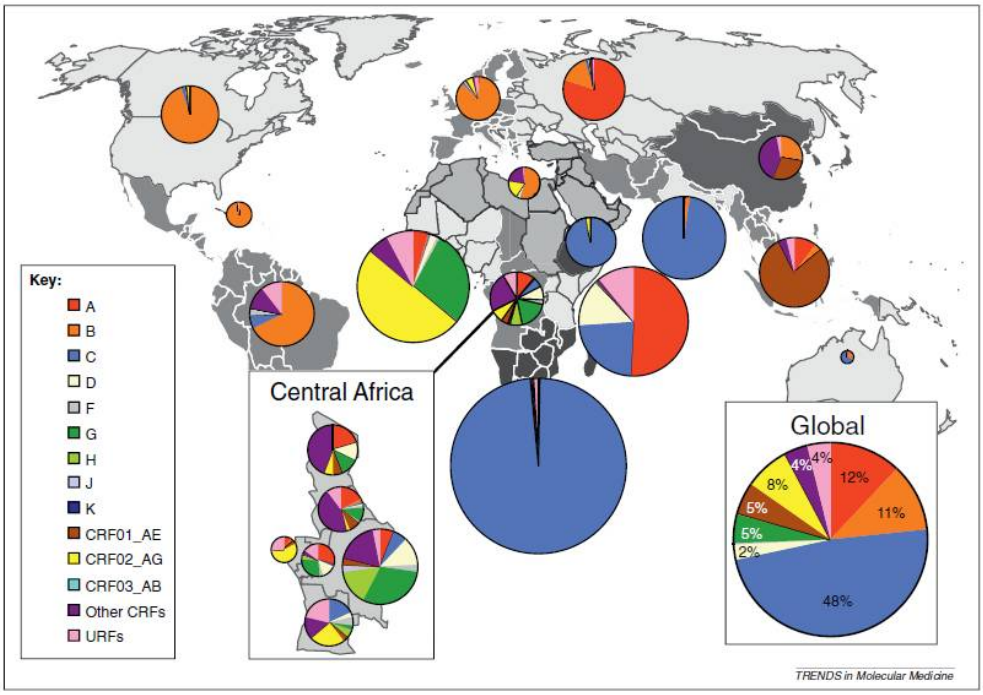

Figure 1 Global distribution of HIV-1 subtypes and recombinants from 2004-2007.
(HEMELAAR 2012)

1.3. HIV Structure & Genome

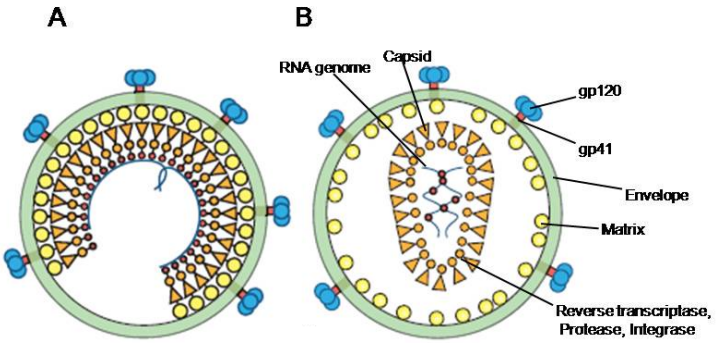

Figure 2 Organization of the immature (A) and mature (B) virion.
modified from (SUNDQUIST and KRAUSSLICH 2012)

HIV particles have a diameter of 100 nm and are surrounded by an envelope, a lipoprotein rich membrane. Trimers of external surface gp120 and the transmembrane spanning gp41 glycoproteins are located in the envelope. Inside, a matrix protein is anchored to the viral lipoprotein membrane. The HIV genome is composed of two identical copies of single-stranded RNA molecules combined with a nucleoprotein that are enclosed by a conical capsid (p24). Additionally, the capsid contains the enzymes reverse transcriptase, integrase and protease (FANALES-BELASIO et al. 2010; JANEWAY 2001).

The entire HIV genome consists of nine genes flanked by long terminal repeat sequences (LTR). The major genes *gag* (group specific antigen), *pol* (polymerase), *env* (envelope) encode viral structural proteins. The *gag* gene encodes viral core proteins, *pol* encodes the enzymes involved in viral replication and integration and *env* encodes the viral envelope glycoprotein. Additionally, six other smaller genes encode for proteins that are involved in regulatory processes. The *tat* gene encodes for a Tat (Trans-activator of transcription) protein that is expressed very early after infection and promotes the expression of HIV genes. The *rev* gene, encoding for Rev (Regulator of expression of virion) protein, ensures the export of mRNA from nucleus to cytoplasm. The Vpr (Viral Protein R) is involved in the arrest of the cell cycle. The same function is performed by Vpx (Viral protein X) for HIV-2. The Vpu (Viral protein U) is necessary for the correct release of the virus and the *vif* gene encodes for a protein Vif (viral infectivity factor) that enhances the infectiveness of progeny virus particles. Nef (Negative factor) protein has multiple functions, including cellular signal transduction and down regulation of the CD4 receptor on the cell surface to allow virus budding in the late stage of the virus replication cycle (FANALES-BELASIO et al. 2010; JANEWAY 2001).

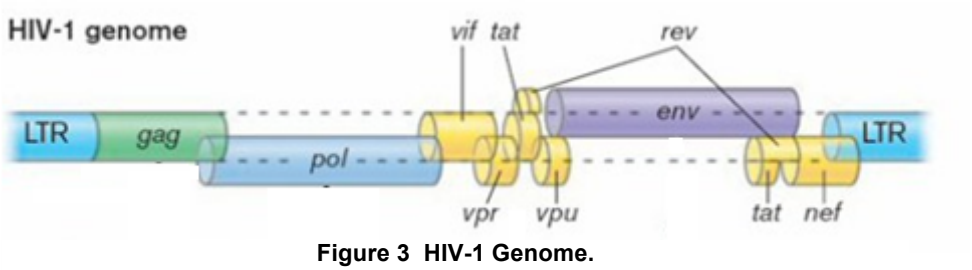

Figure 3 HIV-1 Genome.
(SLONCZEWSKI and FOSTER 2011)

1.4. The HIV Life Cycle

The first phase of the HIV replication cycle begins with the virus entry into the target cell. First, the virion binds to the target cell. This is mediated by the viral envelope protein or host cell proteins incorporated into the virion. Next, the envelope binds to its primary receptor, the host protein CD4 (MADDON et al. 1986). In healthy cells CD4 is used to enhance T-cell receptor mediated signaling. The trimeric subunit gp120 is responsible for this binding and consists of five relatively conserved regions (C1-C5) and five variable loops (V1-V5), which lie predominantly at the surface. The interaction of the envelop with CD4 causes rearrangement of the variable loops (V1,V2 and V3) and results in a conformational change that enables the virion to engage with the co-receptor on the target cell (KWONG et al. 1998).Co-receptor usage allows classifying the virus strains. Those using the chemokine receptor CCR5 are termed R5-tropic and those that use the CXCR4 are termed as X4-tropic. Viruses that can use both are called dual tropic R5X4. CCR5 is found on monocytes/macrophages, dendritic cells, microglia, central and effector memory $CD4^+$ T-lymphocytes. In contrast, CXCR4 is broadly expressed on the majority of hematopoietic, including T-cells, monocytes/macrophages and on many parenchymal cells as well (BERGER et al. 1998).

The final step of virus entry is the membrane fusion, which is mediated by the envelope protein. The co-receptor binding causes the exposure of transmembrane gp41 which inserts into the host cell membrane (CHAN et al. 1997). This results in the formation of a fusion pore that allows the subsequent uncoating and delivery of the viral contents into the host cell cytoplasm (FANALES-BELASIO et al. 2010; WILEN et al. 2012a, b).

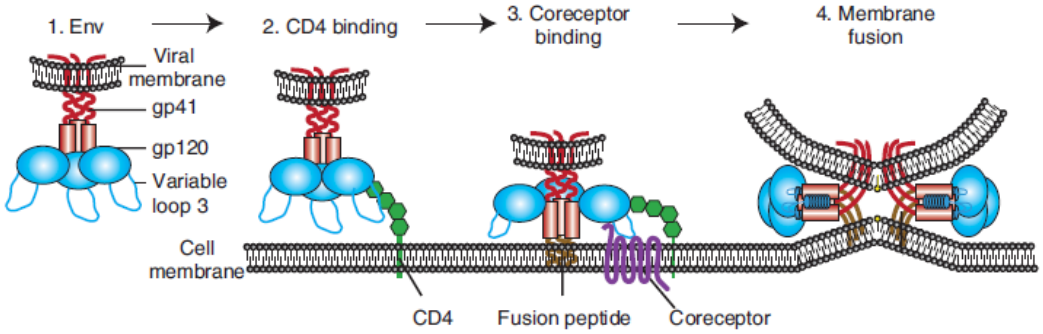

Figure 4 Overview of HIV entry.

(1) The virion attaches to the target cell, binding CD4 (2). The resulting conformational change allows co-receptor binding (3). This iniates mebrane fusion and the delivery of viral contents into the target cell (4) modified from (WILEN et al. 2012a)

Once in the cell, reverse transcription of the viral RNA takes place, generating RNA-DNA duplexes. Then, RNase H breaks down the RNA strand in the duplex, enabling the generation of a complementary DNA strand to form a double helix DNA molecule. With the help of the enzyme integrase the proviral dsDNA gets integrated into the host cell genome. This step requires the target cell to be in an active state, so that transcription of proviral DNA into mRNA can occur. First, regulatory proteins such as Tat and Rev are synthesized, followed by longer mRNA fragments that migrate into the cytoplasm to synthesize structural viral particles like the precursors for Gag, Pol and Env. The formation of an infectious particle is a stepwise process. First, two viral RNA strands assemble together with the enzymes used for replication. Core proteins accumulate around them forming the virus capsid. Then, the particle migrates towards the cell wall. HIV-protease cleaves the previously formed precursor molecules to generate infectious viral particles. The virus is released into the extracellular space by budding through the host cell membrane, thereby building the new envelope. Host cell proteins may be incorporated into the virion during this process (FANALES-BELASIO et al. 2010; SUNDQUIST and KRAUSSLICH 2012).

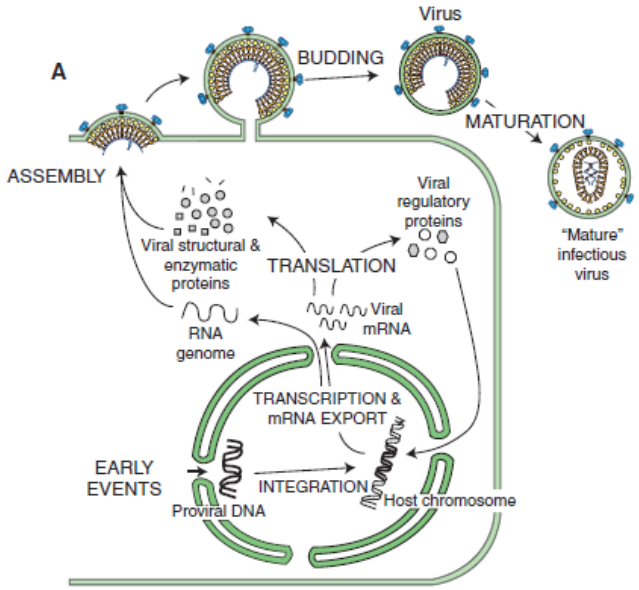

Figure 5 Different stages of virus assembly, budding and maturation.
(SUNDQUIST and KRAUSSLICH 2012)

1.5. Clinical Course of Infection

HIV transmission is dependent on the biological properties of the virus, concentration in the exposed body fluid and host susceptibility. Infection can result from virus exposure at mucosal surfaces, percutaneous inoculation or maternal-infant exposure. Heterosexual mucosal transmissions are responsible for 70% of all HIV-1 infections worldwide, whereas R5-tropic viruses are preferentially transmitted by all routes. In most patients, initial infection occurs through a single virus (LEVY 2009; SHAW and HUNTER 2012).

The majority of people who get infected with HIV first experience non specific self-limiting symptoms like fever, sore throat or lymphadenopathy. In the first few weeks of infection, levels of serum antibodies against HIV proteins are usually not high enough to allow diagnosis of infection by enzyme-linked immunosorbent assay (ELISA), but high levels of viral RNA are detectable in the plasma. Two to three weeks after infection, the virus becomes well established in a lymphatic reservoir (LACKNER et al.2012).

This reservoir evolves into the major virus production and persistence site. During this time, profound destructions of $CCR5^+$ $CD4^+$ memory cells, especially in the gut, take place as well as a destruction of follicles and lymphatic tissue architecture. With the elicitation of specific immune responses the symptoms vanish and high-level virema decreases to a steady “set-point”. This time point is usually associated with seroconversion and a cellular immune response to HIV is generated that partially controls viral replication. $CD4^+$ T cells recover, but to levels lower than those present before infection.

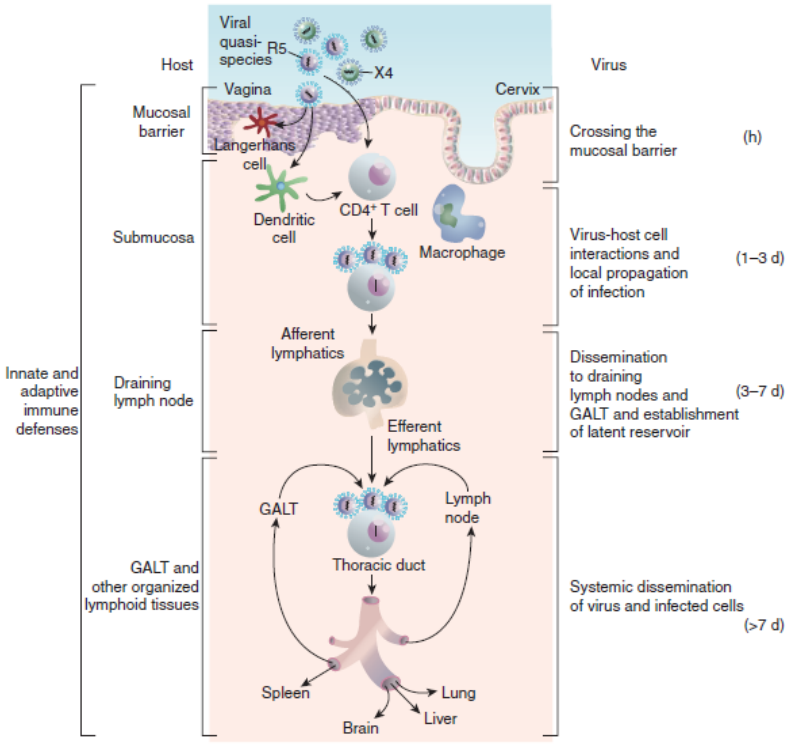

Figure 6 Model of cervicovaginal infection by HIV-1.

After crossing the cervicovaginal mucosal barrier, dendritic cells, $CD4^+$T cells and macrophages in the underlying submucosa are infected. Infection is propagated and disseminated, thereby establishing the lymphatic tisue reservoir that spreads infection to other organs and peripheral tissue. Innate and adaptive immune responses are directed at different stages, trying to prevent transmission. modified from (SHAW and HUNTER 2012)

Although the majority of infected people will not encounter signs or symptoms of disease, HIV continuously keeps replicating, inducing a state of chronic systemic infection. This asymptomatic period is marked by progressive $CD4^+$ T-cell depletion. Infection of monocyte/macrophage lineage cells also plays an important role, as they serve as a major reservoir for viral replication and persistence and thus also contribute to disease progression (LACKNER et al. 2012; POPE and HAASE 2003).

The onset of AIDS is defined as a $CD4^+$ T-cell count of 200 cells/µl or lower. During this phase, CCR5 viruses can evolve to enter cells using CXCR4, allowing the infection of new target cells. The emergence of X4 viruses is typically associated with a rapid progression to AIDS. Untreated, clinical complications like infections or malignancies occur due to the pronounced impairment of the T-cell population. Generally, an 11 year life expectancy is estimated after the first infection (FANALES-BELASIO et al. 2010) .

Interestingly, some untreated HIV infected individuals stay healthy beyond the typical “asymptotic” time and retain normal $CD4^+$ cell counts. These people are termed long term non progressors (LTNP) and represent about 5% of the HIV infected population. Different features might explain this occurrence like mutations in the infecting virus, host genetic background (certain genetic polymorphisms) or strong cell mediated anti-HIV immune response. If anti-retroviral treatment is initiated early enough, the predicted survival of an HIV infected patient can approach that of the general population (LACKNER et al. 2012).

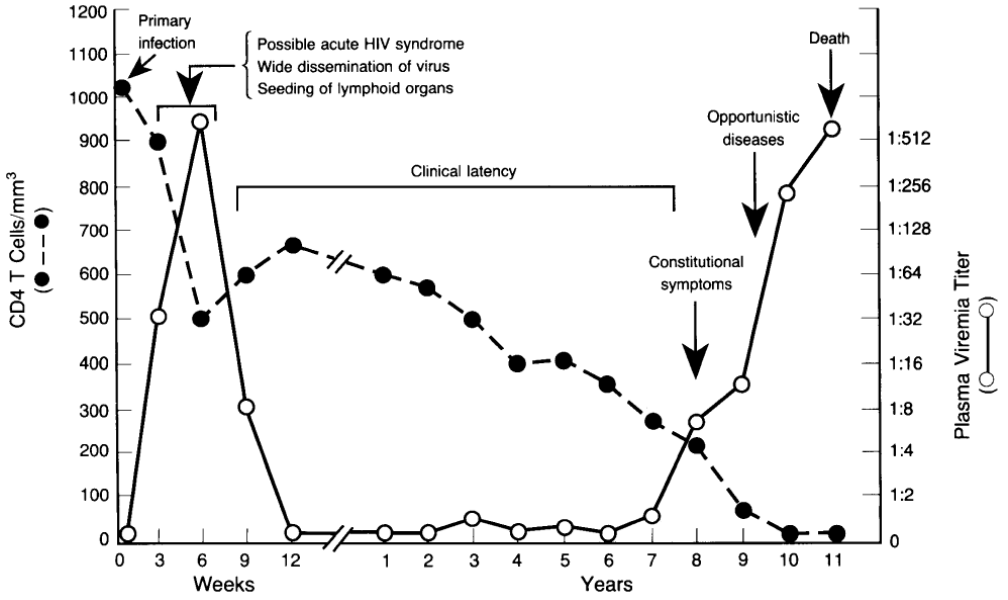

Figure 7 Clinical Course of HIV Infection.

(PANTALEO et al. 1993)

1.6. Host Immune Response

The host immune response to an HIV infection consists of humoral and cellular components of the innate and adaptive immune system. The major difference lies in the response time to an incoming pathogen. Whereas the innate response is very quick and occurs within minutes to hours, therefore also often referred to as the first line of defense, the adaptive immune response needs days to weeks (AKIRA et al. 2006).

The innate immune system recognizes conformational pathogen associated molecular patterns (PAMPs) through germline encoded pattern recognition receptors (PRR) found

on many cells like macrophages, dendritic cells (DC), B-cells, T-cells and on non immune cells like fibroblasts and epithelial cells. During infection, viral structural components (eg: surface glycoproteins) are recognized as PAMPs and trigger a so called “cytokine storm” in which interleukin (IL)-15, interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), IL-18 and IL-10 are produced. Some of these cytokines have antiviral activity (eg IFNs) but at the same time they may also promote viral replication. DCs detect viruses in peripheral tissue sites and following activation and uptake, migrate to draining lymph nodes where they trigger adaptive immune (T-cell) responses and promote natural killer (NK) cell activation. NK cells stimulate anti-viral immunity by producing pro-inflammatory cytokines (such as IFNs and IL-12) and lysing infected cells (ALTFELD et al. 2011; MCMICHAEL et al. 2010).

The adaptive immune response is characterized by specificity and develops a clonal selection of lymphocytes bearing antigen specific receptors. It tries to eliminate pathogens during the late phase of infection (via cytotoxic $CD8^+$ T cells and antibody production) and generates immunological memory (AKIRA et al. 2006).

Additionally the host produces intrinsic restriction factors like apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G), Tripartite motif-containing protein 5 α (TRIM5α), tetherin and SAM domain and HD domain-containing protein 1 (SAMHD1) that have to be overcome by the virus for effective reproduction (KILLIAN and LEVY 2011).

1.7. Escape Mechanisms of HIV

HIV has different mechanisms by which it can resist and counteract anti-HIV means established by the host or the effects of therapeutic drugs. These include incorrect presentation of viral peptides by antigen-presenting cells (APC), defective expression by infected target cells and mutations of the viral peptide, in particular the envelope epitope (LEVY 2009).

HIV-1 sequences vary a lot and this variability is a very powerful weapon of the virus. The viral reverse transcriptase as well as the cellular RNA polymerase II, transcribing proviral DNA into RNA, both lack a proof reading function, thereby making them “error-

prone". The rapid replication of HIV, with the generation of 10^9 to 10^{10} virions every day, leads to the accumulation of many mutations and the emergence of numerous variants of HIV, called quasi species. Additionally, the occurrence of recombination processes between two or more different virions within the same individual, contribute to the mutation rate that is estimated to be $2x10^{-5}$ per nucleotide per replication cycle (HU and HUGHES 2012; JANEWAY 2001).

