VetCore-Facility for Research

University of Veterinary Medicine Vienna

Head: Ao. Univ.-Prof. Dr. med. vet. Dieter Klein

and

Department of Virology and Immunology

Texas Biomedical Research Institute, San Antonio, Texas

Adaptation of a Novel Simian-tropic HIV-1 Clade C Progeny Virus

BACHELOR'S THESIS

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Christopher M. Veeck

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Internal Supervisor:
Ao. Univ. Prof. Dr. med. vet. Dieter Klein
Head of VetCore – Facility for Research
University of Veterinary Medicine
Vienna, Austria

External Supervisor:
Ruth M. Ruprecht, M.D., Ph.D.
Scientist, Department of Virology and Immunology
Infectious Disease Unit Leader
Southwest National Primate Research Center
Director of Texas Biomed AIDS Research Program
San Antonio, Texas, USA

Reviewer:
Univ. Prof. Dr. med.vet. Till Rümenapf
Head of Institute of Virology
University of Veterinary Medicine
Vienna, Austria

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1. INTRODUCTION

1.1. The Spread of HIV

Even 35 years after its discovery in humans, HIV is still one of the most dangerous pathogens on earth. Currently, more than 36 million people are living with HIV infection, and the numbers are rising. In 2016, 21 million carriers were treated with antiretroviral therapy (ART). Still each year, approximately one million people die of acquired immunodeficiency syndrome (AIDS)-related complications. Every year, 1.6 to 2.1 million people become newly infected with HIV, which makes the pathogen one of the most infectious viruses of all time. More than three decades after the discovery of HIV, we are still looking for a vaccine (World Health Organisation, 2016).

The first case of a person infected with HIV was documented in 1968 (Garry et al., 1988). By this time, nobody knew what HIV was, where it came from, or how dangerous it was going to become. Today we know that HIV made a zoonotic jump from chimpanzees, Western gorillas and Sooty mangabeys (Sharp et al., 2011). The pandemic officially started in 1981, when the Center of Disease Control and Prevention (CDC) reported an incidence of five previously healthy homosexual men suffering from *Pneumocystis carinii* pneumonia, short PCP (CDC, 1981). As PCP is an opportunistic infection associated with cellular immune deficiency, this discovery led to intense research on the cause of this incidence. In 1983, Sinoussi and colleagues identified a retrovirus, the human immunodeficiency virus type 1, as the causative agent of AIDS (Barre-Sinoussi et al., 1983). Since then, more than 76 million people have become infected and more than 35 million people have died from AIDS-related illnesses (UNAIDS 2017).

Although HIV infection is still one of the most dangerous diseases on earth, it is not a death sentence anymore. Improvements in treatment and medication enable low viremia and inhibit progression to AIDS for years. The number of AIDS-related deaths has decreased over time. In 1999, the first breakthrough in HIV research was documented. The ‘Berlin Patient’, Timothy Ray Brown, was cured from HIV, and he has remained off ART (Lisziewicz et al., 1999).

Much effort has been put into the development of a protective vaccine. One of the most famous studies was the clinical trial RV144. In this experiment 16,000 Thai volunteers have received either a placebo or a vaccine combination of two reagents, ALVAC and AIDSVAX, achieving an overall protection of 31.2% (Rerks-Ngarm et al., 2009).

HIV-1 clade C is the most prevalent form of HIV-1 infections all over the world and mostly appears in Sub-Saharan Africa, Southern Africa and India. Although nearly 50% of all HIV-1-infected people carry a clade C virus, research on this virus is not as advanced as for clade B. The latter is mainly responsible for infections in the western world, such as Europe, the Americas and Australia. Most of the initial worldwide research was performed HIV-1 clade B. Figure 1 depicts the worldwide distribution of all HIV-1 clades.