1.8. HIV Origin and Non-Human Primate Models

One of the major limitations in HIV research is the lack of an animal model that recapitulates all the features of HIV infection in humans. HIV-1 is a direct progeny of SIVcpz, the simian version of HIV that infects Central African chimpanzees (*Pan troglodytes*). Multiple cross-species transmissions during the preparation of bush meat in the early 20th century in Africa gave rise to the HIV-1 epidemic. However, SIV/HIV infection of chimpanzees hardly ever causes disease. More than 40 species of African apes and monkeys are natural hosts for SIV, but interestingly, despite the development of high levels of viral replication, they usually do not acquire AIDS. This phenomenon can be explained by the virus-host co-evolution that endured over thousands of years, making these animals unsuitable as pathogenicity models. Of the natural hosts, the sooty mangabey (*Cerocebus atys*) is of special interest, because cross-species transmission of SIVsmm gave rise to HIV-2 and SIVmac in macaques. In contrast to chimpanzees, infection with certain SIV strains of Asian macaques that are not SIV natural hosts, results in AIDS-like symptoms resembling those seen in humans. These include high viral loads, $CD4^+$ T-cell depletion, and opportunistic infections. Disease progression, however, is much faster in macaques, leading to AIDS within 1-2 years of infection. Asian macaques have become the most commonly used and widely accepted model for HIV/AIDS research. They are used extensively in studies directed towards finding vaccines and drugs for prevention of HIV-1 infection. In this context, three species are routinely used as animal models: the rhesus macaque (*Macaca mulatta*), the pig-tailed macaque (*Macaca nemestrina*) and the cynomologus macaque (*Macaca fascicularis*). Rhesus macaques of Indian origin are the best-characterized and most often used model for AIDS. SIVmac251 and SIVmac239 are the most used SIV

challenge strains, and are the best adapted to replicate efficiently in these animals. Importantly, while these strains have all been developed through accidental or deliberate infection of macaques with SIVsmm, they differ from HIV-1 in crucial ways. For example, SIVmac does not have a *vpu* gene, but a different accessory gene instead named *vpx*. Furthermore, HIV-1 and SIVmac display only 53% nucleotide sequence homology and have overlap differences in their open reading frames (ORFs)(CHAHROUDI et al. 2012; HATZIIOANNOU and EVANS 2012).

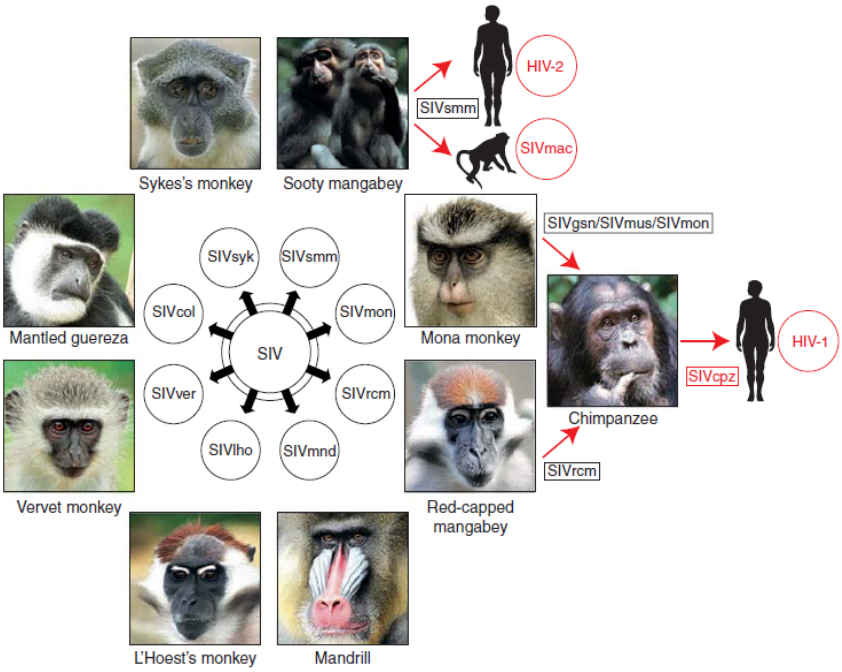

Figure 8 Origins of Human AIDS Viruses.

Old world monkeys are naturally infected with more than 40 different SIVs. Several of these have crossed the species barrier to humans generating new pathogens. Known examples of cross species transmissions are indicated by red arrows. Adapted from (SHARP and HAHN 2011)

Another fundamental difference between SIV and HIV is that HIV-based vaccine immunogens cannot be tested directly by challenging with SIV. Moreover, SIV is not sensitive to many drugs designed to inhibit HIV-infection and/or replication. In addition, although most SIV and HIV-1 isolates use CCR5 as a co-receptor, HIV-1 is able to switch co-receptor usage to CXCR4, a feature not seen in SIV. On the other hand, SIV can use other alternative co-receptors, not used by HIV-1 (eg: CXCR6, GPR15 and GPR1) (RIDDICK et al. 2010).

In order to overcome these issues, SIVs expressing HIV-1 envelope glycoproteins have been constructed. These hybrids, termed Simian-Human Immunodeficiency Virus (SHIV) allow testing of HIV-1 envelope-specific vaccines and drugs in non-human primate models. Most of these hybrid viruses were cloned by replacing the *rev, tat* and *env* genes of SIVmac239 with the analogous *rev, tat, vpu* and *env* genes from HIV-1 isolates. Although these viruses initially replicate poorly in macaques, after extensive serial blood passages (adaptation), they eventually become highly pathogenic and are able to cause disease. The first cloned SHIVs contained Clade B envelopes and were X4-tropic (eg: SHIV89.6P). Hence, they did not infect the usual target cells of SIV or HIV-1 which express CCR5. Additionally, these X4-tropic SHIVs were incapable of mucosal transmission, which is, considering that more than 70% of all HIV transmissions occur through this route, a major drawback. Recent efforts focused on developing biological more relevant SHIVs that are R5-tropic, mucosally transmissible and Clade C Env-expressing constructs, since this is the most prevalent HIV-subtype found worldwide (eg: SHIV1157ipd3N4). SHIVs nowadays offer an excellent tool opportunity to test the efficacy of vaccines targeting relevant HIV-1 envelopes to prevent mucosal transmission (HATZIIOANNOU and EVANS 2012).

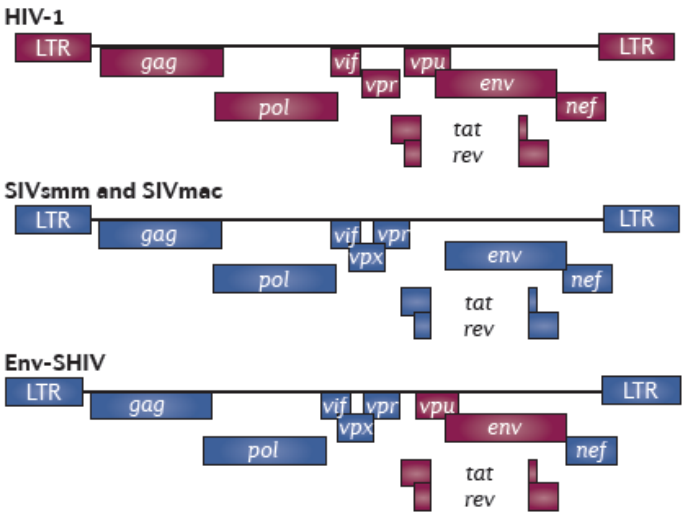

Figure 9 Comparison of HIV, SIV and SHIV Genome.
Blue and red boxes indicate HIV-1 and SIV derived sequences respectively. (HATZIIOANNOU and EVANS 2012)

1.9. Broadly Neutralizing Antibodies and Approaches to HIV Vaccines

Neutralization of infectivity is thought to be the main mechanism of protection by antibodies. They work by blocking the binding to cellular receptors (eg: CD4 or CCR5) or interfering with the fusion machinery. Additionally, they can recruit effector cells or complement, which can kill infected target cells or lyse virus particles. The majority of HIV-1 infected individuals show a strain specific neutralizing humoral immune response within weeks to months of primary infection. This strain specific neutralization activity is usually directed against the variable domains of the trimeric viral envelope complex. The virus has evolved to keep its most vulnerable conserved regions hidden and uses mechanisms like conformational masking, amino acid or glycan shield changes to escape from neutralizing antibodies (nAb) (CORTI and LANZAVECCHIA 2013). According to their neutralization profile HIV-1 strains can be divided into three groups: tier 1 (most sensitive), tier 2 (moderately sensitive) and tier 3 (most resistant). As the virus population diversifies with time, the immune response matures. During the first three years of infection about 20-30% of HIV-1 infected people develop Ab with such a broad activity that viruses from different subtypes are able to be neutralized. However, these HIV-1 infected individuals do not benefit from these broad neutralizing Ab responses. This can be explained in part by the rapid virus escape but also by the fading humoral response during disease progression. So far, several broadly nAb from infected individuals have been isolated and well characterized (Figure 10).

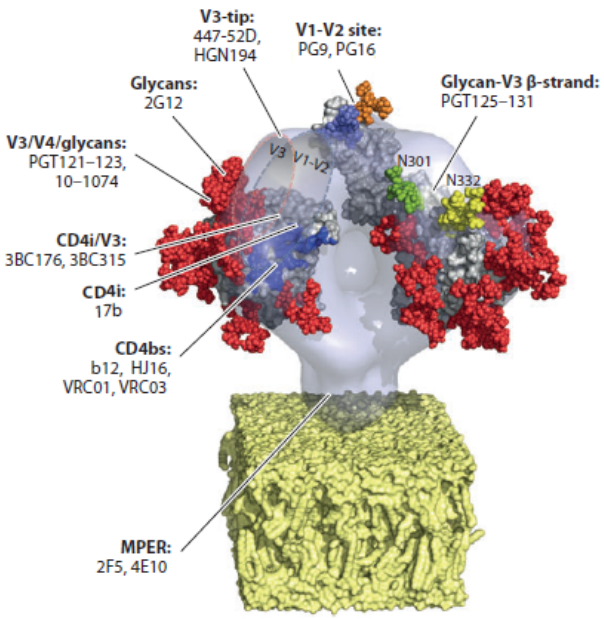

Figure 10 Model of HIV-1 Env Trimeric Glycoprotein bound to Broadly Neutralizing Antibodies.
(CORTI and LANZAVECCHIA 2013)

First attempts to build an HIV vaccine were based on inactivated virus or recombinant gp120 and failed to show efficacy in non-human primate models. Other approaches using attenuated SIV showed efficacy, but due to safety issues were never translated into human trials. Another failed attempt was the generation of a so called “T-cell” vaccine. It aimed to generate protective T-cell responses that would result in viral attenuation once infection occurred. The fact that nAb has shown to prevent infection in non-human primates brought forth the idea that an effective HIV-1 vaccine must also produce protective broadly neutralizing anti-Env antibodies. However, several approaches to elicit nAb using different immunogens delivered as proteins, viral vectors or naked DNA were not successful. With the phase 3 Thai trial (RV144), where two vaccines that failed individually were combined, a modest efficacy of 31,2% after 3,5 years was seen, suggesting for the first time, that prevention of HIV-1 infection is an achievable goal. Since the efficacy in the first year was approximately 60%, the limited success two and a half years later might be due to the failure to maintain sufficiently high antibody levels. More studies are required to understand the mechanism behind long-lived serum antibody levels and the B-cell biology. Many research groups focus these days on the identification of correlates of protection. Now, the focus of several research groups is on the identification of correlates of protection, the discovery of which, would promote HIV-1 vaccine development enormously (CORTI and LANZAVECCHIA 2013; O'CONNELL et al. 2012; VAN GILS and SANDERS 2013).

1.10. Measurement Neutralizing Antibody Responses Against HIV-1

It is believed that a broadly neutralizing antibody response is required in order to develop a protective HIV vaccine. However, significant challenges have hindered the achievement of this goal. Even 30 years after the discovery of HIV, a vaccine remains elusive. A crucial aspect in assessing the neutralizing antibody activity in serum or other body fluids from vaccine recipients (human or animal) is the requirement for a standardized, high-throughput in vitro assay. It is important that the assay is able to reflect the in vivo breadth and potency of the neutralizing antibody response generated

in the vaccinated host. Several different assays have been developed to measure neutralization of HIV-1, differing with respect to the virus used, the target cells, inhibition and read-out of infection, and whether the virus can give rise to only a single cycle of infection or is replication-competent (OCHSENBAUER and KAPPES 2009).

1.10.1 First-Generation Neutralizing Antibody Assays

The first generation of neutralizing antibody assays used immortalized T-cell lines as both the virus source and for the cellular target. Antibody activity was measured by monitoring endpoints such as viral protein expression or cell survival. However, propagation of these viruses in immortalized cells increased their sensitivity to antibody inhibition when compared with primary cells, making the assay biologically irrelevant (MASCOLA et al. 1996).

With the aim of obtaining a more physiologically-relevant measurement, the PBMC (Peripheral Blood Mononuclear Cells) assay was developed. This cell-based assay made it possible to infect HIV-seronegative PBMC (fresh or frozen) from different healthy human donors. It has long been known that individual donors show variable susceptibility to HIV-1 infection. This might be due to many factors such as host genetics, the number of $CD4^+$ T cells, the level of CD4 expression and host genetic polymorphism in chemokines and/or chemokine receptor. Due to these multiple factors, significant intra- and inter-assay variation is observed when this approach is used. The PBMC-based assay measures HIV-1 p24 antigen as the endpoint and requires extensive washing steps in order to avoid artifacts although this also reduces read-out sensitivity. In summary, the assay is very labor-intensive, expensive, and not practical for high-throughput analysis (OCHSENBAUER and KAPPES 2009; POLONIS et al. 2008).

1.10.2 Second-Generation Neutralizing Antibody Assays

One of the newer approaches towards the development of HIV neutralizing antibody assays makes use of “Pseudovirions”, which are molecularly cloned HIV-1 envelopes

engineered into defective virus particles that allow only a single round of infection. They are generated by co-transfecting a cell line (usually 293T) with plasmid DNA encoding the full-length envelope gene of interest and an envelope-lacking (*env*-minus) viral backbone. The resulting virus is infectious but replication defective. Pseudovirions can be used to infect a cell line that expresses a reporter gene in response to an infection (eg: TZM-bl). Different envelope genes can be easily derived from plasma viral RNA using PCR, allowing one to test rapidly for neutralization against primary patient envelopes from multiple clades. This assay system also has a high degree of inter-experiment reproducibility, is enabled for high throughput and is easy and safe to use.
A different approach using a pseudoviral assay uses an *env*-minus viral backbone encoding for the reporter gene firefly luciferase in place of *env*. The reporter gene is expressed upon target cell infection, making it possible in principle to infect primary cells. Although, it affords only a very weak luciferase-to-noise ratio, limiting its use to highly infectable, genetically modified cell lines. Moreover, it has been shown that pseudovirions do not resemble in every respect viruses that have been produced in primary (target) cells. In 293T cells, for example, pseudoviruses are reported to have an excess of unprocessed envelope protein gp160. Also, envelope glycosylation and host cell proteins that are incorporated into the budding virion depend on the producer cell line, resulting in varied composition and thereby potentially affecting infectivity and/or neutralization sensitivity (OCHSENBAUER and KAPPES 2009).

Use of genetically engineered reporter cell lines as infection targets has considerably improved assay standardization. The reporter cells stably express defined levels of CD4, CCR5 and CXCR4. The Hela-derived TZM-bl cell line, for example, expresses the reporter gene firefly luciferase in response to Tat expression following infection with either pseudovirions or (primary or cloned) replication-competent HIV-1. This modified HeLa cell line allows sensitive and quantitative detection of the expressed reporter gene over several orders of magnitude and is an easily automated end point measurement (luminometer). These characteristics have led to wide acceptance of this easily transferable method to determine neutralizing antibody inhibition (MONTEFIORI 2009). However, it should be noted that discrepancies in neutralizing antibody inhibition are

sometimes observed in results from reporter cell line assays versus PBMC-based assays. This might be due to difference in the mode of virus entry between these assays. Thus, in the PBMC assay the majority of HIV-1 enters the T cells via CD4 and co-receptor binding and fusion. By contrast, it has been reported that 85% of virus entry in HeLa cells occurs via endocytosis (MARECHAL et al. 1998). Additionally, it is reported that TZM-bl cells express 100 times more CCR5 on the cell surface compared to primary PBMC, possibly altering the interaction between the antibody and the host-cell and raising a questioning about the biological relevance of the system (POLONIS et al. 2008).

1.10.3 Reporter Gene *Renilla* Luciferase Expressing Viruses in Neutralizing Antibody Assays

In 2010, Edmons et. al. (2010) published an approach that combined the advantages of PBMC-based assays with those of replication-competent reporter viruses. A recombinant HIV-1 proviral backbone was generated that encoded all viral open reading frames (ORFs), was replication competent and stably expressed the reporter gene *Renilla reniformis* luciferase (LucR) over multiple cycles of virus replication.

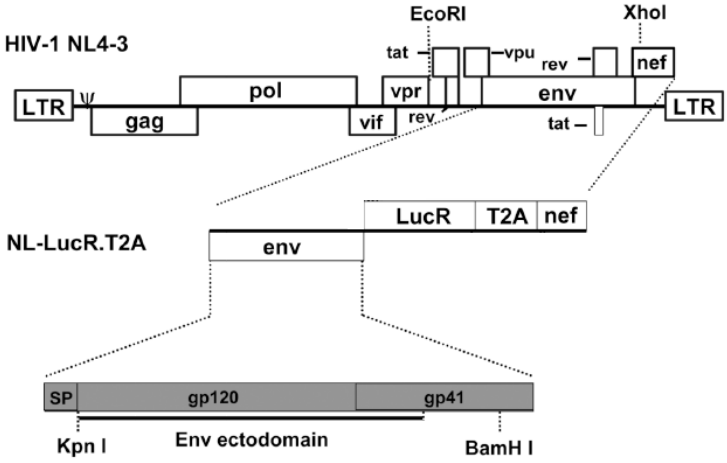

Figure 11 Generation of NL-LucR.T2A.

Schematic representation of the insertion of the *Renilla* luciferase gene into the NL4-3 viral backbone and the "shuttling in" of heterologous envelope sequences. (EDMONDS et al. 2010)

This reporter gene, originating from the sea pansy, was selected because it consists of fewer nucleotides than the firefly luciferase (935 vs 1652), a characteristic thought to favor its retention within the viral genome during replication. The reporter gene LucR was inserted into the genome of the HIV-1 NL4-3 proviral backbone at the position of *nef* and was linked in-frame at the 5'-end with a "self-cleaving" T2A sequence, allowing the generation of two proteins from a single RNA transcript. The resulting vector

pNL-LucR.T2A allows envelope sequences from different HIV-1 strains to be “shuttled in”, enabling the construction of a virus reference panel. The primary purpose of generating this reporter proviral DNA backbone is to facilitate the analysis of neutralizing antibodies in primary cells. The easy, highly specific and sensitive readout (Relative Fluorescence Units-RLU) permits the analysis of large sample numbers and is a significant advance towards the development of a standardized high-throughput neutralization assay (EDMONDS et al. 2010).

1.11. Background of the Thesis

1.11.1 SHIV-1157ipd3N4 & SHIV-115ipEL

In 2006, the Ruprecht laboratory generated a highly replication-competent, mucosally transmissible R5-tropic SHIV encoding a relatively recently transmitted Clade C HIV-envelope. This envelope originates from a 6-month old Zambian infant born to an HIV positive mother. Interestingly, after a long follow up period, the infant turned out to be a long-term non-progressor. The first molecular clone generated from this envelope was named SHIV-1157i (where “i” stands for infant) and contains the backbone of SIVmac239. This clone was used to intravenously infect an infant macaque. After serial blood passages in other rhesus monkeys (giving rise to SHIV-1157ip, “p” for passaged or monkey-adapted), SHIV-1157ipd (“d” for animal with disease) was isolated from a monkey with AIDS, defined by a persistent CD4^{+}T-cell count of <200 cells/µl, and molecular clone SHIV-1157ipd3 (for clone #3) was generated. To further increase pathogenicity, an additional NF-kB site was introduced into the 3’LTR by site-directed mutagenesis. The final

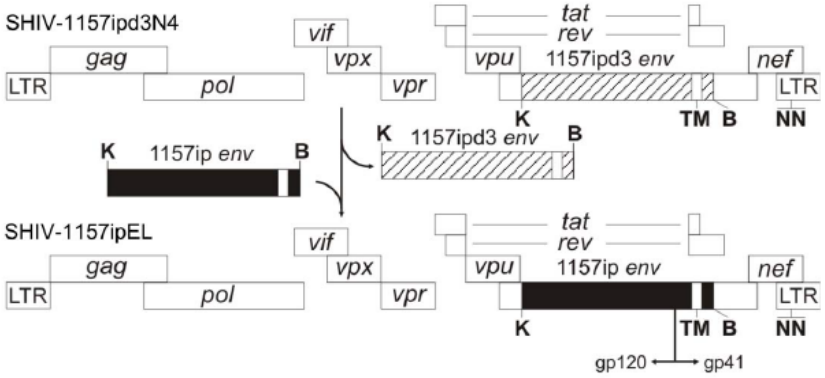

Figure 12 SHIV-1157ipd3N4 Genome and Construction of SHIV-115ipEL. The KpnI-BamHI fragment of “early” SHIV-1157ipd3 was cloned into the backbone of SHIV-1157ipd3N4 generating SHIV-1157ipEL. (SIDDAPPA et al. 2010)

construct was characterized as a tier 2 virus and named SHIV-1157ipd3N4 (SONG et al. 2006).