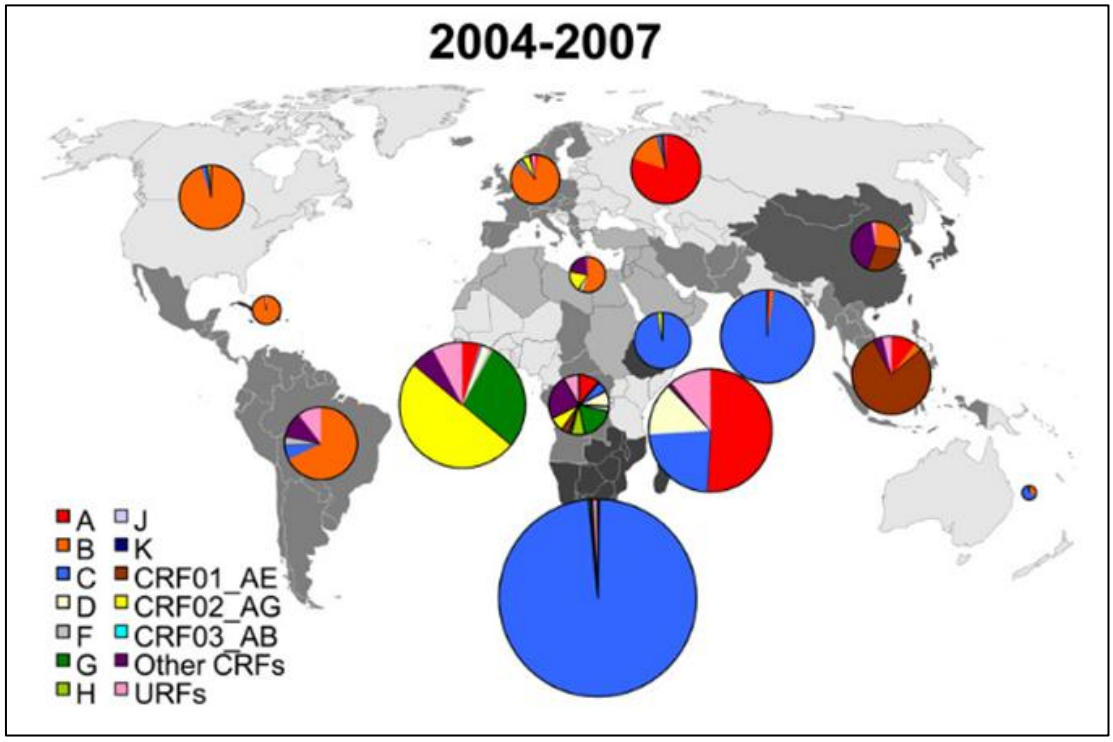


Fig. 1. Prevalence of HIV-1 subtypes (Hemelaar et al., 2011). HIV-1 clade C is the most prevalent form of the epidemic. All subtypes of the graph belong to HIV-1 group M, which is the group responsible for the worldwide epidemic. Clade B is the subtype with the highest prevalence in Europe and the Americas.

Overall, 0.8% of the human population carries HIV-1. Approximately 30% do not know their status. Over 19 million carriers, which are 44% of all HIV-1-infected individuals, live in Southern or East Africa. Only 2.1 million, which sums up to not more than 5.7%, live in Central and Western Europe and North America.

1.2. The Human Immunodeficiency Virus (HIV)

HIV is a retrovirus (Group VI in the Baltimore Classification) in the subfamily of *Orthoretrovirinae* and belongs to the genus of lentiviruses as depicted in Figure 2. HIV is an enveloped virus, composed of two copies of positive-sense, non-covalently linked, single-stranded RNAs that are embedded in a nucleocapsid.

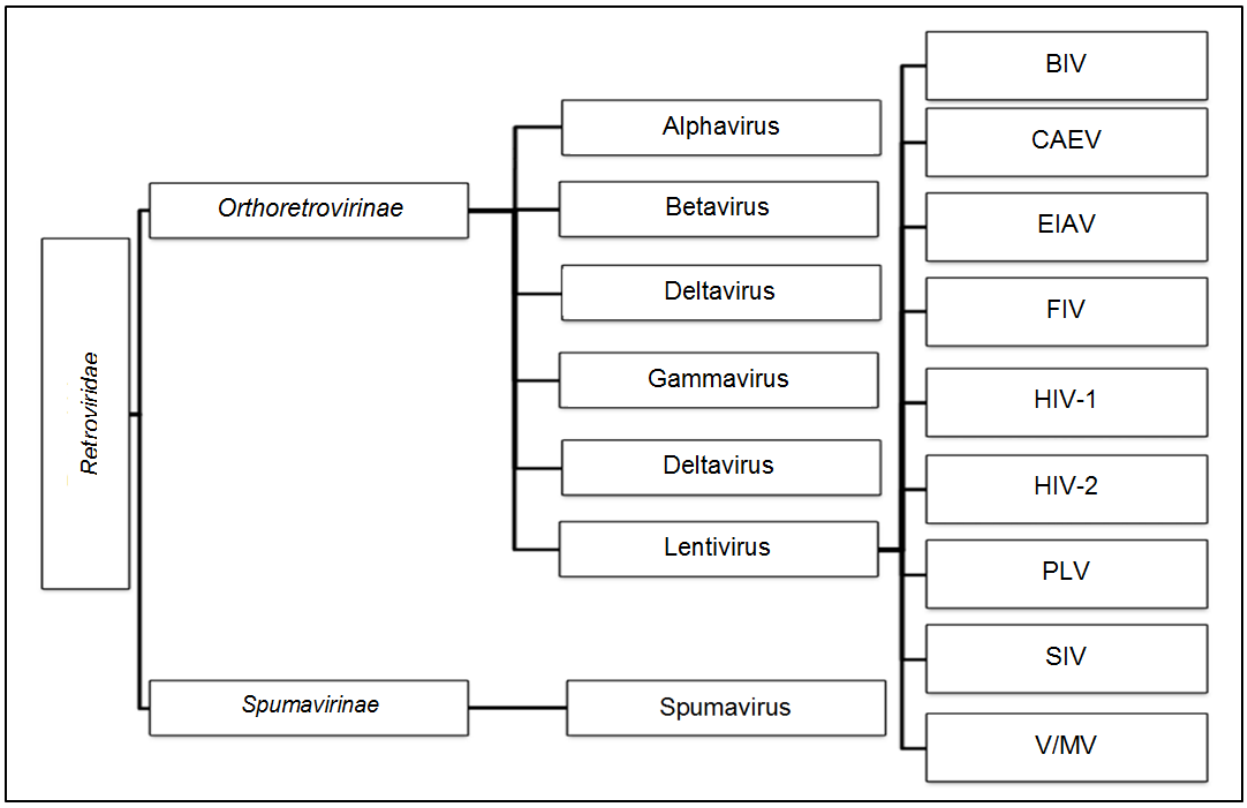


Fig. 2. Taxonomy of Retroviridae. BIV, bovine immunodeficiency virus; CAEV, caprine arthritis encephalitis virus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; PLV, puma lentivirus; SIV, simian immunodeficiency virus; V/MV, Visna/Maedi virus.

HIV is divided into two types, HIV-1 and HIV-2, which share only 40-50% of their genome. HIV-1 can be subdivided into four groups – M, N, O and P. Group M is the most important group as it represents the pandemic form of HIV-1 (Figure 3, red). This group can further be subdivided into several clades – termed A to K and circulating recombinant forms (CRFs; Buonaguro, Tornesello & Buonaguro, 2007). Currently, clade C is the most prevalent form of HIV-1 worldwide, mostly found in Sub-Saharan Africa, India and some parts of China. This thesis will focus on HIV-1.

HIV-2 is the less pathogenic than HIV-1 and mostly appears in Western Africa. We distinguish between several HIV-2 genetic subtypes – A through H (Hemelaar, 2012) (Figure 3, turquoise). These subtypes show a genetic difference of 15-20% (Robertson et al., 2000).