Since the approach of inducing antibody-mediated inhibition of tier 2 viruses has been a very difficult task, the Ruprecht laboratory generated a new neutralization-sensitive tier 1 virus. Once neutralization was achieved, the idea was to optimize immunogens for neutralization-insensitive tier 2 viruses. Thus, the chimera SHIV-1157ipEL was generated, in which the *env* from the "early" neutralization-sensitive SHIV-1157ip biological isolate, derived via rapid passage through five rhesus monkeys, was engineered into the "late" backbone of SHIV-1157ipd3N4 (SIDDAPPA et al. 2010).

1.11.2 SHIV-2873Nip

To test neutralizing antibody response against different relevant HIV isolates, another tier 2 SHIV was generated in 2009. This SHIV contains an HIV Clade C envelope from a 2-month old rapid progressor Zambian infant. The envelope was swapped into the backbone of SHIV-1157ipd3N4, generating the molecular clone SHIV-2873Ni. The latter was serially passaged through five RM, to obtain the adapted biological isolate SHIV-2873Nip. In order to demonstrate mucosal transmissibility, six RM were repeatedly inoculated intra-rectally with different SHIV-2873Nip concentrations (SIDDAPPA et al. 2009). While all animals became viremic, monkey RTb-11 actually developed AIDS and the AIDS-related opportunistic infection cryptosporidiosis. Infected PBMCs from RTb-11 were isolated when its $CD4^+$ counts were <200 cells/µl and co-cultured with naive rhesus monkey PBMCs. The virus, now the biological isolate SHIV-2873Nipd, was isolated. The entire envelope regions from

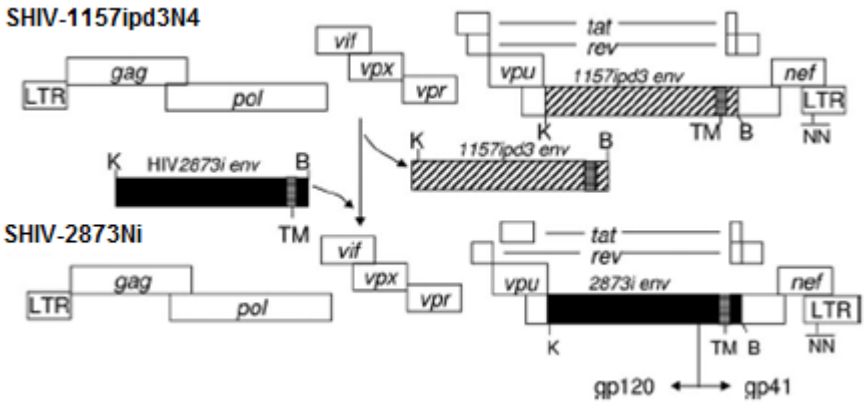

Figure 13 SHIV-1157ipd3N4 Genome and Construction of SHIV-2873Ni. The KpnI-BamHI fragment of HIV 2873Ni was cloned into the backbone of SHI-1157ipd3N4 generating SHIV-2873Ni. modified from (SIDDAPPA et al. 2009)

different biological virus isolates were amplified by PCR and cloned into the vector pcDNA6/myc-His B. To check for functional envelopes, the pcDNA6 vector, now containing the SHIV-2873Nipd envelope, was co-transfected with a reporter HIV vector, lacking the *env* gene and encoding GFP in place of *nef* (HIV- ΔEN), into 293T/17 cells. An infectious virus particle is able to be formed only when a functional envelope is present. Cell-free supernatant containing pseudotyped virus was used to infect CEM.NK^{R}-CCR5 cells. If the supernatant contains infectious virus particles, the cells turn green due to GFP expression by the virus. The infectious clone pcDNA6+SHIV-2873NipdTb-14 (Tb-14 for clone #14) was selected in this manner and was used for further cloning steps. All these experiments had been performed previously by Ruprecht laboratory members before the present study was conducted (unpublished data).

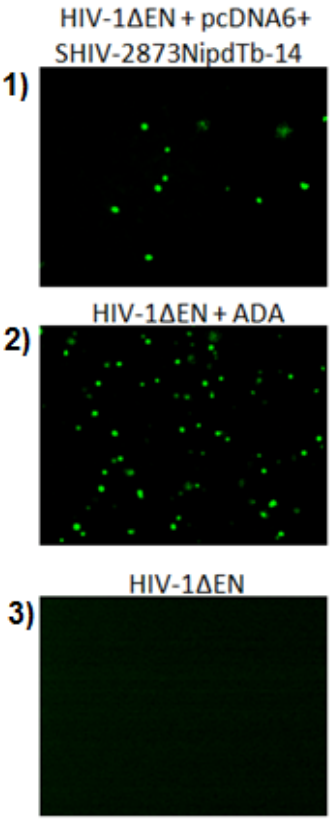

Figure 14 Screening for Infectious Envelope Clones. (1) CloneSHIV-2873NipdTb-14 was selected to be used for further cloning. (2) Positive control (HIV-1ΔEN+ADA) (3) Negative control (HIV-1ΔEN only) (figures provided by Dr.Swati Thorat,DFCI)

Strain	Description
SHIV-1157i	Infectious molecular clone (IMC), SIV backbone + HIV-C *env* from Zambian infant (6 months old, long term non progressor) ("i"for infant, not yet adapted)
SHIV-1157ip	Early biological isolate obtained after passage through five RM ("p" for passaged or monkey adapted)
SHIV-1157ipd3	Late stage IMC, isolated from monkey with AIDS ("d" for disease, "3" for clone number)
SHIV-1157ipd3N4	SHIV-1157ipd3 engineered to contain additional NF-kB site
SHIV-1157ipEL	IMC "early-late" chimera generated by swapping neutralization sensitive *env* from SHIV-1157ip into the backbone of SHIV-1157ipd3N4
NL-LucR.1157ipEL	*env* from SHIV-1157ipEL swapped into reporter proviral DNA backbone pNL-LucR.T2A
SHIV-2873Ni	IMC, SHIV-1157ipd3N4 backbone + HIV-C *env* from Zambian infant (2 months old, rapid progressor)
SHIV-2873Nip	adapted biological isolate, passaged through five RM
SHIV-2873Nipd14	*env* from SHIV-2873Nipd (from RM RTb-11) swapped into the backbone of SHIV-1157ipd3N4 to generate IMC ("14" for clone number)
NL-LucR.2873Nipd14	*env* from SHIV-2873Nipd14 swapped into reporter proviral DNA backbone pNL-LucR.T2A

Table 1 Virus Strain Nomenclature and Origin.

2. Aim of the Thesis

The first aim of the thesis was to facilitate and standardize the application of neutralization assays in Prof. Ruprecht's laboratory by cloning two different relevant SHIV-C envelopes into the reporter proviral DNA backbone pNL-LucR.T2A. The cloned viruses were characterized and their suitability was tested in different assays.

The second aim of the thesis was to generate and characterize an infectious molecular clone of SHIV-2873Nipd using the infectious *env* clone Tb-14 from the monkey-adapted biological virus isolate.

3. Animals, Materials and Methods

3.1. Animals and Animal Care

Sera from eight Indian-origin RMs (*Macaca mulatta*) were used to conduct neutralizations assays in hPBMCs. The animals were housed at the Yerkes National Primate Research Center (YNPRC), Atlanta, Georgia, USA. YNPRC is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Approval for all procedures was received from the Institutional Animal and Care and Use Committees of Dana-Farber Cancer Institute and Emory University. Two RM groups, each consisting of 8 animals (Group 1 and Group 2), were vaccinated in 2010 with two different strategies. Serum samples were collected and send to the Dana-Farber Cancer Institute for evaluation.

PBMCs from naive RM for the screening of SHIV-2873Nipd14 were isolated from maximum allowable survival bleeds and stored frozen in liquid nitrogen. They were obtained from Advanced BioScience Laboratories, Inc (ABL), Rockville, Maryland. ABL's animal facility is USDA-registered and is accredited by the American Association for the Accreditation of Laboratory Animal Care International (AAALAC). ABL complies with all policies of the "Guide for the Care and Use of Laboratory Animals," DHHS (NIH 85-23), Animal Welfare (DHHS-TN 73-2) the NIH Manual Issuance 4206 and 6000-3-4-58, "Responsibility for Care and Use of Animals CDC/NIH 4th edition", "Biosafety in Microbiological and Biomedical Laboratories," and Public Health Service Policy on Humane Care and Use of Laboratory Animals.

3.2. Materials

List of original plasmids:

pBR322: The vector is a commonly used for plasmid cloning in *E. coli.* The molecule is a double-stranded circle 4,361 base pairs in length and contains the genes for resistance to ampicillin and tetracycline. It was purchased from New England Biolabs (Ipswich, MA).

pNL-LucR.T2A: The *Renilla reniformis* luciferase reporter gene (LucR) from the phRL-CMV vector (Promega) and a T2A peptide-coding sequence were fused in frame by PCR and inserted between the HIV-1 NL4-3 *env* and *nef* genes. This plasmid was kindly provided by Dr.Christina Ochsenbauer, University of Alabama, Birmingham.

List of primers: all primers were obtained from Life Technologies (Grand Island, NY)

2873Nipd env primers -Forward	Primer Name	Designed by
AGCAATAGTTGTGTGGTCCATAGTAA	Nip-F1	Vivekanandan Shanmuganathan
GTTGACCTCACTCTGTGTCACT	Nip-F2	
ATAAACATTACGTGTACAAGACCAG	Nip-F3	
GGAGGCAACACAGAAGAGAATA	Nip-F4	
GGAAAAACATGACCTGGATG	Nip-F5	
TTGTAACGAGGACTGTGGAAC	Nip-F6	

2873Nipd env primers -Reverse		
CTTCAGCTGGGTTTCTCCAT	Nip-R1	Vivekanandan Shanmuganathan
TTCGATTCCTCTGAGCCTGT	Nip-R2	
TTAATGCCCCAGACCGTAAG	Nip-R3	
TATATGGGTCCCCTCCACTG	Nip-R4	
ACATTCTGGCATGGTCCTGT	Nip-R5	
GAGCATTTCTTGTGGGTTGG	Nip-R6	

1157ipEL env primers–Forward		
TAAGACAAAGAAAAATAGACAGGTTAATTAAT	A109	Dr.Swati Thorat
GGAAGTAAAAAATTGCTCTTTCAATGTAA	A110	
GTGATATAATAGGAGACATAAGACAAGCA	A111	
AAGCCATTGGGAATAGCACCCACTAAGG	A112	
AGATTTATTAGCATTGGACAGTTGGAAAAA	A113	
TAGCAGTAAGACAATATGGGTGGAGCTATT	A114	

Table 2 Sequencing Primers and Corresponding Sequence

List of Antibodies:

Fm6	Kindly provided by Prof. Wayne A. Marasco, MD, PhD (DFCI)
VRC0I	NIH AIDS Reagent Program (Dr. John Mascola)
IgG1 b12	NIH AIDS Reagent Program (Dr. Dennis Burton and Carlos Barbas)
2G12	NIH AIDS Reagent Program (Dr. Hermann Katinger)
2F5	NIH AIDS Reagent Program (Dr. Hermann Katinger)
4E10	NIH AIDS Reagent Program (Dr. Hermann Katinger)
HGN194	Kindly provided by Dr.Davide Corti (Institute for Research in Biomedicine,Switzerland)
SHIVIG	Kindly provided by Dr. Anton Sholukh (DFCI)
Rhesus Monkey IgG	SouthernBiotech, Birmingham, AL

List of Growth Medias (GM):

293T/17 cells	DMEM
	10% Fetal Bovine Serum
	2 mM L-glutamine
	1%Penicillin/ Streptomycin

TZM-bl cells	DMEM
	10% Fetal Bovine Serum
	50 µg/ml Gentamicin
	25 mM HEPES

hBMC cells	RPMI
	15% Fetal Bovine Serum
	2 mM L-glutamine
	1% Penicillin/Streptomycin

A3R5, SupT1.R5 & CEMx-174-GFP cells	RPMI
	10% Fetal Bovine Serum
	2mM L-glutamine
	1% Penicillin/ Streptomycin
	1 mg/ml Geneticin (G418)

U87.CD4 cells	DMEM
	15% Fetal Bovine Serum
	2 mM L-glutamine
	1% Penicillin/ Streptomycin
	1 µm/ml Puromycin
	300 µg/ml Geneticin (G418)

List of Restriction Enzymes:

All enzymes used were purchased from Thermo-Fisher Scientific (Waltham, MA)

<u>Enzymes used for the NL-LucR cloning and their recognition sites:</u>

FastDigest EcoRI 5'...G↓A A T T C...3'

FastDigest BamHI 5'...G↓G A T C C...3'

FastDigest KpnI 5'...G G T A C↓C...3'

FastDigest AvrII 5'...C↓C T A G G...3'

FastDigest HindIII 5'...A↓A G C T T...3'

Enzymes used for SHIV-2873Nipd14 cloning and their recognition sites:

FastDigestSphI 5'...G C A T G↓C...3'

FastDigestNotI 5'...G C↓G G C C G C...3'

FastDigest BamHI see previous site

FastDigest KpnI see previous site

FastDigest PmlI 5'...C A C↓G T G...3'

List of Products:

293 T/17 cells	ATCC ®CRL-11268 (Manassas, VA)
0.45 micron sterile syringe filter	Corning Incorporated (Corning, NY)
1.5 ml safe-lock tubes	Eppendorf (Westbury, NY)
12-well plate	Beckon Dickinson Labware (Franklin Lakes, NJ)
14 ml polypropylene tubes	BD Biosciences (Bedfored,MO)
1kb DNA ladder	NEB (Ipswich, MA)
24 well multiple well plate	Corning Incorporated (Corning, NY)
3/20 ml Syringe Luer-Lok™Tip	BD Biosciences (Bedfored,MO)
6x DNA Loading Dye	Thermo-Fisher Scientific (Waltham, MA)
96-well flat bottom tissue culture plates with low evaporation lid	BD Biosciences (Bedfored,MO)
Costar* 96-Well Black/White Clear-Bottom Plates	Thermo-Fisher Scientific (Waltham, MA)
A3R5 Cells	NIH AIDS Reagent Program (Germantown, MD)
Ambion® Nuclease Free Water	Life Technologies (Grand Island, NY)
Azidothymidine (AZT)	Sigma-Aldrich (St.Louis,MO)
BD Falcon™ Conical Tubes	BD Biosciences (Bedfored,MO)
BD Falcon™ Petri Dish	BD Biosciences (Bedford, MA)
Britelite Plus reagent	Perkin Elmer (Waltham, MA)
CEMX-174-GFP Cells	B. Felber (National Cancer Institute, Frederick, MD)
Concanavalin A (Con-A)	Sigma-Aldrich (St.Louis,MO)
Cryotubes ™Vials	Nunc A/S(Roskilde,Denmark)
DEAE-Dextran	Sigma-Aldrich (St.Louis,MO)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich (St.Louis,MO)
FastDigest 10x Buffer	Thermo-Fisher Scientific (Waltham, MA)
Ficoll-Paque™	GE Health Care Life Science (Pittsburgh,PA)
Gibco® DMEM	Life Technologies (Grand Island, NY)

Gibco® Fetal Bovine Serum (FBS)	Life Technologies (Grand Island, NY)
Gibco® PBS	Life Technologies (Grand Island, NY)
Gibco® RPMI	Life Technologies (Grand Island, NY)
Gibco®Carbenicillin	Life Technologies (Grand Island, NY)
Gibco®Trypan Blue Stain	Life Technologies (Grand Island, NY)
Glycerol	Sigma-Aldrich (St.Louis,MO)
Hemocytometer KOVA® GLASSTIC® SLIDE	Thermo-Fisher Scientific (Waltham, MA)
HIV-1 p24 Antigen Capture Assay	ABLinc (Rockville, MD)
Interleukin-2 (IL-2)	Roche Applied Science (Indianapolis, IN)
LB-Agar Medium (Capsules)	MP Biomedicals (Solon, OH)
MAX Efficiency ®Stbl TM Competent Cells	Life Technologies (Grand Island, NY)
Mr.Frosty Freezing Container	Thermo-Fisher Scientific (Waltham, MA)
Phytohaemagglutinin (PHA)	Sigma-Aldrich (St.Louis,MO)
QIAGEN®Plasmid Midi Kit	QIAGEN (Valencia, CA)
QIAGEN®Plasmid Mini Kit	QIAGEN (Valencia, CA)
QIAquick® Gel Extraction Kit	QIAGEN (Valencia, CA)
Rapid DNA ligation Kit	Roche Applied Science (Indianapolis, IN)
SIV p27 Antigen Capture Assay	ABLinc (Rockville, MD)
SupT1.R5 Cells	Kindly provided by Dr. James Hoxi (University of Pennsylvania School of Medicine, PA)
T-75 flask	Corning Incorporated (Corning, NY)
T-75cm2 Cell Culture Flask	Corning Incorporated (Corning, NY)
TZM-bl Cells	NIH AIDS Reagent Program (Germantown, MD)
U87.CD4 CXCR4 and CCR5	NIH AIDS Reagent Program (Germantown, MD)
UltraPure™ Ethidium Bromide	Life Technologies (Grand Island, NY)
UltraPure™Agarose gel	Life Technologies (Grand Island, NY)
UltraPure™DNA Typing Grade® TAE buffer	Life Technologies (Grand Island, NY)
ViviRen™ Live Cell Substrate	Promega Corporation (Madison, WI)
x- Gel Extraction Tool	USA Scientific (Ocala, FL)
X-tremeGENE 9 DNA Transfection Reagent	Roche Applied Science (Indianapolis, IN)

3.3. Molecular Cloning Strategy

Cloning Strategy for SHIV-2873Nipd14

The envelope region of the biological isolate SHIV-2873Nipd from clone Tb-14 (in the pcDNA6 vector) was cloned into the backbone of SHIV-1157ipd3N4. The cloning was performed as described by Siddappa et al. (2009). Briefly, the *env* region from pcDNA6+SHIV-2873NipdTb-14 was cloned into the 3'-backbone of SHIV-1157ipd3N4 by using KpnI and BamHI restriction enzymes. The new plasmid 3'SHIV-2873NipdTb-14 was confirmed by digestion with PmlI and KpnI. Following confirmation, the 3'-region of SHIV-2873Nipd containing the *env* gene (ca. 4,000 bp) was then extracted and cloned into the 5'-region of SHIV-1157ipd3N4 (ca. 14,000 bp) with SphI and NotI. The final construct, SHIV-2873Nipd14, was confirmed using PmlI and NotI digestion.

Cloning Strategy for NL-LucR.1157ipEL and NL-LucR.2873Nipd14

The envelope regions of SHIV-1157ipEL and pcDNA6+SHIV-2873Nipd14 were cloned into the proviral DNA backbone pNL-LucR.T2A.

Using the EcoRI/ BamHI sites, the backbone region (3984bp band) of pBR322 and the envelope region (2722,bp band) of pNL-LucR.T2A were ligated to produce the shuttle vector pBR322(NL4-3) (6706 bp). The new pBR322(NL4-3) plasmid was confirmed by EcoRI and EcoRI/BamHI double digestion. The envelope regions of SHIV-1157ipEL (2,100 bp band) and SHIV-2873Nipd14 (2,133 bp band) were extracted using KpnI/BamHI sites and ligated to the backbone of the newly constructed shuttle vector pBR322(NL4-3) (4,588 bp band) to generate the vectors pBR322-1157ipEL and pBR322-2873Nipd14 respectively (ca. 6,733 bp each). The plasmids were confirmed by AvrII/EcoRI double digestion. Finally, using the EcoRI/BamHI sites, the envelope regions of pBR322-1157ipEL and pBR322-2873Nipd14 (ca. 2,750 bp each) were swapped into the backbone of pNL-LucR.T2A (13,128 bp band) to obtain NL-LucR.1157ipEL and NL-LucR.2873Nipd14 (ca. 15,870 bp each). To confirm the final constructs a double digest of EcoRI/BamHI and AvrII/EcoRI was performed.