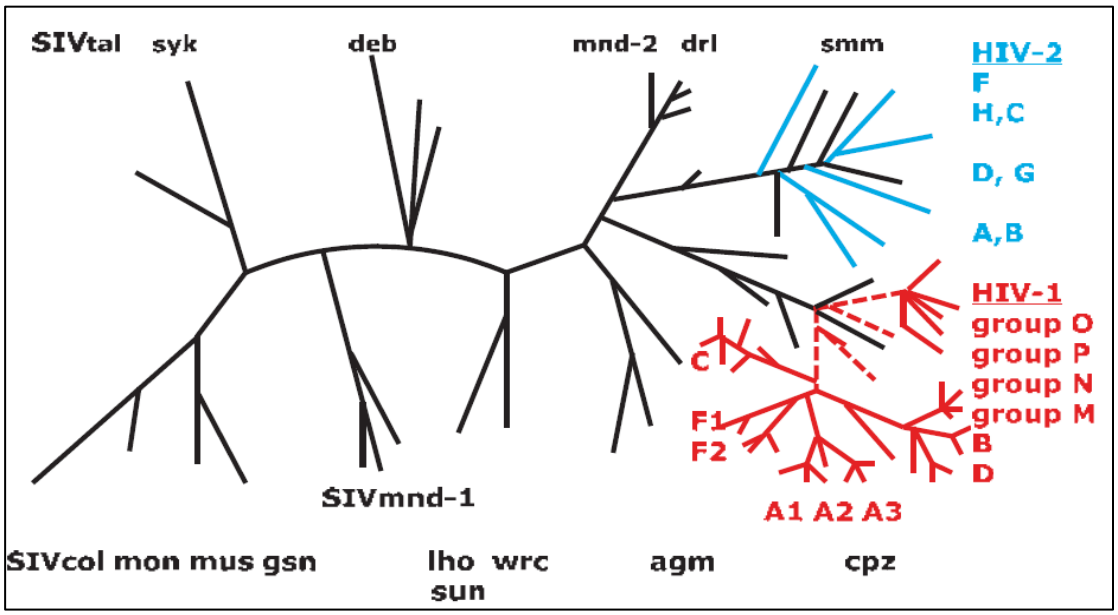


Fig. 3. Phylogenetic Tree of Human and Simian Lentiviruses (German Advisory Committee Blood, 2016). The graph shows that the origin of HIV-1 was a zoonotic jump from SIVcpz, which is SIV that can be found in chimpanzees. This is another explanation why chimpanzees are the only non-human primates (NHPs) that can be infected with HIV (more in chapter 1.4.).

Top row: SIVtal, SIV in northern talapoins; SIVsyk, SIV in Sykes monkeys; SIVdeb, SIV in de brazza monkeys; SIVmnd-2, SIV in mandrills type 2; SIVdrl, SIV in drill monkeys; SIVsmm, SIV in sooty mangabeys

Bottom row: SIVcol, SIV in guereza colobus monkeys; SIVmon, SIV in mona monkeys; SIVmus, SIV in mustached monkeys; SIVgsn, SIV in spot-nosed monkeys; SIVmnd-1, SIV in mandrills type 1; SIVlho, SIV in L'Hoest's monkeys; SIVsun, SIV in sun-tailed monkeys; SIVwrc, SIV in western red colobus monkeys; SIVagm, SIV in African green monkeys; SIVcpz, SIV in chimpanzees

1.3. Structure and Genome

The HIV particle has a diameter of 100-120 nm and is surrounded by a lipoprotein-rich envelope. The latter contains trimeric spikes composed of the glycoprotein – gp120 (surface glycoprotein). These spikes are attached to gp41 (transmembrane glycoprotein). Both glycoproteins are important antibody targets for vaccine development. Furthermore, the envelope comprises host cell proteins, such as HLA-Class I and HLA-Class II proteins. The matrix proteins (p17/MA) are attached to the membrane underneath the envelope. Inside, the conical nucleocapsid (p24/CA) is anchored to the membrane and encloses the nucleoproteins, two RNA copies and several enzymes that are essential for the viral replication. These enzymes include reverse transcriptase (p51/RT), integrase (p32/Int), and protease (p12/Pro). Figure 4 represents a simplified version of the genetic and physical structure of HIV-1.

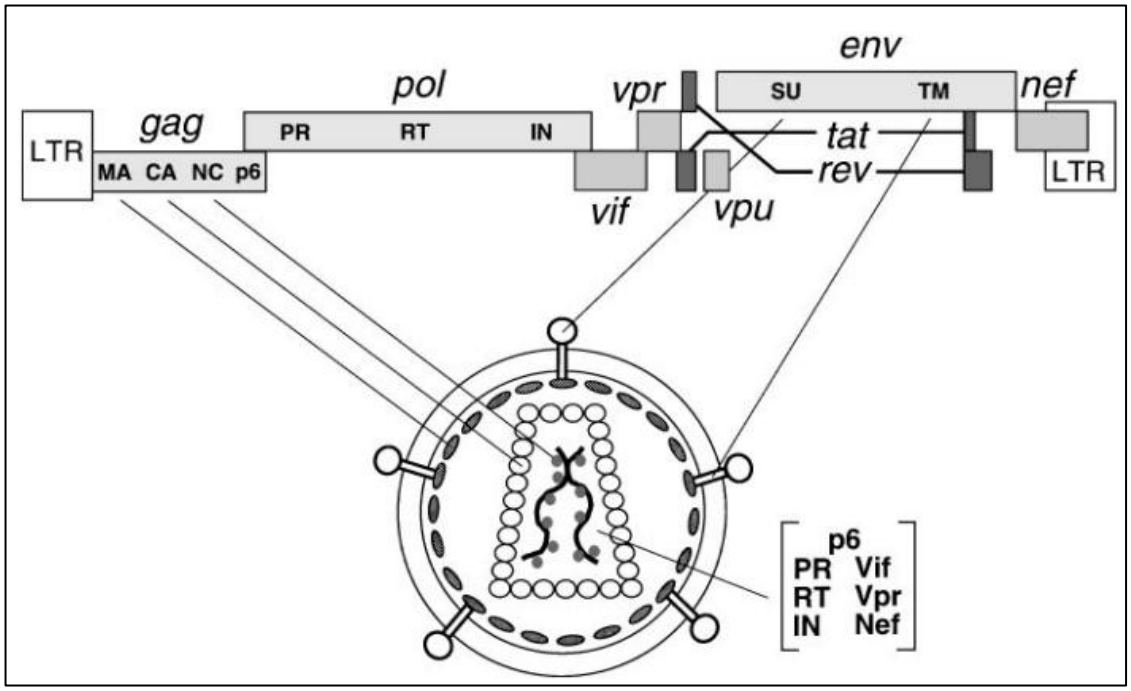


Fig. 4. Organization of the HIV-1 Genome (German Advisory Committee Blood, 2016). The HIV-1 genome is composed of three major gene groups, encoding for large polyproteins, flanked by two long terminal repeats (LTRs) and multiple accessory genes in between.

Abbreviations: MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface glycoprotein; TM, transmembrane glycoprotein