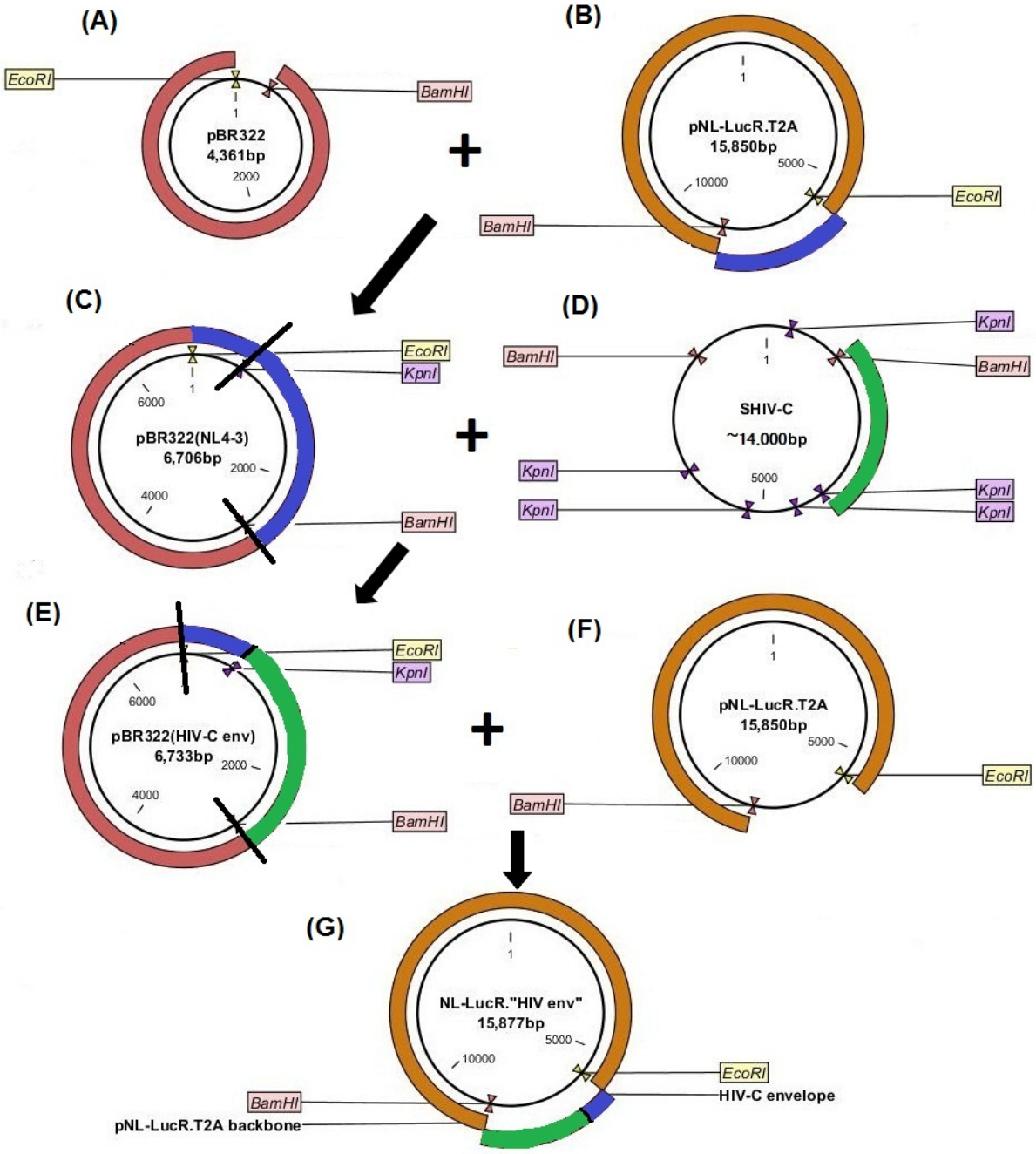

Figure 15 Reporter virus cloning strategy.

First backbone of pBR322 (A) and envelope of pNL-LucR.T2A (B) were ligated to form the shuttle vector pBR322 (NL4-3) (C). Then the envelope sequence of the desired SHIV-C (D) was cloned into the shuttle vector, generating pBR322(HIV-C env) (E). As a last step the HIV-C envelope (F) from the shuttle vector was extracted and swapped into the backbone of pNL-LucR.T2A generating the *Renila* luciferase expressing, HIV-C carrying reporter Virus (G).

3.4. Proviral Cloning

3.4.1 Generation of Plasmids Stocks

Before cloning, a stock of pBR322 and pNL-LucR.T2A was produced by transformation and subsequent plasmid purification. Additionally, the plasmids pSP73+3'SHIV-1157ipd3N4, SHIV-1157ipd3N4 and pcDNA6+SHIV-2873NipdTb-14 were inoculated and isolated.

3.4.2 Transformation

All transformations in this study were performed using MAX Efficiency ®Stbl ™ Competent *E.Coli* Cells following the manufacturer's protocol. Briefly, 100 µl of cells were mixed with 10 µl of plasmid in chilled 14 ml polypropylene tubes and kept on ice for 10 min. Cells were transformed by heat shock treatment at 42°C, followed by 2 min incubation on ice. 900 µl of S.O.C.(Super-optimal broth with catabolite repression) medium was added and the mixture was incubated for 75 min at 30°C in a bacterial shaker (150 rpm). 10 µl and 100 µl respectively of the mixture were distributed evenly on Luria-Bertani (LB) agar plates containing 50 µg/ml carbenicillin and were incubated overnight at 30°C and 37°C for cells transformed with retroviral sequences and non-retroviral sequences (pBR322), respectively.

3.4.3 Inoculation and Plasmid Isolation

Single colonies from each transformation were selected and inoculated in 10 ml of LB-medium containing 50 µg/ml carbenicillin. The inoculated single colonies were incubated overnight in a bacterial shaker (150 rpm) at 30°C. The next day glycerol stocks were prepared from each culture by combining 700 µl of bacterial suspension with 300 µl of glycerol. The prepared glycerol stocks were stored at -80°C. Subsequently, plasmid isolation was performed from the remaining culture using the QIAGEN®Plasmid Mini Kit following the manufacturer's protocol and DNA was eluted in 50 µl Elution Buffer (EB).

3.4.4 DNA Measurement

Purified DNA was measured with the NanoDrop1000 Spectrophotometer. Using the 'Nucleic Acid' application module (DNA-50), 1.5 µl of each sample was pipetted on the fiber optic cable of the instrument's measurement pedestal. Absorbance at the wavelength of 260 nm determined the sample concentration in ng/ml.

3.4.5 Plasmid Verification

In order to verify the isolated plasmids, digestion was performed using restriction enzymes that gave unique pattern of bands allowing unequivocal identification. All verification digests used the same protocol. Briefly, 350 ng of plasmid DNA was digested with 0.5 FastDigest Unit (FDU) of the required FastDigest Enzyme and 2 µl of FastDigest 10x Buffer. Nuclease-free water was added to a final volume of 20 µl. The samples were digested 5-10 minutes at 37°C and then combined with 6x DNA loading dye before being loaded onto a 1.2% agarose gel containing 300 ng/ml ethidium bromide. The gel was run in 1x TAE buffer containing 0.5mg/ml ethidium bromide for ca. 40 min at 100V. After the run, the gel was photographed with the Molecular Imager® Gel Doc™ XR System and analyzed with the Quantity One 1-D Analysis Software (Version 4.6.3, BioRad). Using the 1kb DNA ladder, the samples were identified according to their digested fragments.

3.4.6 Plasmid Digests for Cloning

After the isolated plasmids had been confirmed, new digests were set up to obtain higher concentrations of the required fragments (Chapter 3.3) for the following cloning steps. All digests were performed according to the same protocol. A DNA amount of 2 µg, an enzyme amount of 2 FDU and 5 µl of 10x FastDigest Buffer were used. Then nuclease free water was added to a final volume of 50 µl. The samples were digested for 5-10 minutes at 37°C and then run on a 1.2% agarose gel.

3.4.7 Extraction of Plasmid Fragments for Cloning

After the samples were run on the gel, the desired fragments (see chapter 3.3) were gel extracted under a hand-held Ultraviolet (UV) lamp (Model ENF-260, Spectroline). DNA isolation of the extracted gel fragments was done using the QIAquick® Gel Extraction Kit.

3.4.8 Ligation and Transformation of Newly Generated Plasmids

Using the Rapid DNA ligation Kit, the isolated fragments were ligated to generate new plasmids. In short, vector (usually referred to as “backbone”) and insert (usually referred to as “envelope”) were mixed in a molar ratio of 1:3 and diluted with DNA elution buffer to a final volume of 10 µl. Then 10 µl of T4 DNA ligation buffer and 5 units of T4 DNA ligase were added. The ligation mixture was incubated for 30 min at room temperature. Then, 5-10 µl of the mixture were used to transform MAX Efficiency ®Stbl ^{TM} Competent *E.coli* cells and distributed onto LB-agar/carbenicillin plates, which were incubated at 30°C overnight. The new construct was named as described earlier (see chapter 3.3). Approximately 5-10 single colonies were selected from the agar plates and inoculated in LB/carbenicillin media. The next day, glycerol stocks were generated from each culture and the remaining bacterial suspensions were used for plasmid isolation. For confirmation, the newly generated plasmids were digested according to the protocol for plasmid verification described earlier.

3.5. Sequence Analysis of Cloned Envelope Regions

For confirmation, all plasmid *env* regions were sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core. For this purpose 12 µl DNA (100 ng/µl) of each plasmid was mixed with 2.4 µl of primer (10 pmol/µl) and sent for sequencing. Specific forward and reverse primers for each envelope have been designed previously in Prof. Ruprechts’s laboratory. Sequence analysis was done using DNA STAR SeqBuilder™ software (version 10.1.2) For comparison, sequences were aligned with the *env* regions of SHIV-1157ipd3N4, SHIV-2873Ni and SHIV-2873Nip consensus sequence using

Clustal W. Additionally, a phylogenetic analysis was performed using the DNA STAR MegAlign™ software (version 10.1.2).

3.6. Screening and Virus Production

3.6.1 293T/17 Transfection and Virus Stock Preparation

Different proviral plasmid clones were screened for their infectivity. Small cell-free virus stocks were obtained by transfecting 293T/17 cells with infectious proviral DNA and harvesting supernatant. All transfection steps were performed using the X-tremeGENE 9 DNA Transfection Reagent according to the manufacturer's protocol.

Briefly, three 6-well plates were seeded with $1x10^6$ cells per well in a total volume of 2 ml growth media (GM) and incubated overnight. Transfection was carried out when cells became 70-90% confluent. A transfection mixture containing 2 µg DNA, 6 µl transfection reagent was prepared for each well, and the required amount of serum-free media DMEM medium was added to reach a final volume of 100 µl. The DNA-reagent complex was incubated at room temperature for 15 minutes. Meanwhile, 293T/17 cells were washed with 1x Phosphate buffered saline (PBS) and 1 ml of fresh GM was added to each well. The transfection mix was added to the cells in a dropwise manner and incubated for 3 hours at 37°C, 5% CO_2 before another 1 ml of (GM) was added to each well and the cells were incubated for a final 72 hours at 37°C, 5% CO_2.

After incubation, the supernatant from each well was filtered using a 0.45 micron filter. Aliquots of 0.5 ml filtered virus supernatant per cryotube were prepared and stored frozen in liquid nitrogen.

To prepare a large virus stock from transfection, a scaled-up version of the protocol described above was followed. A T-75 flask was seeded with $8x10^6$ 293T/17 cells in 15 ml GM. The next day, 20 µg DNA was mixed with 60 µl transfection reagent, DMEM was used to achieve a final volume of 1000 µl, and the mixture was incubated. In the meantime, the cells were washed and 3 ml GM was added to the flask. After adding the transfection mix and following a 3 hour incubation, 11 ml of fresh GM was added and

the cells were kept incubated for 72 hours at 37°C, 5% CO_2. Virus containing cell-free supernatant was collected using a 0,45 micron filter, divided into aliquots, and stored in liquid nitrogen.

3.6.2 Infectivity Screening in TZM-bl Cells

The infectivity of the virus clones was determined using the TZM-bl reporter cell line. Briefly, 100 µl of TZM-bl GM was added into each well of a 96-well-flat-bottom culture plate. A 25 µl aliquot of virus clone to be tested was transferred in duplicate to two adjacent wells of column 1. A serial 5-fold dilution (achieved by transferring 25 µl each time) was performed for a total of 11 dilutions (up to column 11). Wells in column 12 received no virus and served as controls for background luminescence. Next, 100 µl TZM-bl cells (containing 10^4 cells) were added to each well. Cells receiving azidothymidine (AZT) were incubated 1 hour prior to the addition of the cells to the wells with 20 µM AZT so that the final concentration of 10 µM AZT per well was achieved after adding the cells. Specifically, for the SHIV-2873Nipd14 clones, dextran was added to the TZM-bl cells to enhance infectivity, so that a final concentration of 10 µg/ml was present in each well.

The assay plates were incubated for 48 hours at 37°C, 5% CO_2. After 2 days, 100 µl of culture medium was removed from each well and replaced with 100 µl of Britelite Plus reagent, followed by incubation for 2 min at room temperature. Following a quick mixing (by pipette) 150 µl from each well was transferred to a corresponding black 96-well plate and read immediately in a luminometer. The average RLUs was calculated for each virus sample and plotted according to the corresponding dilution.

3.6.3 Determining the Tissue Culture Infectious Dose 50 (TCID50)

The TCID50 values were calculated for each virus stock with and without dextran. Since SHIVs replicate poorly in TZM-bl their TCID50 value was determined only with dextran. The assay was performed as described by David C. Montefiori (see link next page).

Briefly, 100 µl of TZM-bl GM was added to each well of a 96-well plate (flat bottom), followed by 25 µl virus into the first 4 wells of a dilution series (column 1, rows A-D for

virus without dextran and rows E-H virus with dextran). A serial 5-fold dilution was then performed up to column 11, and 25 µl were discarded from each well of this final row. As before, column 12 served as the background luminescence control. 100 µl cell suspension (containing 10^4 cells) with and without dextran (final concentration 10 µg/ml) was added, to each well and after 48 hour incubation at 37°C, 5% CO_2 the RLU was measured. The TCID50 values were calculated with the help of the "TCID" macro (see link). (http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm)

3.7. Co-receptor Tropism

3.7.1 CEMx 174-GFP Co-receptor Assessment

For the CEMx-174-GFP co-receptor assay, a six-well plate was seeded with 1 ml GM containing $1x10^6$ cells, 350 µl of each virus to be tested was added followed by 1 ml of fresh GM. One well received the X4-tropic positive control HIV-1 NL4-3. Another well with no virus served as the negative control. After three-day incubation at 37°C, 5% CO_2 cells were visualized by fluorescence microscopy (FITC channel).

3.7.2 U87.CD4 Co-Receptor Assessment

For further co-receptor testing, U87.CD4.CXCR4 and U87.CD4.CCR5 cell lines, each expressing the co-receptor CXCR4 or CCCR5 respectively, were used. A 24-well plate was seeded with $0.5x10^6$ cells in 1 ml GM and incubated at 37°C, 5% CO_2 for 24 hours. The next day, each cell line was infected with 450 µl of virus. HIV 196USSN20 was used as a positive control. The virus was incubated with the cells for 3.5 hours at 37°C, 5% CO_2. Then, the entire supernatant, including virus, was removed and each well was washed three times with 1 ml of 1x PBS, 1.2 ml of fresh GM were added, and 200 µl supernatant per well was collected (=sample day 0). The 24-well plate was incubated at 37°C, 5% CO_2 with another 200 µl supernatant being collected on days 1, 3, 5, 7 and 10. After each collection, 200 µl of fresh GM was added. Once samples from all time points had been collected the p24/p27-assays were performed by measuring Gag concentration to determine viral replication.

3.7.3 HIV-1 p24 and SIV p27 ELISA

For quantification of virus in the collected supernatants double-antibody sandwich enzyme immunoassays were performed (HIV-1 p24/SIV p27 Antigen Capture Assay). Both assays quantify the amount of a capsid protein in the sample (p24 for HIV and p27 for SHIV) which correlates well with the in vitro viral load. Both assays were performed according to the manufacturer's protocol. Briefly, 25 µl of disruption buffer was added to each well to be used in the assay. Then, a specific standard was prepared for each assay by serially diluting the provided standard antigen. Standard range was 3.1-100 pg/ml for p24 and 62.5-2000 pg/ml for p27. A 100 µl volume of each standard, along with an appropriately diluted test sample, was added to each well. As a negative control, 100 µl of GM was added into 2 wells. The microelisa plate was covered with a plate sealer and incubated for 1 hour at 37°C then washed 3 times with 300 µl wash buffer per well in an automated plate washing system. Next, 100 µl conjugate solution was added to each well and the plate was incubated for one hour incubation at 37°C followed by a washing step as described above. Subsequently, 100 µl of peroxidase substrate was added to each well and incubated for 30 minutes at room temperature in the dark. Finally, the reaction was terminated using 100 µl stop solution, and absorbance at 450 nm was measured in an ELISA plate reader. The test samples absorbance values should fall into the range of the standard curve to give valid results. Values above/below this range have to be re-determined using higher/lower dilutions of the test sample. The p24/p27 concentration in each test sample was calculated by interpolation from a standard curve.

3.8. Reporter Virus Titration and Neutralization Assays

3.8.1 Titration and Neutralizing Antibody Assay in A3R5 cell line

Before performing neutralization assays in the A3R5 cell line, each virus was titrated in the same cell line. Briefly, 100 µl of GM/well was placed in each well of a 96-well flat-bottom tissue culture plate. Then, a 1:10 dilution of each virus was prepared and 100 µl of this dilution was transferred to the first 3 wells of a dilution series (row A, column 1-3; for second virus row A, column 4-6). A serial 2-fold dilution was performed for a total of 7 dilutions up to row G, by serially transferring 100 µl and finally discarding 100 µl from the 7^{th} dilution. Wells in row H received cells but no virus and served as controls for background luminescence. Next, a 100 µl aliquot of cells (containing a total of 90,000 cells) was added to each well and the plates were incubated for 96 hours at 37°C, 5% CO_2.

After incubation, 72 µl of supernatant was discarded from each well. The remaining cells were suspended and 75 µl were transferred from each well to the corresponding well of a white 96-well plate. Separately, a 10 µl aliquot of ViviRen Live Cell Substrate was diluted in 3.5 µl of GM, and 30 µl of the diluted ViviRen Reagent was dispensed to each well. The plates were incubated at room temperature for 4 min and then read in a luminometer using 0.5 sec/well protocol in the Wallac Software. The average RLU of each triplicate was calculated and plotted.

All neutralizations assays in A3R5 were performed according to the following protocol. First, a working dilution of each monoclonal or polyclonal Ab was prepared at 40 µg/ml. Then 67 µl of prepared antibody was transferred to the first 3 wells of a dilution series (row A, column 1-3, second Ab column 4-6 etc.) of a 96-well flat-bottomed tissue culture plate. 50 µl of GM was added to all other wells of the dilution series as well as to the virus only control (row G). 100 µl of GM was added to each well in row H for the cells only control. A 4-fold serial dilution was then performed by transferring 16.7 µl from row A to row B until row F, from which 16.7 µl was discarded. 50 µl of virus was added to each well (except for row H) at a dilution affording ca. 50,000 RLU, based on the initially performed virus titration. Then the virus and Ab mixture was incubated for one hour at 37°C, 5% CO_2 and 100 µl of cell suspension (containing 90,000 cells) was added to

each well, followed by incubation for 96 hours at 37°C, 5% CO_2. After 4 days the plates were read using the protocol described earlier. The average RLUs of each triplicate was calculated. Valid results require that the average RLU of virus control is >10 times the average RLU of control wells (uninfected cells only).

3.8.2 Calculation of Neutralization and IC50

Percent neutralization was determined by calculating the difference in average relative luminescence units (RLU) between test wells (cells + Ab + virus) and cell control wells (cells only), dividing this result by the difference in average RLU between virus control (cell + virus) and cell control wells (cells only), subtracting from 1 and multiplying by 100.

Neutralizing antibody titers are expressed as the amount of antibody required for reducing RLU by 50%. This half-maximal inhibitory concentration (IC50) is determined as the lowest Ab dilution that can achieve this result. Antibodies failing to obtain at least 50% reduction of RLU at any dilution are denoted as >20 µg/ml.

3.8.3 Isolation of Mononuclear Cells from Human Peripheral Blood

Human whole blood was obtained from the Kraft Family Blood Donor Center at Dana-Farber Cancer Institute. Whole blood from one blood donor was transferred into 50 ml tubes and diluted 1:1 with 1x PBS. The diluted blood was carefully layered onto 15 ml Ficoll-Paque in new 50 ml tubes. The tubes were centrifuged for 30 min at 2500 rpm (room temperature) in a swinging bucket rotor without braking. Following separation, the upper layer was aspirated carefully in order to leave the interphase buffy layer containing the mononuclear cells undisturbed. The mononuclear cell layer was transferred carefully into a new 50 ml tube and washed twice with 1x PBS. After removal of the supernatant, the pellet was resupended in 50 ml of GM, and the cells were counted using a hemocytometer. Cells were centrifuged again and resuspended in freezing medium (10% DMSO, 90% FBS) to a final cell concentration of $50x10^6$ cells/ml. Finally, aliqouts of the cells (1 ml/cryotube) were placed in a Mr. Frosty freezing container at -80°C and one day later stored in liquid nitrogen.

3.8.4 Activation and Titration of hPBMC

Before titration and neutralization assays were performed, the previously isolated hPBMC had to be activated. $100x10^6$ cells were thawed, washed and resuspended in 50 ml GM. The cells were activated with PHA (final concentration 1mg/ml) and IL-2 (final concentration 20U/ml). The cells were incubated for 3 days at 37°C, 5% CO_2.

For titration, a working dilution of 1:5 was first prepared for each virus to be tested. Then 100 µl of virus was added in triplicates to row A of a 96-flat-bottom well plate. To dilute the virus, 50 µl of GM was added to each well from row B to row G. Row H received 100 µl GM/well and served as background control. Then, 50 µl of virus suspension was transferred from each well in row A to row B. This serial 2-fold dilution was continued until row G, from which 50 µl were discarded. A 100 µl volume of cell suspension (containing $0,2x10^6$ cells from the previously prepared donor stock) was added to each well (GM contained 40U/ml IL-2) and the plate was incubated for 96 hours at 37°C, 5% CO_2 and read in a luminometer.