The HIV-1 genome consists of nine genes, flanked by long terminal repeats (LTRs). The three largest genes are termed *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope). The *gag* gene encodes structural proteins, mainly core proteins, which are specific for the group to which the virus belongs. The *pol* gene contains the genetic information for functional enzymes that are crucial for viral replication, including reverse transcriptase (RT), which is needed to turn viral RNA into DNA, integrase, which integrates the viral information into the host cell DNA, and protease, an enzyme that cleaves the large precursor proteins into the final compounds. The *env* gene encodes the large glycoprotein gp160, which is cleaved into the envelope glycoproteins gp120 and gp41. Furthermore, six additional genes are part of the HIV-1 genome that are involved into regulatory and survival processes of the viral life cycle. The *tat* gene is crucial for the viral replication as it encodes the Tat protein (transactivator of transcription), which is expressed very early after the integration of the viral genome and promotes the transcription all HIV-1 genes. Rev (regulator of expression of the virion), encoded by *rev*, ensures that viral mRNA is able to leave the nucleus can be processed into the cytoplasm. Vpr (viral protein R), encoded by *vpr*, enables the virus to arrest the host cell in its life cycle. Vpu (viral protein U), is encoded by the *vpu* gene, makes sure that the virus is released properly. The v*if* gene encodes for Vif (viral infectivity factor), which is a protein that increases the progeny viral particles' infectivity. Lastly, *nef* encodes the protein Nef (negative actor), an important accessory gene. Nef has several functions, including the down regulation of CD4 receptors (Garcia & Miller, 1991) on the host-cell surface, allowing budding of the virus during late stages of the viral replication and cellular signal transduction (reviewed in Basmaciogullari & Pizzato, 2014).

1.4. Animal Models in HIV Research

One of the biggest challenges in HIV-1 research is the lack of an animal model that fits all the requirements to represent HIV-1 infection in humans. Looking for an appropriate model for HIV-1 infection, scientists would immediately think about mice or rats. Unfortunately, attempts to establish HIV-1 replication in mice, rats, or rabbits were unsuccessful. Although cats are hosts for the feline immunodeficiency virus (FIV; Hartmann, 2012), which is a relative to HIV-1, cats did not support HIV-1 replication. In addition, FIV infection of cats significantly differs from HIV-1 infection of humans. Therefore, efforts were made to establish a model among non-human primates (NHPs). Although there are more than 40 different SIV strains that are endemically infecting African primate species, efforts to develop AIDS disease model in NHPs remained unsuccessful for a long time. This can be explained by the coexistence of virus and host over centuries, making African monkeys and apes resistant to SIV/HIV-1 disease progression (Klatt,

Silvestri and Hirsch, 2012). This theory can be vindicated by the fact that SIV causes severe AIDS, including $CD4^+$ T-cell depletion, high viral loads and opportunistic infections in rhesus macaques (*Macaca mulatta*, RM) of Indian origin (Marthas et al., 2003) – and to a lesser degree – in Chinese-origin rhesus macaques (Zhou et al., 2013). Asian rhesus macaques are not natural hosts for SIV.

Taxonomically, HIV-1 is a direct progeny of SIVcpz, endemically infecting mainly Central African chimpanzees (*Pan troglodytes*). Interestingly, cross-species transfer of SIVcpz from chimpanzees to humans opened the door to the HIV-1 pandemic. Later, a jump of SIVsmm from sooty mangabeys (*Cerocebus atys*) to humans in West Africa resulted in the outbreak of HIV-2 (Sharp and Hahn, 2011). The SIVmac is pathogenic in Asian rhesus macaques and will gain more importance later in this thesis.

Pig-tailed macaques (*Macaca nemestrina*, PM, Figure 5) are natural hosts for SIVmac. Interestingly, they progress significantly faster to AIDS than RMs. Also, they have a very important genetic difference to RMs, which will gain more importance later in this thesis. This genetic difference is crucial for the development of a macaque model for HIV-1 infection (see chapter 1.6; Hatziioannou and Evans, 2012; Evans & Silvestri, 2013).

Fig. 5. Pig-tailed Macaque – *Macaca nemestrina* (Universal-Protein-Database)

1.5. Simian-human Immunodeficiency Virus (SHIV)

Challenging macaques with SIV does not provide enough insights into how HIV-1 works. Drugs developed for HIV-1 may not be able to be evaluated using SIV. For instance, SIV is not susceptible to some inhibitors of protease, RT, and integrase. Furthermore, most SIV strains use CCR5 (chemokine coreceptor 5), CXCR4 (CXC-motive-chemokine-receptor 4), BOB/GPR15 (G-protein-coupled receptor 15), Bonzo/STRL33/CXCR6 and GPR1 as second coreceptors. HIV-1 almost exclusively uses CCR5 in human transmission events and may switch to CXCR4 later during the course of infection (Grivel, Shattock & Margolis, 2011). HIV-1 is not usually entering cells via 'BOB and Bonzo' (Pöhlmann, Krumbiegel and Kirchhoff, 1999).