3.8.5 Neutralization Assays in hPBMC with RM-Serum

Activated hPBMC were incubated with RM serum from a vaccine study performed in 2010. For each RM-serum two time points, before (pre-immune) and after (post-immune) immunization, were selected. Before the assay, all serum specimen were heat-inactivated by incubating them for 1 hour at 56°C to inactivate complement. Then, the inactivated serum specimens were diluted with hPBMC GM to achieve a 1:10, 1:20, 1:40 and 1:80 dilutions. A 50 µl aliqout of each serum dilution was transferred in triplicates to a 96-flatt bottom well plate, followed by addition of diluted virus (50µl) to each well. A virus dilution (based on the prior titration) was chosen that would yield a final virus concentration of 50,000 RLU. The plate was incubated for 1 hour at 37°C, 5% CO_2 and 100 µl of cell suspension (containing $0.2x10^6$ cells) was added to each well. An additional pate was set up in which one row served as cell-only control and one row served as cell plus virus control.

Percentage neutralization was determined by calculating the difference in average RLU between wells containing post-immune samples and wells containing pre-immune samples from the same RM for the same dilution. For a valid run the average RLU of virus control had to be >10 times the average RLU of cell control wells.

3.9. Complement Mediated Antibody Dependent Enhancement Assays (C'ADE)

Before performing C'ADE assays in SupT1.R5 cells, the viruses to be used were titrated. Briefly, 100 µl GM was added to each well of a 96-flat bottom well plate. Next, 25 µl of each virus was added to three wells of a dilution series. From there a serial 5-fold dilution (by transferring 25 µl each time) was done for a total of six dilutions up to row F. Then, 100 µl cell suspension (containing 10^5 cells) was added to each well except for row H, which served as a medium-only control. Wells in row G received no virus and served as cell-only controls. The plate was incubated for three days at 37°C, 5% CO_2 and luminescence was measured.

C'ADE assays were performed using 96-flat bottom plates for the viruses NL-LucR.2873Nipd14, NL-LucR.2873Ni and NL-LucR.1157ipEL, in 96-well plates (flat-bottomed). Each virus was incubated with serial dilutions of SHIVIG or naive rhesus monkey IgG (NRM IgG) in duplicates in the presence of 10% fresh human serum (as a source of complement-C') or heat-inactivated serum/complement (HIC) for 1 h at 37°C in 96-well flat-bottom plates. Before the assay was set up, the required amount of HIC was prepared immediately prior to each assay by incubating fresh human serum for 1 hour at 56°C to inactivate complement activity.

Briefly, 150 µl of GM was added to each well in column 1 (containing 10% fresh human complement-C') and column 12 (containing heat-inactivated complement-HIC). These wells served as cell-only controls. Then, 100 µl of C'GM was added to each well from column 2 to 6 and 100 µl of HIC-GM was added to each well from column 7 to 11. Additionally, 40 µl of the respective GM was added to each well from row H columns 3 to 10.

Next, 11 µl of SHIVIG (26 mg/ml) was added to each well in row H columns 3 to 7 and 11 µl of naive IgG (22,5 mg/ml) was added to each well in row H columns 7 and 8. Samples in row H were mixed and 50 µl was transferred to row G. This serial 3-fold dilution was repeated up to row A, from which 50 µl was discarded from each well.
50 µl of the tested virus was then added to each well from column 2 to 11. Based on prior titration a virus working dilution was selected that yielded ca. 50,000 RLU in its final well. After 1 hour incubation at 37°C, 5% CO_2, 100 µl cell suspension (containing 100,000 cells) in C' or HIC GM was added to each well respectively. The plate was then incubated at 37°C, 5% CO_2 for three days and luminescence was measured.

Percent neutralization was determined by calculating the difference in average relative luminescence units (RLU) between test wells (cells + serum sample + virus) and cell control wells (cells only), dividing this result by the difference in average RLU between virus control (cell + virus) and cell control wells, subtracting from 1 and multiplying by 100. Negative values are indicative of infection-enhancement.

3.10. Infection of SHIV-2873Nipd14 in RM-PBMC Donors

PBMC from eight randomly selected rhesus monkeys (previously isolated in the Ruprecht laboratory), were thawed and activated. Cells were resuspended in 10 ml GM and counted, then they were centrifuged for 10 min at 1250 rpm (RT) and resuspended in the required amount of GM containing 5 µg/ml Con-A and 40U/ml human IL-2 to obtain a cell count of $4x10^6$ cells/ml. Cells were kept in T-25 flasks and incubated for 3 days at 37°C, 5% CO_2.

After activation, all eight PBMC RM donors were infected with SHIV-2873Nipd14. Donor cells were transferred into a 15 ml tube and centrifuged for 10 min at 1250 rpm (RT). Next the supernatant was removed and the pellet washed twice with 10 ml GM and counted using Trypan Blue staining. Cells were spun down one more time and resuspended in the required amount of GM containing 40 U/ml IL-2 to obtain a final cell concentration of $2x10^6$ cells/ml. A 1 ml aliquot of donor cells was transferred to a new 15 ml tube and 500 µl of SHIV-2873Nipd14 virus supernatant was added undiluted to

each tube. The virus+cell mixture was incubated for 2 hours at 37°C, 5% CO_2 with tapping of the tubes every 15 min. Then, the tubes were centrifuged and the supernatant was removed. The pellet was washed twice with 10 ml GM, and resuspended in 1.2 ml GM containing IL-2 and transferred to one well of a 12-well plate. 200 µl supernatant was collected from each well for the day 0 sample and subsequently the plate was incubated at 37°C, 5% CO_2. Every second or third day, 200 µl supernatant was collected and replaced with fresh GM and IL-2. After 22 days, the experiment was terminated and a p27 assay performed to quantify the virus present in the supernatant samples.

4. Results

4.1. Construction of the Reporter Viruses NL-LucR.1157ipEL and NL-LucR.2873Nipd14

To construct the new reporter viruses, the HIV-C envelopes from SHIV-115ipEL and infectious SHIV-2873Nipd *env*, reisolated from RM (RTb-11) diagnosed with AIDS, were cloned into the pNL-LucR.T2A backbone containing reporter gene *Renilla* luciferase (Fig.16).

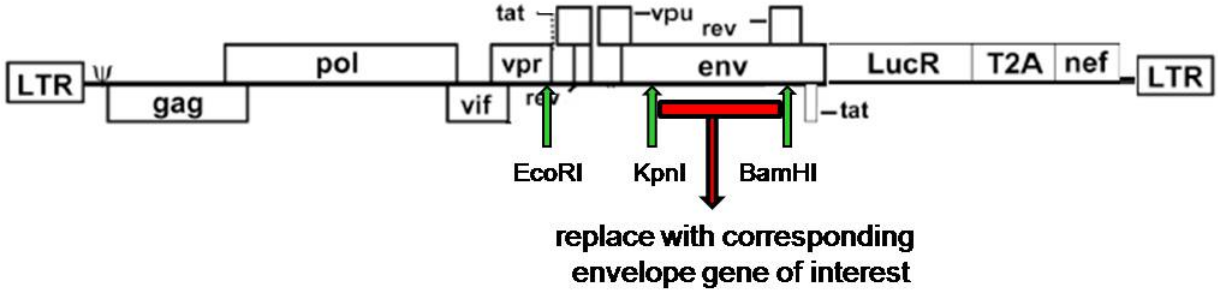

Figure 16 NL-LucR.T2A Genome with highlighted restriction sites (green arrows).
modified from (EDMONDS et al. 2010)

The SHIV envelopes used do not contain an EcoRI site to swap the envelope directly with BamHI into pNL-LucR.T2A. Also, there are multiple KpnI restrictions sites in the reporter virus genome making a direct swap with EcoRI not possible. Therefore, a shuttle vector based on the pBR322 backbone had to be generated. First, the envelope region of pNL-LucR.T2A was cloned into the backbone of pBR322 using EcoRI/BamHI. The construct was named pBR322(NL4-3). Six clones were selected and double digested with EcoRI/BamHI for confirmation. All six displayed the expected band pattern (Fig.17).

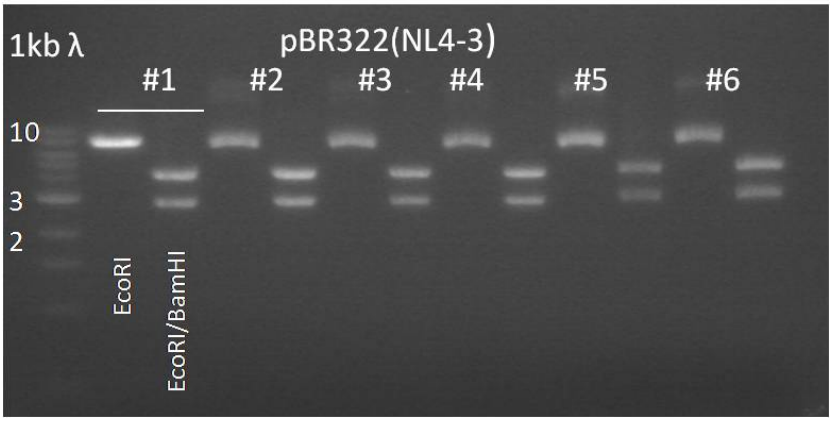

Figure 17 Confirmation of pBR322(NL4-3).

EcoRI and EcoRI/BamHI double digest of six selected clones. All display bands of expected size (6,706 bp and 3,984 bp/2,722 bp), indicating an successful envelope swap from pNL-LucR.T2A into pBR322 to create the shuttle vector pBR322(NL4-3).

Next, the envelope regions of SHIV-1157ipEL and pcDNA6+SHIV-2873Nipd14 were isolated by using KpnI/BamHI sites and ligated to the backbone of the newly constructed shuttle vector pBR322(NL4-3). The new constructs were named pBR322-2873Nipd14 (Fig.18) and pBR322-1157ipEL (Fig.19), respectively. For confirmation, clones were isolated and double-digested with AvrII/EcoRI. The unique restriction site of AvRII is present only in the envelopes of SHIV-1157ipEL and SHIV-2873Nipd14 (and not in SHIV-1157ipd3N4 or pcDNA6) and can therefore be used for clone identification. All tested clones displayed the expected bands.

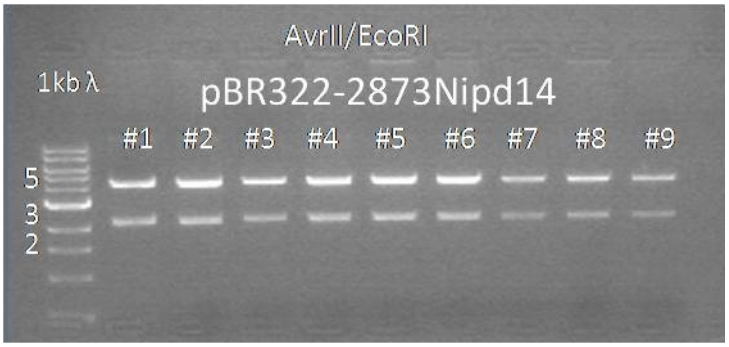

Figure 18 Confirmation of pB322-2873Nipd14.

All 9 clones were double-digested with AvrII/EcoRI. AvrII is only present in the 2873Nipd14 envelope and the presence of two bands (ca. 2,270 bp/ 4,460 bp) therefore indicates a successful swap into the shuttle vector pBR322(NL4-3).

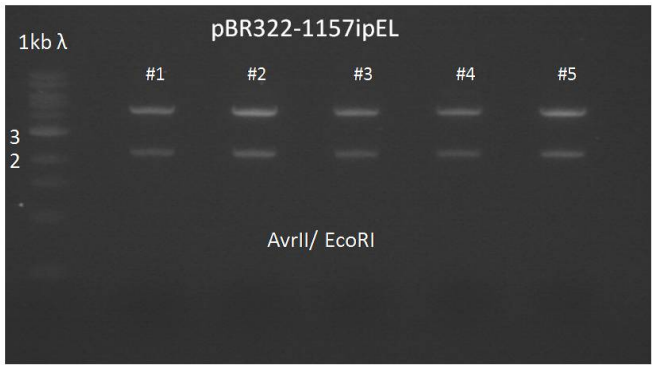

Figure 19 Confirmation of pBR322-1157ipEL.

All 5 clones were double digested with AvrII/EcoRI (AvrII is only present in the 1157ipEL envelope and the presence of two bands (ca. 2,270 bp/4,460 bp) therefore indicates a successful swap into the shuttle vector pBR322(NL4-3).

Finally, using the EcoRI/BamHI sites, the envelope regions of pBR322-1157ipEL and pBR322-2873Nipd14 were swapped into the backbone of pNL-LucR.T2A generating the final constructs NL-LucR.1157ipEL and NL-LucR.2873Nipd14. To confirm these, a double-digest of EcoRI/BamHI and AvrII/EcoRI was used for NL-LucR.1157ipEL and to confirm NL-LucR.2873Nipd14 a single digest of HindII was performed.

For NL-LucR.1157ipEL, #1 showed the expected bands and was confirmed positive (Fig.20). Of the 10 NL-LucR.2873Nipd14 clones digested with HindIII, all except #2 and #9 could be confirmed as positive, the pattern displayed by them being clearly distinguishable from that of the parental NL-LucR.T2A plasmid (Fig.21).

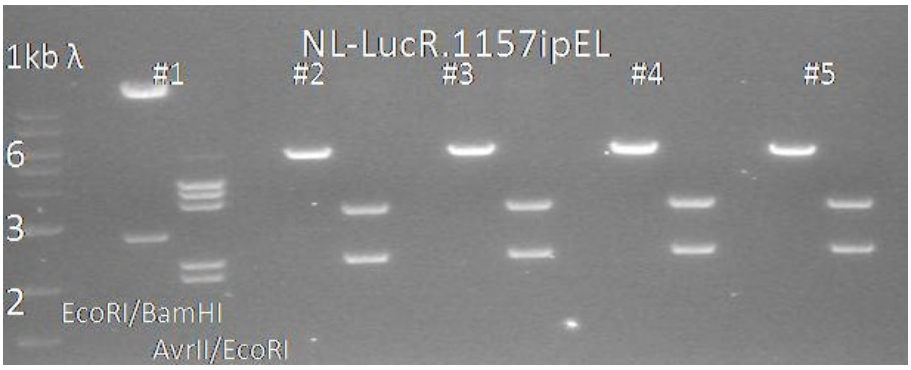

Figure 20 Confirmation of NL-LucR.1157ipEL.

All selected clones were double digested with EcoRI/BamHI and AvrII/EcoRI. Clone#1 display the expected band pattern (approx. 4,086 bp, 3,767 bp, 3,420 bp, 2,290 bp, 2,018 bp; the smaller ones: 230bp and 82bp were too small to be visualized)

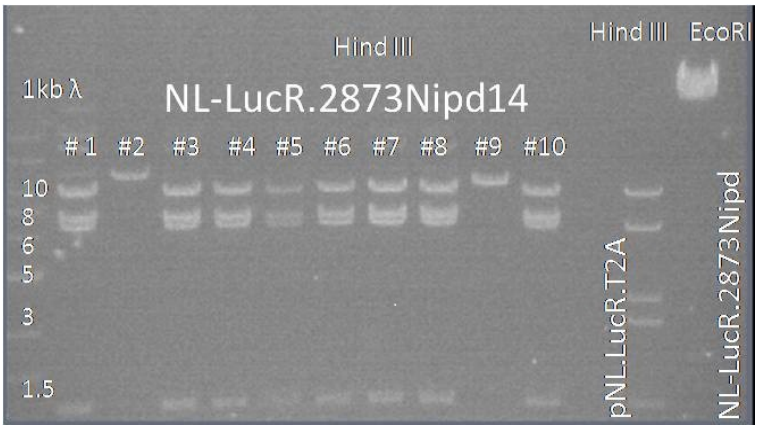

Figure 21 Confirmation of NL-LucR.2873Nipd14.

10 selected clones were digested with HindIII. Clones with successful ligation of the *env* display a different pattern to its parent pNL-LucR.T2A and allows therefore a clear discrimination. Positive clones are number 1,3-8 and 10 (approx. 5,773 bp, 4,609 bp, 4,314 bp, 1,181 bp).

4.2. Construction of the Infectious Molecular Clone SHIV-2873Nipd14

As SHIV-1157ipd3N4 contains multiple BamHI and KpnI sites, a direct insertion of the SHIV-2873Nipd envelope was not possible. Therefore, the 3' part of SHIV-1157ipd3N4 which had been cloned into the vector pSP73 earlier (SONG et al. 2006) was used as a shuttle vector. Using the sites of KpnI/BamHI the envelope in the vector pcDNA6+SHIV-2873NipdTb14 was isolated and cloned into the 3'SHIV-1157ipd3N4 plasmid (after the excision of the 1157ipd3 *env*) (Fig.22,A).

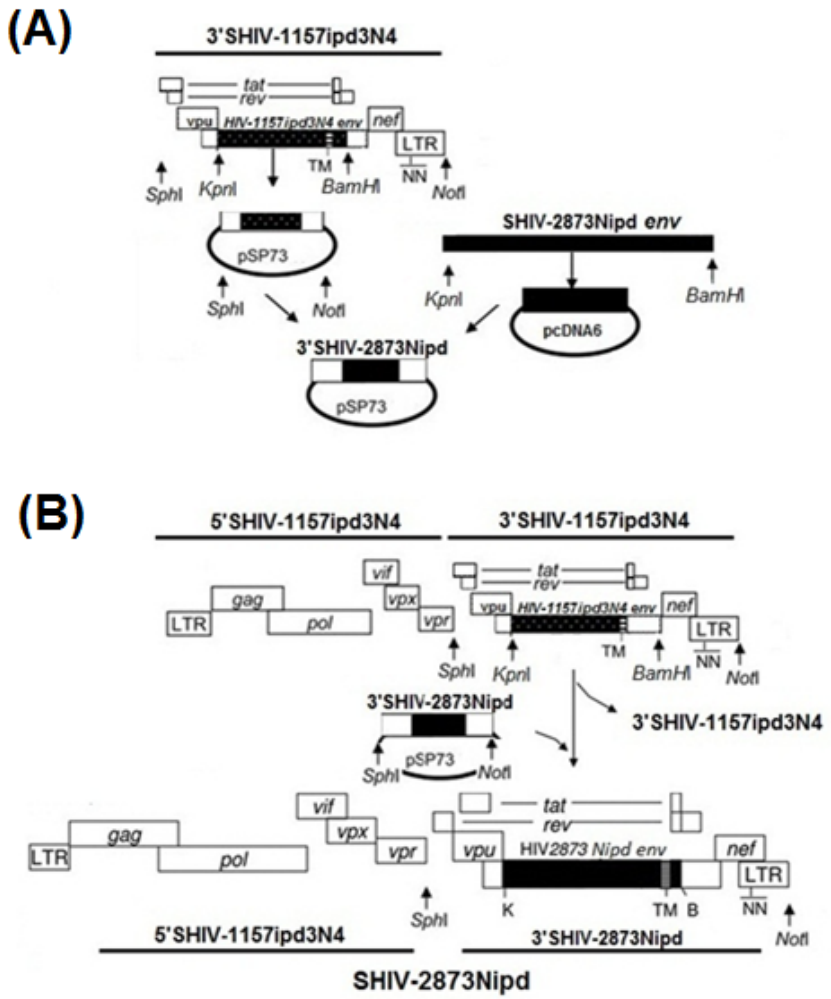

Figure 22 SHIV-2873Nipd Cloning strategy.

(A) First the envelope region (KpnI-BamHI) from SHIV-2873Nipd (in the pcDNA6 vector) was cloned into the 3'SHIV-1157ipd3N4 (In the pSP73 vector) generating 3'SHIV-2873Nipd.

(B)Then the SpHI-NotI part from 3'SHIV-2873Nipd was cloned into 5' part of SHIV-1157ipd3N4 generating SHIV-2873Nipd (now with the "backbone" from SHIV-1157ipd3N4).

The new plasmid was named 3'SHIV-2873NipdTb-14. Ten single colonies were selected for screening. For confirmation, the clones were double digested with PmlI/KpnI. PmlI is a unique site only found in the SHIV-2873Nipd envelope and not in SHIV-1157ipd3N4. The appearance of 2 bands in the gel therefore identifies a positive clone. Ten clones were tested and all displayed the expected band pattern for 3'SHIV-2873Nipd (Fig.23). Clone #8 was selected for further cloning.

Next, the 3'part of SHIV-2873Nipd was extracted from clone #8 and cloned into the 5'part of SHIV-1157ipd3N4 using SphI/NotI sites (Fig.22,B). The final construct was called SHIV-2873Nipd14. Again, ten clones were isolated and for confirmation digested with PmlI/KpnI. All except clone #5 displayed the expected bands and could be identified positive (Fig.24).

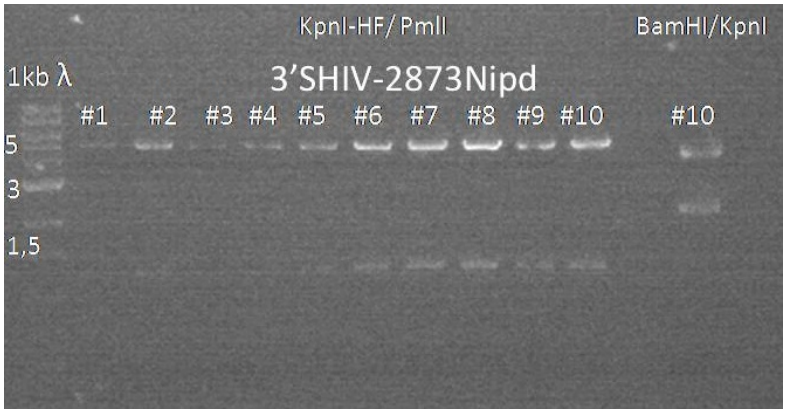

Figure 23 Confirmation of 3'SHIV-2873Nipd.

All 10 clones were double digested with KpnI/PmlI. The restriction site for PmlI is only present in 2873Nipd *env* that's why the presence of two bands (5200bp and 1254bp) indicates the successful swapping of the SHIV-2873Nipd envelope into 3'SHIV-1157ipd3N4. Although only faintly visible all digested clones were confirmed positive.

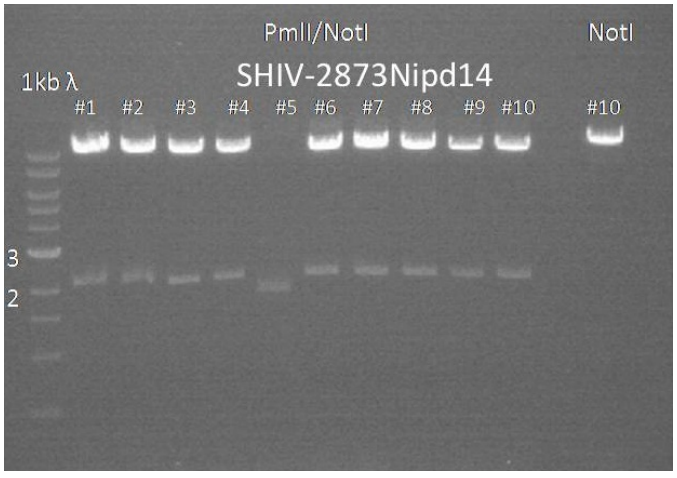

Figure 24 Confirmation of SHIV-2873Nipd14.

10 clones were selected and double digested with PmlI/NotI. Except for clone #5 all cones display the expected band size (ca. 2224bp and 10 000bp),indicating the successful ligation from 3'SHIV-2873Nipd14 with 5'SHIV-1157ipd3N4.

4.3. Characterization of Generated Viruses

4.3.1. Screening in TZM-bl

To identify functional, infectious virus clones, an infectivity assay to assess replication was performed in the TZM-bl cell line. Expression of the reporter gene firefly luciferase is induced by the viral Tat protein shortly after infection. Luciferase activity is measured by luminescence (=RLU) and is directly proportional to the number of virus particles present in the inoculum. To estimate the virus infectivity, TZM-bl cells were incubated with different dilutions of the virus clones in the presence or absence of azidothymidine (AZT). The addition of AZT was to make sure that the measured RLU corresponded to de novo infection of the cells and was not due to artifacts of cell lysis and subsequent viral Tat activation. 4 Clones of SHIV-2873Nipd (#1, #2, #3 and #4), 4 clones of NL-LucR.2873Nipd12 (#1, #3, #4 and #5) and 1 clone of NL-LucR.1157ipEL (#1) were tested (Fig.25). All clones tested replicated well in TZM-bl cells. Clones SHIV-2873Nipd14 #2, NL-LucR.2873Nipd14 #5, and NL-LucR.1157ipEL #1 were selected due to their high degree of replication and their plasmid DNA was used to prepare bigger virus stocks. The produced virus stocks were given the name NL-LucR.2873Nipd14, SHIV-2873Nipd14 and NL-LucR.1157ipEL, respectively. Additionally, it has to be mentioned that all viruses incubated with AZT displayed no viral replication, indicating that the measured replication activity is due to infectious particles present in the virus supernatant.

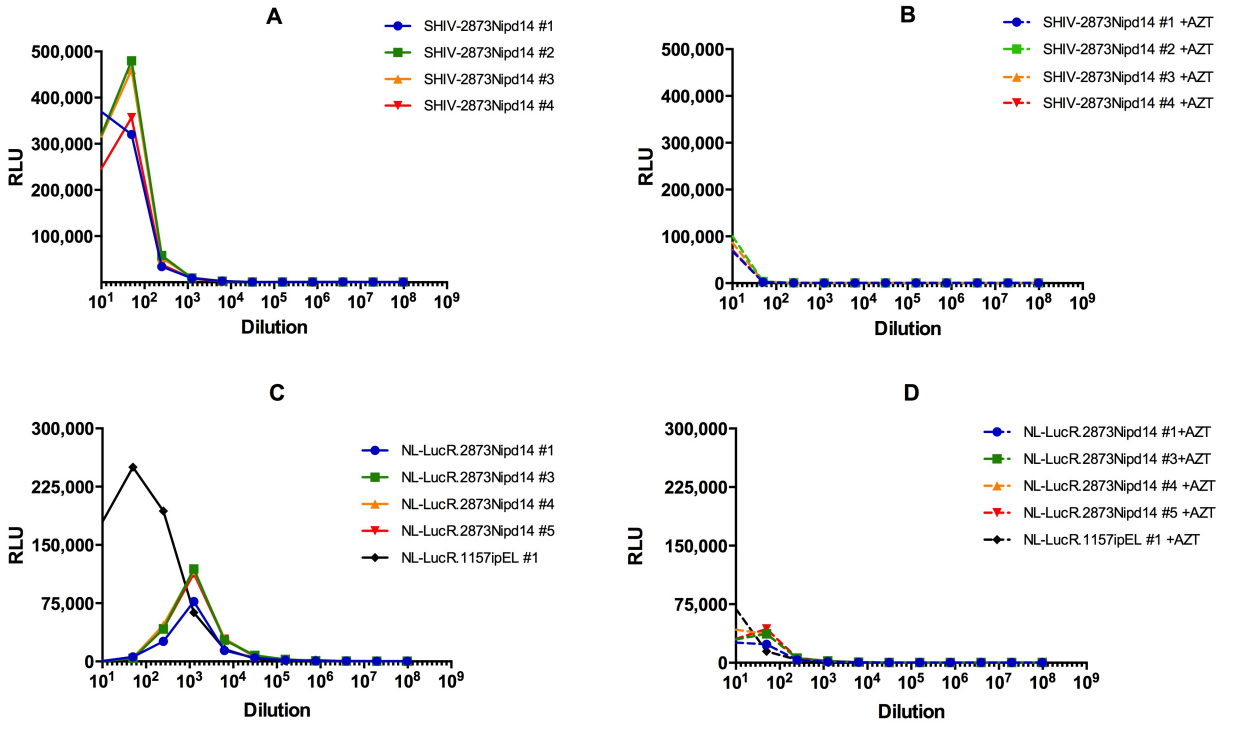

Figure 25 Virus screening in TZM-bl.

TZM-bl cells incubated with different virus dilutions with (B+D) and without (A+C) AZT. (A+B) SHIV-2873Nipd14 clones #1-4. (C+D) NL-LucR.2873Nipd14 #1,#3,#4,#5 and NL-LucR.1157ipEL. All viruses replicated well in TZM-bl cells when incubated alone, whereas with no replication was detectable when incubated with AZT.

4.3.2. Sequence Analysis of 1157ipEL env and 2873Nipd14 env

Sequence analysis confirmed the envelope identity of SHIV-2873Nipd14 with NL-LucR.2873Nipd14, showing that no mutations had occurred during the cloning process. Similarly, NL-LucR.1157ipEL *env* was found to be identical to the parent SHIV-1157ipEL *env* sequence (data not shown). A detailed sequence analysis of SHIV-1157ipEL and its parental clone SHIV-1157ipd3N4 was already performed by Siddadappa et al. (2010) and will not be discussed at this point. To further validate the evolution of the 2873Nipd envelope during the process of adaptation, its sequence was aligned with its parental envelope clones 2873Ni and 2873Nip (consensus sequence). Interestingly, some major mutations were found in the gp120 glycoprotein. The amount of deletions in the variable regions of gp120 increased drastically in the 2873Nipd14 envelope, with a 3-aa deletion in V1, a 6-aa deletion in V4 and a 5-aa deletion in V5, all

in close proximity to CD4 binding sites. Still, the virus was functional as was tested by the GFP-pseudotyped virus assay and all the infectivity assays performed in this study. Increased accumulation of mutations in the HIV genome is not surprising given the rapid virus replication cycle with its error prone reverse transcriptase (SMYTH et al. 2012).

The phylogenetic analysis shows the relationship of 2873Ni, 2873Nip and 2873Nipd. It can be seen that the 2873Ni and 2873Nip envelopes are more closely related. This finding is not unexpected given the time intervals before the viruses were reisolated. SHIV-2873Nip was reisolated one year after inoculation, whereas SHIV-2873Nipd was reisolated 3 years after inoculation from RTb-11, thereby giving the virus much more time to adapt (unpublished data).

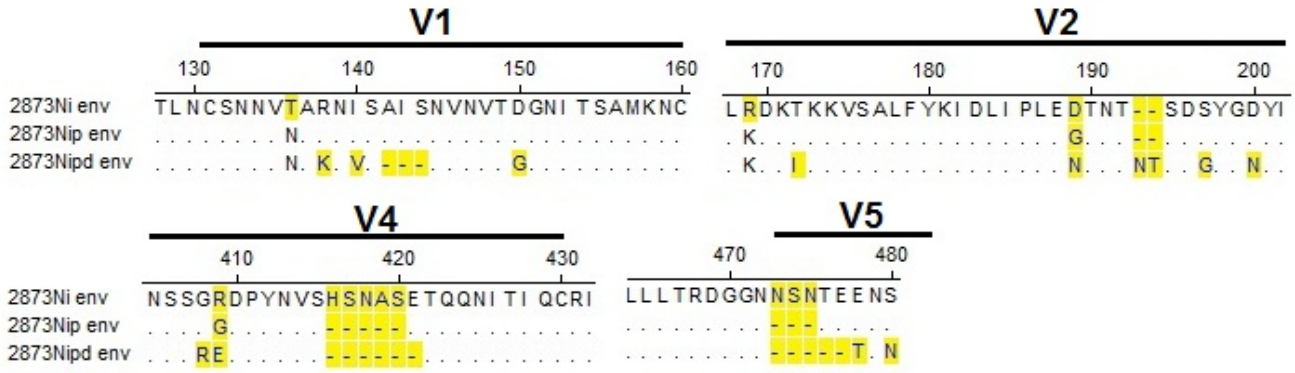

Figure 26 Envelope sequence alignment of 2873Ni, 2873Nip and 2873Nipd.

Mutations occurred mainly around the variable regions V1, V2, V4 and V5 and are highlighted in yellow.

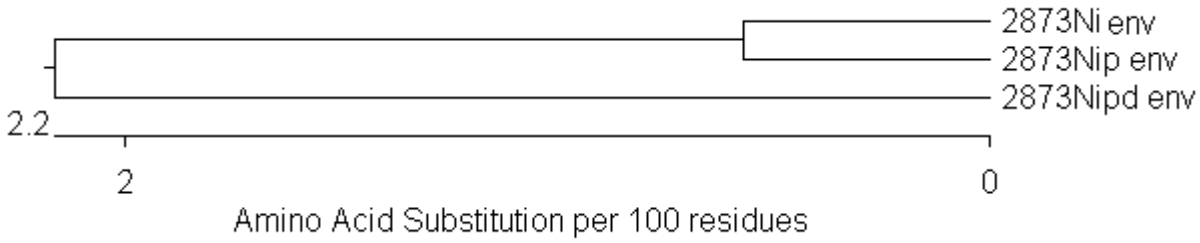

Figure 27 Phylogenetic tree analysis.

The phylogenetic tree shows the evolutionary relationship between the different 2873 envelopes. The closest relationship is found between 2873Ni and 2872Nipd.

4.3.3. TCID50 Measurement

To characterize the newly prepared virus stocks (NL-LucR.1157ipEL, NL-LucR.2873Nipd14 and SHIV-2873Nipd14) the Tissue Infectious Dose 50 (TCID50) was determined with and without dextran. These values determine the amount of virus particles (TCID50/ml) or the virus dilution needed to infect 50% of the TZM-bl cells. Hence, it is a measure for virus infectivity and it can be used to compare different stocks. For this thesis, two other reporter viruses (NL-LucR.2873Ni and NL-LucR.1157ipd3N4, generated by Dr.Samir Lakhashe, DFCI) were included in the comparison and their TCID50 values are summarized in the table below (Table 3). Not surprisingly, when incubated with the infectivity enhancing reagent dextran, all reporter viruses displayed much higher TCID50 values. SHIV-2873Nipd14 had the lowest TCID50 and was measured only with dextran, since SHIV infection without is very inefficient (Table 3).

Virus	stock date	TCID 50 no dextran	TCID50 + dextran
NL-LucR.2873Nipd14	15Feb13	53,437	488,281,250
NL-LucR.2873Ni	6May13	13,975	8,734,641
NL-LucR.1157ipEL	15Apr13	13,975	11,421,944
NL-LucR.1157ipd3N4	15Feb13	18,000	1,750,000
SHIV-2873Nipd14	26Apr13	/	10,687

Table 3 TCID50 values with and without dextran.

4.3.4. Determination of Tropism

Co-receptor usage in CEMx 174-GFP

To study the virus tropism of the cloned viruses the CEMx-174-GFP and the U87.CD4 cell lines were used. The CEMx 174-GFP cell line is a T-B hybrid cell line that exclusively expresses CXCR4 and not CCR5. It contains a green fluorescent protein gene under HIV-1 LTR regulation. Upon infection with a X4-tropic virus the florescent protein becomes expressed and can be detected using fluorescence microscopy. However, all tested viruses (NL-LucR.1157ipEL, NL-LucR.2873Nipd14 and SHIV-2873Nipd14) displayed no fluorescence, indicating that they are negative for CXCR4 co-receptor usage (Fig.28).

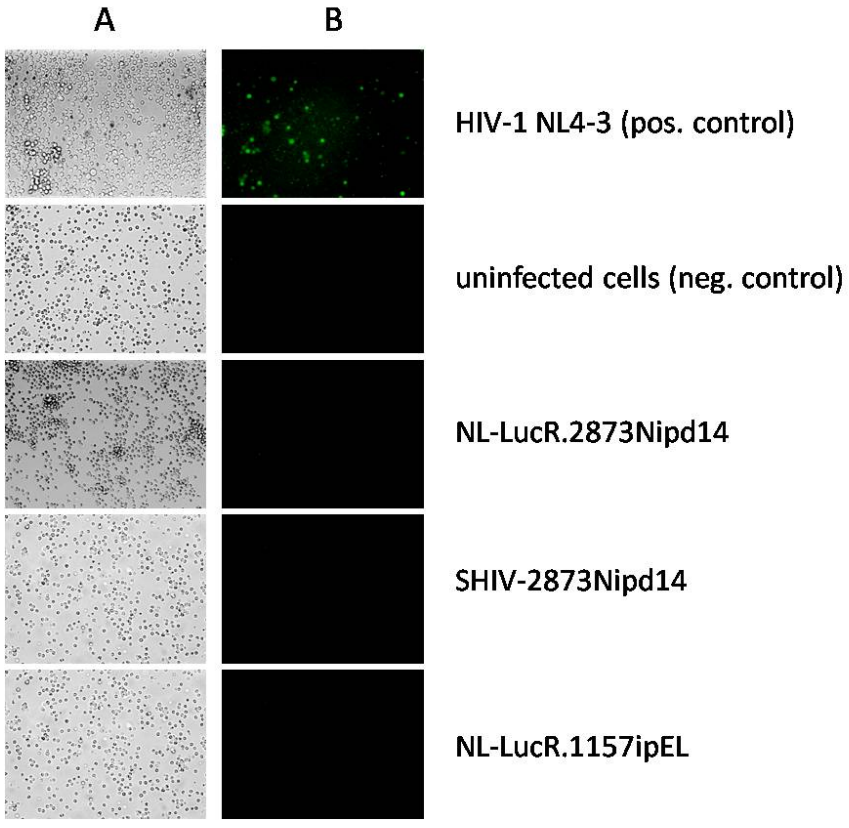

Figure 28 CEMx-174 GFP Co-receptor assay.

CXCR4 expressing CEMx-174 GFP cells incubated with cloned viruses, positive (HIV-1 NL4-3) and negative control (uninfected cells). Pictures taken with (A) light microscope and (B) fluorescence microscope. All viruses tested (except the pos.control) were not able to infect CEMx-174 GFP cells, indicating CCR5 tropism.

Co-receptor usage in U87.CD4

To check if the newly generated viruses use CCR5 as co-receptor, the U87.CD4 cell line was used. It originates from a human glioma cell line that has been engineered to express CD4 and CXCR4 or CCR5. Cells were infected and the supernatant was collected on day 0, 1, 3, 5, 7 and 10. In addition to NL-LucR.1157ipEL, NL-LucR.2873Nipd14 and SHIV-2873Nipd14 the parental LucR clones NL-LucR.1157ipd3N4 and NL-lucR.2873Ni were included in this co-receptor study. Using microtiter ELISA plates that have been coated with specific antibodies against the virus capsid protein, the virus amount present in the supernatant was quantified and displayed over time. The calculated p24/p27 values show that all tested viruses are exclusively using the CCR5 co-receptor (Fig.29 and Fig.30).

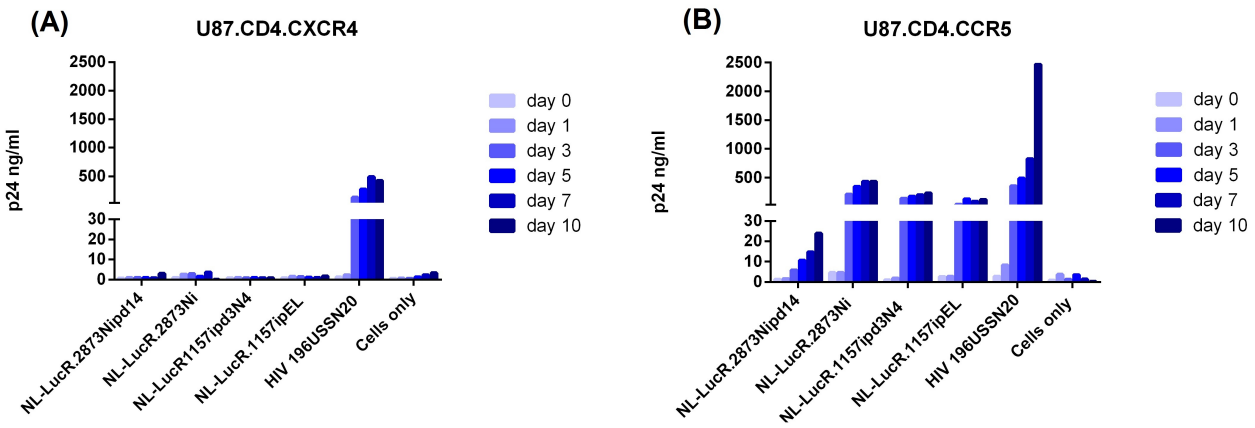

Figure 29 U87.CD4 Co-receptor test for reporter viruses.

(A) CXCR4 and (B) CCR5 expressing U87.CD4 cells incubated with NL-LucR.2873Nipd14, NL-2873Ni, NL-LucR.1157ipd3N4 NL-LucR.1157ipEL,HIV 196USSN20 (dual tropic pos control.) and cells only. Supernatant was harvested for 10 days and p24 determined susequently. All reporter viruses use the CCR5 co-receptor exclusively.

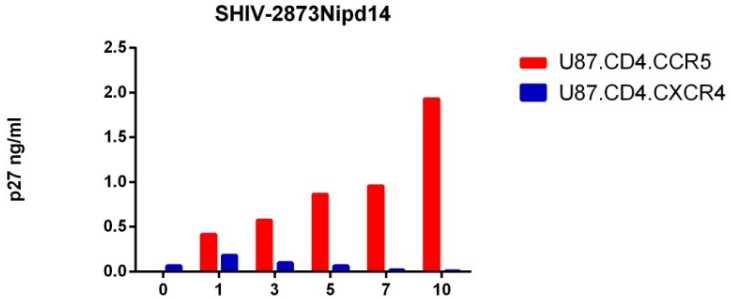

Figure 30 U87.CD4 Co-receptor test for SHIV-2873Nipd14.

CCR5 and CXCR4 expressing U87.CD4 cells incubated with SHIV-2873Nipd14. Supernatant was harvested for 10 days and p27 was determined subsequently. SHIV-2873Nipd uses the CCR5 co-receptor exclusively.

4.3.5. Neutralization Profiles of Reporter Viruses in A3R5 cells

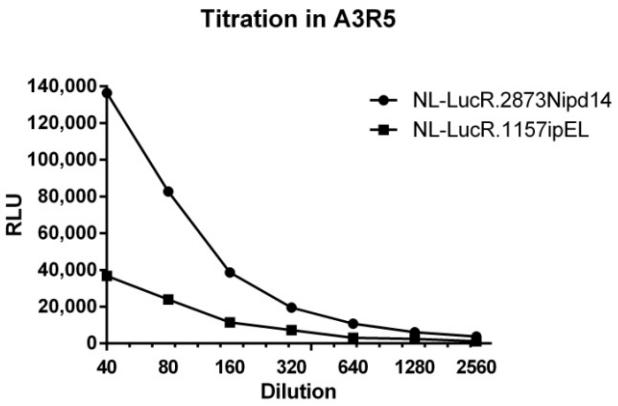

Figure 31 Titration of NL-LucR.2873Nipd14 and NL-LucR.1157ipEL in A3R5 cells.

To be able to perform the assay under properly standardized, optimized and validated conditions, an accurate titration of virus infectivity in the A3R5 cell line was done. This cell line is a derivative of the human lymphoblastoid cell line CEM, which naturally expresses CD4 and CXCR4, and was engineered to additionally express CCR5. A3R5 cells do not contain a reporter gene and therefore are suitable for infection with molecularly cloned viruses that carry a reporter gene like the *Renilla* luciferase. Expression of the reporter gene is induced by viral Tat protein soon after infection. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious virus particles present in the sample. Different virus dilutions were tested to determine the dilution that yielded approximately 50,000 RLU, which was then used for the actual assay (Fig.31).

To determine the neutralization susceptibility of NL-LucR.1157ipEL and NL-LucR.2873Nipd14 they were tested against a standard panel of monoclonal antibodies in the A3R5 cell line. For better comparison, the parental clones NL-LucR1157ipd3N4 and NL-LucR.2873Ni were also included in the assay and all were tested against a standard panel of Ab used in Prof. Ruprechts laboratory. This panel consisted of Fm6, VRC01, IgG1b12, 2F5, 4E10, HGN194, SHIVIG and naive IgG. IgG1b12 and VRCOI target the CD4 binding site, 2G12 recognizes conserved mannan residues on gp120, 2F5 and 4E10 recognize a coiled-coil region on gp41. HGN194 targets an epitope in the V3 loop and Fm6 is an anti-SARS mAb used as a negative control (CORTI and LANZAVECCHIA 2013; SUI et al. 2009).

SHIVIG is a polyclonal preparation of IgG isolated from rhesus monkeys, which were chronically infected with different clade C SHIV strains. It targets different proteins found in the HIV-1 envelope including parts of the V1 and V3 loops.

It is noteworthy that the 2G12 epitope has been found to be missing in many primary HIV-C isolates (LI et al. 2006) and that the epitope recognized by 2F5 was found to have residue substitutions in some of the SHIV-C viruses, thereby affecting its neutralization susceptibility (CAYABYAB et al. 2004).

NL-LucR.2873Ni and NL-LucR.2873Nipd14 were effectively neutralized by VRC01, IgG1b12 and 4E10. All other tested Abs (Fm6, 2G12, 2F5, HGN194, SHIVIG, naive IgG) did not demonstrate any neutralization. Surprisingly, SHIVIG and naive IgG displayed some enhancing effect of infection as shown by the negative neutralization percentage. In summary, NL-LucR.2873Nipd14 and its parental tier 2 clone NL-LucR.2873Ni had similar neutralization profiles (Fig.32), suggesting that further adaptation in RMs did not change the neutralization profile of this *env* to a more neutralization resistant tier 3 virus.

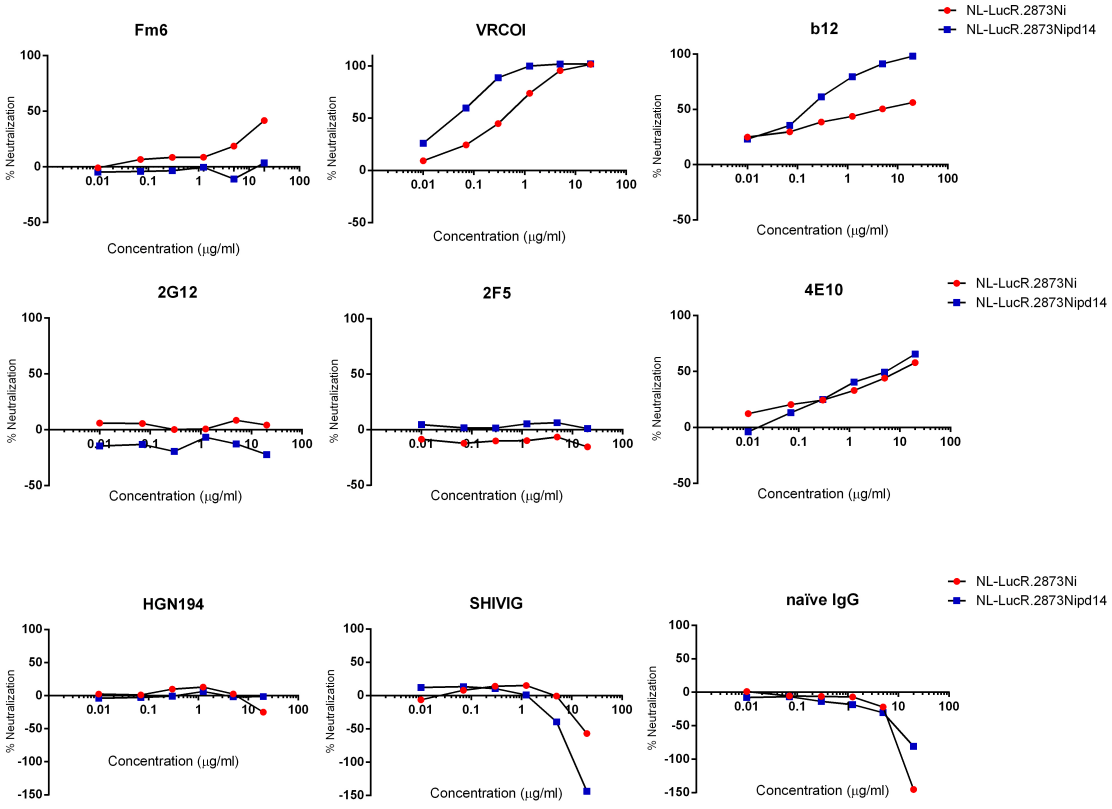

Figure 32 Neutralization profiles of NL-LucR.2873Ni and NL-LucR.2873Nipd14 in A3R5 against a panel of Ab.
VRC01,b12 and 4E10 were able to neutralize both viruses, whereas all other Ab tested displayed no efficacy.

NL-LucR.1157ipEL and NL-LucR.1157ipd3N4 were effectively neutralized by VRC01, IgG1b12 and 4E10. Additionally, NL-LucR.1157ipEL, containing the envelope from a tier 1 virus, was also neutralized by HGN194 and SHIVIG. Both viruses showed no neutralization sensitivity to Fm6, 2G12, 2F5 and naive IgG (Fig.33). These results suggest that the tier classification given to the SHIV derivatives (SHIV-1157ipEL tier 1 and SHIV-1157ipd3N4 tier 2) is identical to the tier classification of the reporter viruses.

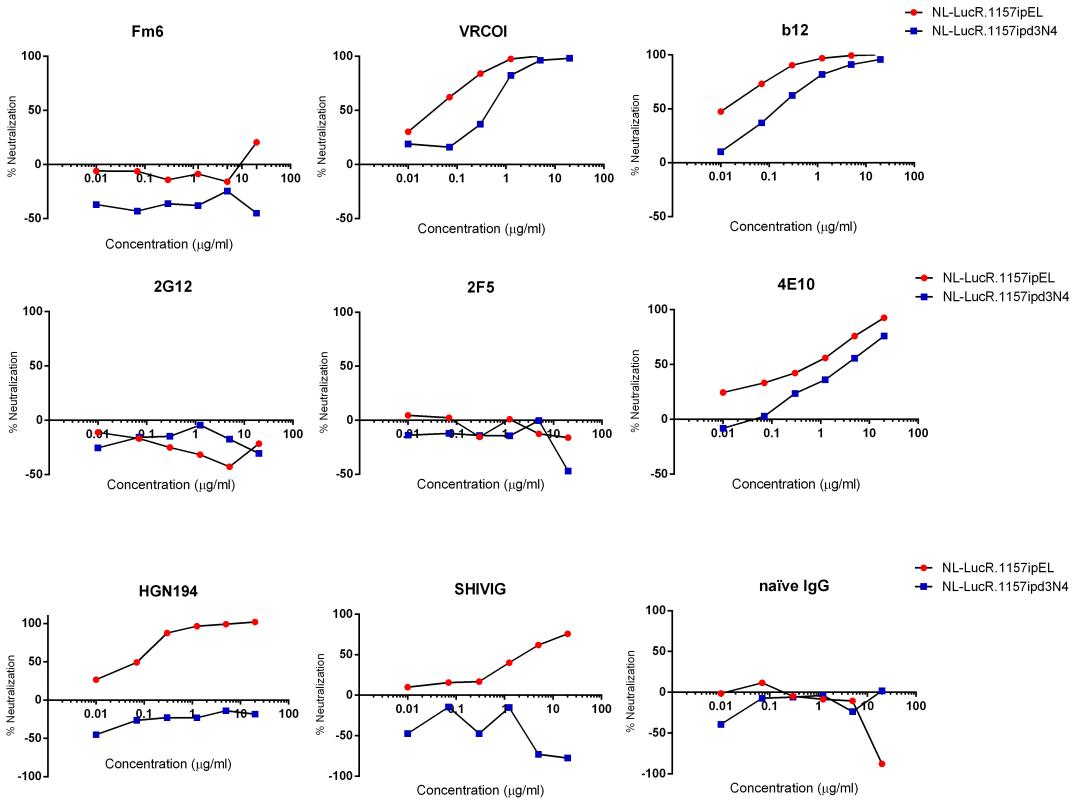

Figure 33 Neutralization profiles of NL-LucR.1157ipEL and NL-LucR.1157ipd3N4 in A3R5 against a panel of Ab.

VRCOI, b12 and 4E10 were able to neutralize both viruses, whereas HGN194 and SHIVIG showed efficacy only against NL-LucR.1157ipEL.

To further illustrate the neutralization efficiency of the antibodies tested, the half maximal inhibitory concentration (IC50) was determined. It is defined by the amount of antibody needed (µg/ml) to inhibit or neutralize 50% of the virus. In this study the lowest antibody dilution needed to neutralize 50% of the virus was defined as the IC50 value (Table 4).

	NL-lucR.2873Ni	NL-LucR.2873Nipd14	NL-LucR.1157ipEL	NL-LucR.1157ipd3N4
Antibody (µg/ml)	IC50	IC50	IC50	IC50
Fm6	>20	>20	>20	>20
VRCOI	0.3	0.07	0.07	1.25
b12	5	0.3	0.01	0.03
2G12	>20	>20	>20	>20
2F5	>20	>20	>20	>20
4E10	5	5	0.03	5
HGN194	>20	>20	0.07	>20
SHIVIG	>20	>20	5	>20
naive IgG	>20	>20	>20	>20

Table 4 IC50 values for all Reporter Viruses in A3R5.

4.4. Application of Generated Viruses

After the cloned viruses were fully characterized, their application was tested in relevant cell-based assays used in Prof. Ruprecht's group.

4.4.1. Using Reporter Viruses for C'ADE Assays

Recently, a study had been performed in Prof. Ruprecht's laboratory to test if passive immunization with polyclonal SHIVIG could protect RMs against repeated intrarectal challenges with SHIV-2873Nip. SHIVIG showed to neutralize SHIV-2873Nip in TZM-bl and hPBMC assays in vitro (Sholukh et al, 2013 submitted).

Interestingly, single-genome analysis revealed a higher number of transmitted variants in one of the tested groups compared to controls implying increased acquisition. To test these findings, assays were performed using two viruses containing envelopes closely related to the challenge virus, NL-LucR.2873Ni and NL-LucR.2873Nipd14.

These viruses were used to perform a complement mediated antibody-dependant enhancement (C'ADE) assay. This assay helps to determine enhancement of HIV

replication due to opsonization of the virus particle with complement and antibodies and complement receptors present on the target cells.

The human T-lymphoblast line SupT1.R5 expressing CD4, CCR5 and complement receptors were used as target cells. The C'ADE assays were performed based on the previously conducted virus titration (data not shown). Each virus was incubated with serial dilutions of SHIVIG or naive rhesus monkey IgG in the presence of normal or heat-inactivated serum for 1 h at 37°C.

Usually, the read-out of a C'ADE assay is to determine p27 concentration of the supernatant from the infected cells at defined time points. Infection of the human originating SuptT1.R5 cell line with SHIV-2873Nip did not work well and did not yield useful results (as tested by Dr. Anton Sholukh,DFCI previously). Hence the same assay was repeated with the HIV-based reporter viruses carrying the 2873 envelope in its early and adapted form. SHIVIG enhanced infection of both viruses, when complement in fresh normal serum was present. Infection caused by the "parental" NL-LucR.2873Ni virus was enhanced by almost 3-fold, while infection by "late" NL-LucR.2873Nipd14 was increased up to 16-fold. No significant enhancement was seen when the complement was destroyed through heat inactivation. IgG isolated from a naive, not infected RM did not show any enhancement.

To check if neutralization could be achieved in this assay, the neutralization-sensitive (tier 1) NL-LucR.1157ipEL that carries an envelope closely related to viruses against which SHIVIG had been raised was tested. SHIVIG completely neutralized NL-LucR.1157ipEL in the presence of either fresh or heat-inactivated complement.

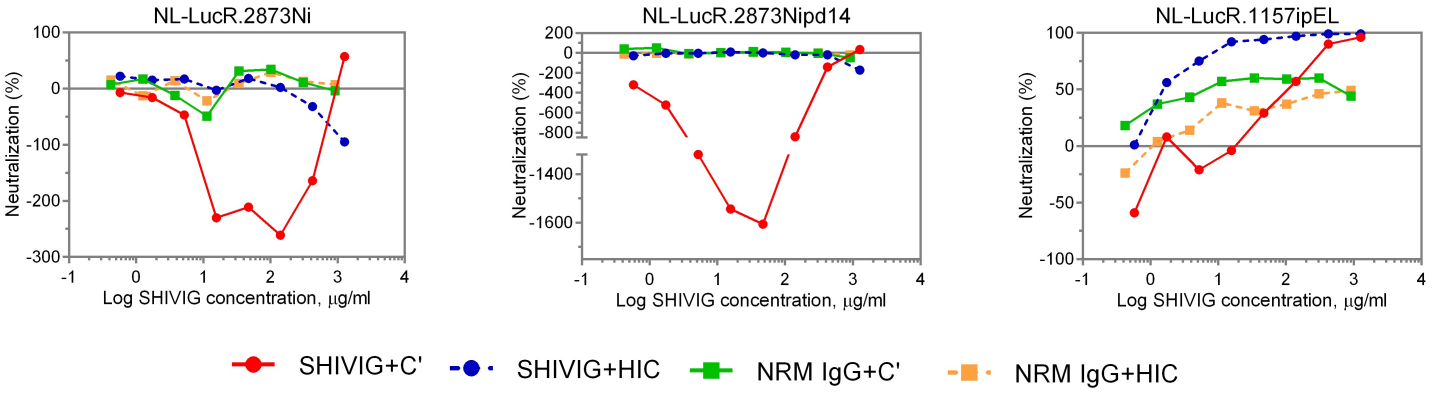

Figure 34 C'ADE Assay.

SuptT1.R5 cells infected with NL-LucR. 2873Ni, NL-LucR.2873Nipd14 and NL-LucR.1157ipEL using normal (C') and heat-inactivated (HIC) SHIVIG or NRM (normal) IgG. Virus neutralization indicated as positive percentage values, enhancement of infection indicated as negative percentage values.

4.4.2. Using Reporter Viruses for hPBMC Neutralization Assays

Before the actual assay was performed, the replication efficiency of all reporter viruses was tested in hPBMC. For this, hPBMC from one blood donor were isolated and a big cell stock was prepared. All following assays used the same donor cells to rule out inter-donor variability. Prior to the assay, human T-cells were activated by adding PHA and IL-2. Different virus dilutions were tested and the average RLU were determined after 4 days of incubation. As seen in Figure 35 all reporter viruses replicated well in hPBMC.

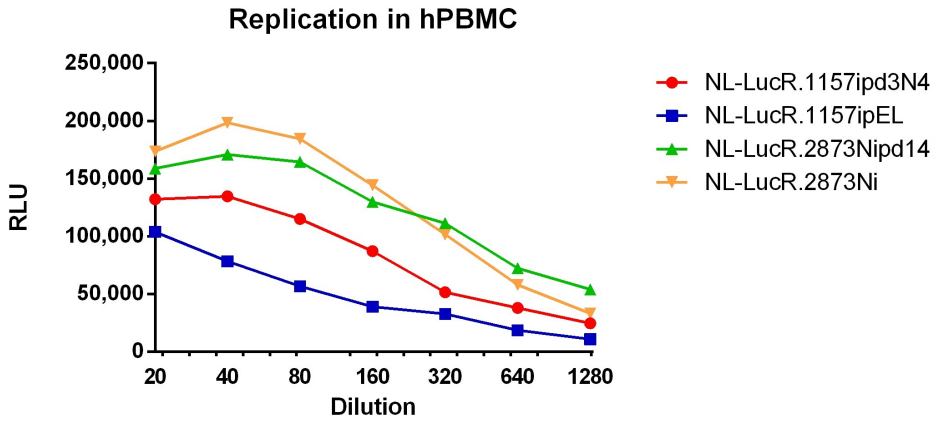

Figure 35 Reporter virus replication in human PBMC.

Different virus dilutions were incubated with hPBMC for 4 days.

To test the efficacy of the reporter viruses in an actual neutralization assay using primary cells, NL-LucR.2873Nipd14 was selected and tested against a panel of RM-sera in hPBMC.

These sera originate from a 2010 vaccine study that was performed in Prof. Ruprecht's laboratory and aimed to test whether vaccine-induced immune responses could protect RM against challenges with SHIV-2873Nip. Two recombinant Env immunogens, both heterologous to the challenge virus, were used to induce immune responses against Gag and Tat. Two strategies were used in this vaccine study, the first involved immunizing with recombinant proteins only (Group 1), whereas the second one involved immunizing by priming with synthetic peptides first, followed by boosting with

recombinant proteins (Group 2). One animal from Group 2 remained aviremic (ROb-12), even after repeated viral inoculations (unpublished data).

In this thesis, neutralization efficiency of the sera collected from Group 2 (8 animals) was assessed. The serum samples were provided by Dr. Samir Lakhashe (DFCI) and have been collected before and after immunization but before virus challenge. For the protected monkey, ROb-12, an additional serum sample (6 weeks after challenge) was included. It was seen that neutralization against the adapted NL-LucR.2873Nipd14 was not very efficient. Serum from ROb-12 reached less than 80% neutralization at the lowest dilution tested. With increasing dilution, all samples decreased dramatically in their neutralization efficiency. No significant difference between sera from protected and unprotected monkeys was seen and likewise, no significant difference could be shown between serum from ROb-12 after immunization and after challenge (Fig.36).

More experiments will be performed to elucidate the outcome of the study but for the sole purpose of testing the cloned reporter viruses in a neutralization assay using primary cells, it can be concluded that they are a reliable and highly sensitive way to facilitate standardized high throughput neutralization assays for future applications.

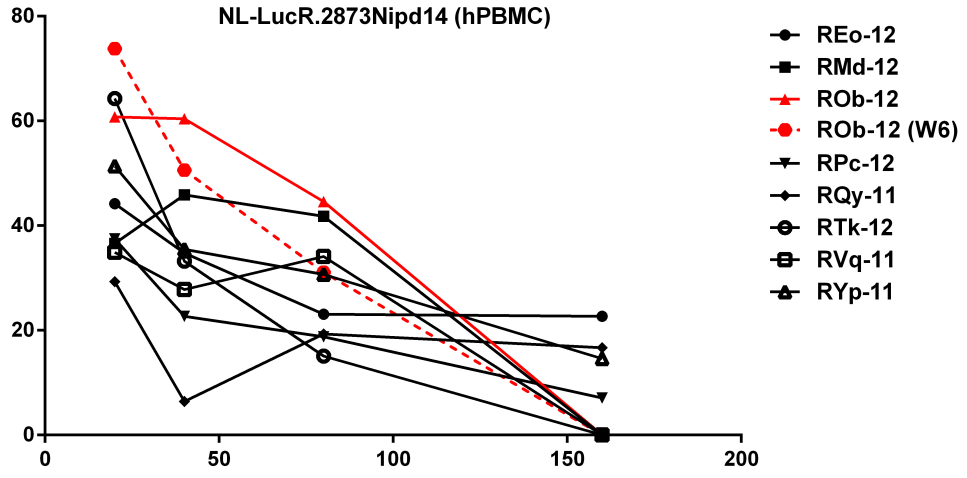

Figure 36 Neutralization Assay of NL-LucR.2873Nipd14 against panel of RM-sera in hPBMC.

4.4.3. Replication of SHIV-2873Nipd14 in random RM-PBMCs

One aim of this thesis was to generate an IMC of a SHIV-C that would replicate reliably in all randomly chosen test animals of the rhesus maquaces species. It is known that the ability of a SHIV to replicate in RM-PBMC in vitro is predictive of its ability to infect RM in vivo (SIDDAPPA et al. 2010). Eight RM were selected randomly as PBMC donors to evaluate the replication kinetics of the newly generated SHIV-2873Nipd14 IMC. Undiluted virus supernatant from 293T/17 transfected cells was used to inoculate the activated PBMC and the replication kinetics was assessed by p27 ELISA. SHIV-2873Nipd14 replicated in all donor PBMC except one (10PP047). However, the virus replicated with varying efficiency in the different donor cells. 10P025 and 10P065 showed higher replication while 10P010, 10P046 and 10P088 showed intermediate replication kinetics. The lowest replication was observed for 10P092 and 10P101 (Fig37).

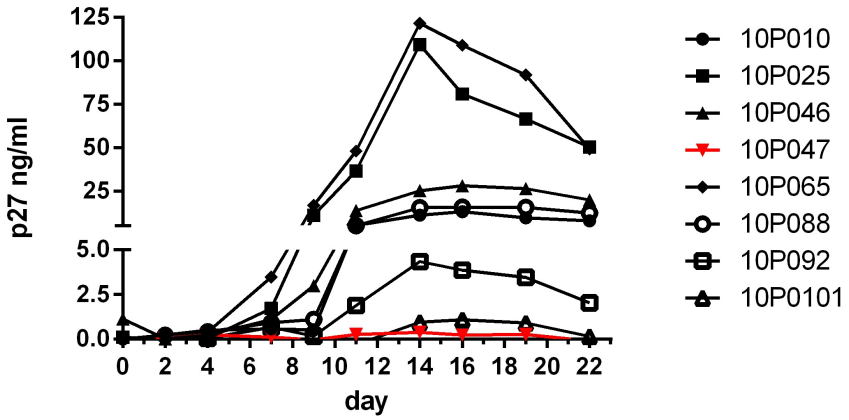

Figure 37 Replication of SHIV-2873Nipd14 in random RM-PBMC donors.

All except one donor (red line) supported replication in RM-PBMC.

5. Discussion

5.1. *Renilla* Luciferase Reporter Viruses carrying Relevant HIV-1 Clade C Envelopes

The focus of this study was the generation of two replication-competent reporter viruses expressing *Renilla* luciferase and carrying relevant HIV-1 Clade C envelopes. With the help of a shuttle vector, the envelope regions of SHIV-1157ipEL and the biological isolate SHIV-2873Nipd from clone Tb14 were cloned into the reporter HIV-1 proviral DNA backbone, pNL-LucR.T2A. In this study, the molecular cloning strategy as well as the virus characterization is described. Additionally, the application of the reporter viruses was tested in primary cell neutralization and complement mediated antibody-dependent enhancement (C'ADE) assays.

The resulting clones NL-LucR.1157ipEL and NL-LucR.2873Nipd14 (i) are replication competent, (ii) express Renilla luciferase (LucR) over multiple rounds of infection, (iii) offer a highly specific, sensitive and quantifiable endpoint allowing standardized high-throughput assays, (iv) use exclusively the CCR5 coreceptor (v) and enable the detection of (neutralizing) antibody responses in different primary and genetically engineered cell lines in different assay formats.

The replication competent pNL-LucR.T2A reporter virus was generated by Edmons et al. in 2010 and described a major improvement towards the establishment of standardized high-throughput neutralization assays that could be used for both, primary and engineered cell lines. Many efforts had been made in the past by introducing different new assay systems like the use of reporter cell lines (TZM-bl) or pseudovirions to detect neutralizing activity of antibodies against HIV-1, but both of them produced results that were not consistent with the data obtained from the PBMC-based neutralization assay. Most strikingly, in a study performed in 2004, the mAb 4E10 neutralized all viruses when tested in the pseudivirion assay but not in the PBMC assay. (BINLEY et al. 2004) This and other similar findings (BROWN et al. 2008; FENYO et al. 2009) stress the importance of using the biologically relevant PBMC assay. However,

this assay is more labor intensive and therefore not practical for high throughput and standardization (OCHSENBAUER and KAPPES 2009). With the generation of pNL-LucR.T2A, a strategy has been developed which made it possible to shuttle different HIV-1 envelopes into a replication-competent reporter gene expressing HIV-1 proviral backbone. By determining the expression of *Renilla* luciferase the assay enables a highly sensitive endpoint measurement that can be applied in different high throughput assay formats.

5.1.1. Cloning and Characterization of NL-LucR.2873Nipd14 and NL-LucR.1157ipEL

Following the cloning strategy the envelope regions of SHIV-2873Nipd14 and biological isolate SHIV-2873Nipd from clone Tb14 were inserted into pNL-LucR.T2A. Due to restriction site limitations, a direct swapping was not possible and the shuttle vector pBR322(NL4-3) had to be used to successfully clone the reporter viruses. A virus stock was produced for each virus clone and characterized.

The TCID50 measurement of each tested stock gave an insight of virus particles present and confirmed infectivity of the stock prepared. Since it is recommended to add dextran to achieve optimal levels of virus infection (MONTEFIORI 2005), TCID50 was measured with and without it to allow the comparison of virus infectivity. Not surprisingly, all virus incubations with dextran yielded much higher TCID50 than without it, demonstrating that dextran enhances virus binding to target cells to increase infectivity. The virus infectivity of a stock depends on various factors like characteristics of the virus envelope and transfection efficiency. When incubated without dextran, the infectivity of NL-LucR.2873Nipd14 was the highest, followed by NL-LucR.2873Ni, NL-LucR.1157ipEL and NL-LucR.1157ipd3N4. The lowest was measured in SHIV-2873Nipd14. These values gave an idea on the virus concentration to be used in future assays.

R5 SHIV infections induce a disease course that is more similar to that which occurs during human HIV-1 infections (which is almost always R5-tropic) than X4 SHIV infections. Therefore, the event of a co-receptor switch to X4 or shift to dual tropism is

undesirable (SINA et al. 2011). Co-receptor usage of both viruses was tested in the CEMx 174-GFP and U87.CD4 CXCR4/CCR5 cell lines. The parental clones NL-LucR.1157ipd3N4 and NL-LucR.2873Ni were included in this study and it was confirmed that all tested viruses exclusively use the CCR5 co-receptor. This data demonstrates that even with extended *in vivo* passage and adaptation (3 years until reisolation), no co-receptor switch was found in the 2873Nipd *env.*

Sequencing analysis confirmed that the envelopes (the KpnI-BamHI fragment) in NL-LucR.2873Nipd14 and biological isolate SHIV-2873Nipd from clone Tb14 posses the same sequence, indicating that no mutations occurred during the cloning process.

To get an idea about the neutralization sensitivity of the reporter viruses, they were tested in an A3R5 cell line based neutralization assay against a panel of monoclonal and polyclonal neutralizing antibodies. The neutralization profile of NL-LucR.2873Nipd14 was compared with its parental clone NL-LucR.2873Ni and except for the broadly neutralizing mAb VRC01 and b12, both reporter viruses displayed an almost identical neutralization pattern with similar IC50 values. Interestingly, the adapted NL-LucR.2873Nipd14 was neutralized better by VRC01 and b12, as determined by the lower IC50 values (VRC01 0.07 µg/ml, b12 0.3 µg/ml).

Subsequent alignment with the envelope regions of its parental clones (2873Ni and 2873Nip consensus sequence) showed how the envelope regions changed over the course of adaptation. Interestingly, the amount of deletions in the variable loops of gp120 increased drastically in the 2873Nipd14 *env* as compared to 2873Ni and were found close to the CD4 binding site (data not shown). The effects of these specific mutations still need to be investigated further, but others have postulated that the variable loop length is important for the determination of neutralization sensitivity. Especially the V1/V2 loops have been hypothesized to act as protective barriers around important antibody binding sites, masking their accessibility to neutralizing antibodies and therefore determining their neutralization profiles (LAIRD et al. 2008; PINTER et al.

2004).Therefore, it is possible that shorter loops might lead to an increase in neutralization sensitivity.

Adapted SHIV spent more time in the monkey host, allowing the virus to adapt under the positive selection pressure by the host immune system, favoring the emergence of neutralization escape variants (SMYTH et al. 2012). The finding that the adapted virus was easier to neutralize than its non adapted clone is rather unusual. However, this was not the first time this finding has been reported. A similar finding was observed in the SHIV SF162 model. The tier 2 SHIV SF162P3 (for passage 3) showed to be harder to neutralize (HAROUSE et al. 2001) than its direct progeny SHIV SF162P4 (for passage 4)(BARNETT et al. 2008).

Also, when thinking about HIV infection one needs to keep in mind that, although most infections are initiated from one single viral particle, the genetic diversity of HIV-1 quasispecies in an infected individual near end stage of disease are comparable to the global annual genetic variation of the influenza virus (KORBER et al. 2001). Therefore, the reisolated SHIV virus might not be representative for the overall virus population.

The neutralization profile of NL-LucR.1157ipEL and NL-LucR.1157ipd3N4 differed markedly. The neutralization sensitive NL-LucR.1157ipEL was effectively neutralized by the broadly neutralizing mAbs VRC01, b12 and 4E10. HGN194, isolated from an individual infected with an HIV-1 clade AG recombinant from neutralized the tier 1 virus as well. The polyclonal SHIVIG isolated from RMs infected with SHIV-1157ipd3N4 and other related 1157i clade C viruses, neutralized NL-LucR.1157ipEL to a lesser extent. The non-neutralization sensitive tier 2 virus, NL-LucR.1157ipd3N4 in contrast was hardly neutralized, except with VRC01, b12 and at higher concentrations also by 4E10. These findings are more in accordance with the general assumption that adapted primate immunodeficiency viruses are more pathogenic and therefore harder to neutralize (KIMATA et al. 1999).

5.1.2. Application in C'ADE Assays

For the C'ADE assay, different dilutions of the antibody SHIVIG were tested for neutralization efficacy in a passive immunization study against the challenge virus

(SHIV-2873Nip) related reporter viruses NL-LucR.2873Ni and NL-LucR.2873Nipd. This assay used the genetically engineered cell line SUPT1.R5 that expresses complement receptors in addition to the (co-) receptors needed for HIV infection. With this assay format, it was possible to detect complement-mediated enhancement of infection with low SHIVIG dosages for NL-LucR.2873Nipd14. Enhancement of infection was also observed for NL-LucR.2873Ni, but to a lower extent. Previous attempts to perform C'ADE assays with SHIV-2873Nip failed due to the inability of the SHIV to infect the human originating SUPT1.R5 cell line as measured by p27 ELISA. Attempts using dextran as an enhancement for infection resulted in unreasonably high p27 values that made it impossible to detect any enhancement that might be due to the complement receptors present on the target cells (experiments previously performed by Dr. Anton Sholukh,DFCI). The assay could be performed readily using the newly generated reporter viruses. They facilitate the C'ADE assay performance and read out tremendously by the simple endpoint measurement of RLU. It is no longer required to collect the supernatant of the infected cells for many days and conduct labor-intensive ELISAs afterwards, thereby saving time and money.

To combat host responses viruses have developed mechanisms to control and take advantage of the complement system. The complement regulatory protein CD59, expressed on the mammalian cell surface, is known to be incorporated by HIV-1 during budding and is used by the virus to evade the complement attack (BERNET et al. 2003). Additionally it has been reported earlier that non-neutralizing antibodies display the potential to inhibit or enhance infection through interactions with complement and/or Fc receptors. Inactivation through opsonisation and lysis of the virion by the complement have been reported, yet when complement receptors (especially CR2) are present on the target cell, antibodies and complement together can enhance viral infectivity (WILLEY et al. 2011). This mechanism can be triggered through different ways. It has been reported that non neutralizing antibodies bound to the viral surface can activate complement or bind directly to Fc receptors on the target cell (WILLEY and AASA-CHAPMAN 2008). Also, it was shown that viral surface-bound antibodies amplify complement activation and the deposition of complement fragments on the virion

envelope (SPEAR et al. 1993). Additionally, it has been reported that HIV can activate complement in the absence of antibodies through direct interaction of the envelope protein and the complement system (SUSAL et al. 1996). In any case, it has been postulated that the mechanism behind enhancement of infection most likely occurs because of an increased physical attachment between the virus and the target cell leading to enhanced entry (LUND et al. 1995; ROBINSON 2006).

In this study, the finding that the reporter viruses NL-LucR.2873Ni and NL-LucR.2873Nipd14 enhanced infection at low SHIVIG concentrations was rather surprising but it might explain the increased virus acquisition in one of the tested animal groups found. At higher SHIVIG concentrations zero or minimal neutralization was observed. This data suggests a dual role for polyclonal anti-HIV-1 envelope antibodies depending on plasma levels upon virus encounter (Sholukh et al, submitted 2013). The potential role of C'ADE definitely needs to be considered more carefully when aiming for future HIV-1 vaccine research. The impending questions whether these enhancing effects can be overcome or if they can be separated from protective neutralizing antibody responses remain to be answered.

5.1.3. Application in hPBMC Neutralization Assays

The application of the reporter viruses in a neutralization assay using primary cells was tested successfully. Before the generation of Renilla luciferase carrying reporter viruses, similar to the C'ADE assay, neutralization was assessed using labor intensive p27 ELISAs.

In this study, RM sera collected after a passive immunization study performed in 2010 was used and tested for its efficacy to neutralize against the challenge virus (SHIV-2873Nip) related NL-LucR.2873Nipd14.

One of the eight sera tested came from a completely protected RM (ROb-12) (unpublished data). In this study, ROb-12 serum even showed a fair amount of protection against the challenge virus progeny NL-LucR.2873Nipd14. The other sera originating from non-protected monkeys from the same group displayed no significant protection. The outcome of this study needs further evaluation, but from this experiment

it can be concluded that the reporter viruses generated work well in neutralization assays using hPBMC.

5.2. R5-tropic SHIV-C Construct carrying an HIV-1 Envelope from an Zambian infant

Here in this study the generation of the infectious molecular clone SHIV-2873Nipd14 is described. This virus has a number of very desirable characteristics: (i) it possesses an HIV-C env originating form a pediatric rapid progressor, (ii) the envelope was cloned into the backbone of SHIV-1157ipd3N4 and contains an extra NF-kB site, (iii) SHIV-2873Nipd14 exclusively uses the CCR5 co-receptor (iv) and replicates in randomly selected rhesus-monkey PBMC donors.

SHIV-2873Nipd14 originates from its parental clone SHIV-2873Ni that carries the envelope gene of a Zambian infant that was born to an HIV-1 clade C infected mother, the most prevalent strain in the world. 2 months after birth, the HIV-C envelope 2873 was isolated and cloned into the backbone of SHIV-1157ipd3N4 (SIDDAPPA et al. 2009).

After the passage of SHIV-2873Ni through five RM, the now passaged biological isolate SHIV-2873Nip was used to intra-rectally infect another set of six monkeys to find the viral dose leading to systemic infection (SIDDAPPA et al. 2009). From this experiment, monkey RTb-11 developed AIDS and an opportunistic infection (Cryptosporidiosis). Its PBMC were used to re-isolate the now adapted envelope, which was cloned into the backbone of SHIV-1157ipd3N4 giving rise to SHIV-2873Nipd14.

Other SHIV strains encoding for HIV-1 clade C have been constructed but most of them failed to mimic “real life” HIV-1 conditions by being dual- or X4-tropic, not being mucosally transmissible or not being able to replicate in RM PBMC (HATZIIOANNOU and EVANS 2012).

SHIV-2873Nipd14, in contrast, directly originates from the mucosally transmissible virus SHIV-2873Nip and is exclusively R5-tropic, as demonstrated by the co-receptor assays in CEMx 174-GFP and U87.CD4 cell lines. Additionally, it replicated in all but one tested RM PBMC donor. However, the replication efficiency displayed was diverse ranging

from very little (RM10P101) to extremely effective (RM10P010 & RM10P065). There are different factors that could lead to such a pattern. It is possible that the donor PBMCs in which virus replication was not supported expressed the resistant TRIM5α phenotype. The TRIM5α protein is one of the host replication factors against HIV. It acts after virus entry into the cell by recognizing the retroviral capsid, disrupting the order of virus uncoating and subsequently blocking transcriptase activity (MALIM and BIENIASZ 2012). Genetic background analysis of the donor RMs has not been performed and therefore might be useful for the future to explain such outcomes. Additionally, the cells used for the assay were stored for more than one year in liquid nitrogen and the donor PBMC that did not show high replication might have encountered either suboptimal freezing/thawing conditions or suboptimal blood processing conditions (like long shipping or PBMC isolation periods)(BULL et al. 2007).

For this virus to be used as a model in non-human primate vaccine studies, a homogenous replication pattern is desired for all test animals that would receive the virus. Therefore, further testing of additional donors is required. If a similar pattern is seen with additional donors, a different clone could be tested or the virus might need further *in vivo* adaptation in the monkey host to reach its full pathogenicity.

5.3. Outlook

5.3.1. The potential of *Renilla* luciferase expressing HIV-1 Reporter Viruses

With the generation of replication competent HIV-1 viruses carrying the reporter gene *Renilla* luciferase, a significant improvement towards assessing neutralizing antibodies triggered by HIV-1 vaccine immunogens was achieved. The application of these viruses is very diverse, allowing them to be used in different cell and assay formats. With the detection of luminescence, emitted by the viruses themselves, a reliable as well as labor and time saving method has been established to detect viral presence. The possibility of using the pNL-LucR.T2A backbone to shuttle different HIV-1 envelopes allows for the convenient construction of a virus reference panel carrying the desired envelopes. In Prof. Ruprecht's laboratory reporter viruses have been constructed carrying the most

relevant HIV-1 envelopes from previous SHIV research (1157ipd3N4, 1157ipEL, 2873Ni and 2873Nipd14).

The application of *Renilla* luciferase expressing HIV-1 reporter viruses will especially facilitate the routine application of standardized neutralization assays in the assessment of sera from human or rhesus monkey HIV-vaccine trials.

5.3.2. Using the SHIV model to test HIV-vaccine efficacy

Replication-competent, R5-tropic mucosally transmissible tier 2 clade C carrying SHIVs are highly desired. The molecular cloning strategy of shuttling the envelope of recently transmitted HIV-1 isolates into the modified SHIV-1157ipd3N4 backbone, allows the generation of biological relevant clade C carrying SHIVs. They are useful tools to study viral pathogenesis *in vivo* and in particular, allow the evaluation of Env-specific vaccines and drugs in non-human primate models.

6. Zusammenfassung

HIV, der Krankheitserreger von AIDS, bleibt auch mehr als 30 Jahre nach seiner Entdeckung, verantwortlich für die Infektion und HIV/AIDS bedingter Todesfälle vieler Millionen Menschen weltweit. Da bis jetzt keine Heilung vorhanden ist und die Anzahl an resistenten Viren gegenüber anti-retroviraler Medikamente immer weiter ansteigt, konzentriert sich die derzeitige Forschung auf die Entwicklung einer Schutzimpfung. Um dieses Ziel zu erreichen wird allgemein angenommen, dass neben einer protektiven zellulären Immunantwort, auch eine breite neutralisierende Antikörperantwort benötigt wird. Allerdings sind Testsysteme notwendig, die eine bessere Beurteilung neutralisierender Antikörperaktivität erlauben. Diese Arbeit beschreibt die Klonierung von zwei replikations-kompetenten Reporterviren basierend auf dem NL-LucR.T2A Plasmid. Diese Viren exprimieren das Reportergen *Renilla* luciferase und besitzen relevante Subtyp C Glykoproteinhüllen, die von relativ kürzlich übertragen HIV-1 Isolaten stammen (NL-LucR.1157ipEL und NL-LucR.2873Nipd14). Die geklonten Viren wurden charakterisiert und ihre Anwendung in verschiedenen Analyseverfahren getestet. Diese Reporterviren werden insbesondere die routinemäßige Anwendung von standardisierten Neutralisationstests bei der Beurteilung von Seren (humanen oder rhesusaffen-artigen Ursprungs) aus Impfstoffstudien stark vereinfachen.

Als ein zweites Ziel, wurde ein infektiöser molekularer Klon aus einem isolierten Rhesusaffen-adaptierten Simian-Human Immunodeficiency Virus (SHIV) generiert. SHIVs sind Hybridviren, welche von SIVs abstammen und so modifiziert wurden, dass sie HIV-1 Glykoproteinhüllen exprimieren. Sie ermöglichen die Evaluierung von Medikamenten und Impfstoffen, die gegen diese Glykoproteine gerichtet sind in nichtmenschlichen Primaten und sind damit wertvolle *in vivo* Modelle in der HIV Forschung. Diese Arbeit beschreibt die erfolgreiche Klonierung eines CCR5 Co-Rezeptor verwendenden und Glykoproteinhüllen Subtyp C tragenden SHIV (SHIV-2873Nipd14). Außerdem wurde der Klon charakterisiert und dessen Replikationsfitness in verschiedenen Rhesusaffen PBMC getestet.

7. Summary

HIV, the causative agent of AIDS, remains to be responsible for the infection and death of millions of people all over the world, more than 30 years after its discovery. A cure remains elusive and with increasing resistance of the virus to anti-retroviral drugs, research has been focused on the development of a protective vaccine. To achieve this goal, it is believed that a broadly neutralizing antibody response is required in addition to protective cellular immunity. However, assay systems that allow better assessment of the neutralizing antibody activity are needed. This thesis describes the generation of two replication competent reporter viruses based on NL-LucR.T2A. These viruses express the reporter gene *Renilla* luciferase and carry relevant Clade C envelopes originating from relatively recently transmitted HIV-1 isolates. The cloned viruses were characterized and their application in different assay system was tested. This approach will especially facilitate the routine application of standardized neutralization assays in the assessment of sera from human or rhesus monkey HIV-vaccine trials.

A second aim was to generate an infectious molecular clone of a monkey adapted Simian-Human Immunodeficiency Virus (SHIV) re-isolated from its host. SHIVs are hybrid viruses, which originate from SIVs that are engineered to express HIV-1 envelope glycoproteins. They allow the testing of HIV-1 envelope specific vaccines and drugs in non-human primates and are therefore valuable in vivo models in HIV research. In this thesis, a R5-tropic virus carrying an infectious Clade C *env* (SHIV-2873Nipd14) was generated and characterized. Additionally, its replication fitness in random rhesus monkey (RM) peripheral blood mononuclear cells (PBMC) was tested.

8. Literature

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