Moreover, anti-SIV antibodies will not recognize HIV envelope antigens. Cross-recognition of neutralizing antibodies (nAbs) cannot be observed. Passive immunization with anti-SIV mAbs against HIV-1 is therefore not possible. Consequently, anti-HIV-1 vaccines can only be developed with a proper HIV-1 model and not SIV.

In order to solve this problem, chimeric strains containing elements of both HIV-1 and SIV have been developed, named simian-human immunodeficiency viruses (SHIVs). These new strains mostly contain parts of SIV but envelope glycoproteins (*env*) are expressed on the base of HIV-1 information (Figure 6). The first chimeric strains cloned contained the SIVmac239 backbone with supplemented *env*, *vpu*, *tat* and *rev* regulatory genes of HIV-1 (HXBc2) and were named SHIV-1 to SHIV-4 (Shibata et al., 1991). These strains facilitated vaccine studies in macaques targeting envelope glycoproteins of HIV-1. Over the years, new SHIV strains have been developed, adapted and improved to cause viremia, $CD4^+$ T-cell depletion and develop AIDS within a few years in their hosts.

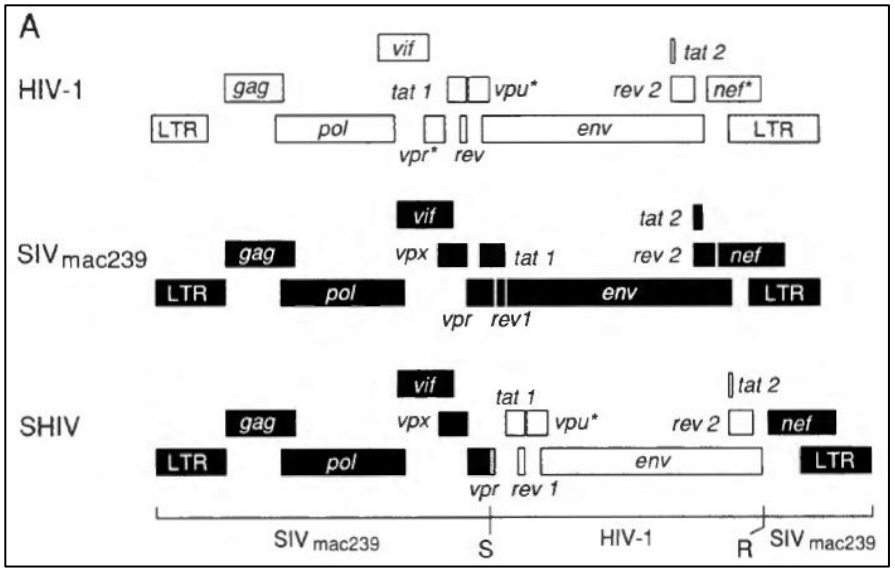


Fig. 6. Cloning of one of the first SHIV strains (Li et al., 1992). The black boxes represent the SIV part of the novel SHIV chimera, whereas the white parts of the genome represent the HIV-1 origin.

Abbreviations: S, SphI; R, RsrII

As mentioned, SHIV-4 was one of the first chimeric strains developed. Soon after, multiple new clones have been generated, such as SHIV89.6P, which was the first clone with HIV-1 *env* from a patient isolate (Reimann et al., 1996). These clones were mostly X4-tropic (using the CXCR4 coreceptor) and were therefore not able to replicate in the typical target cells of HIV-1 or SIV, as they express CCR5 and not CXCR4. Furthermore, these strains were not capable of mucosal transmission, which was unfortunate as 70% of all infections are transmitted over a mucosal route (Hatziioannou et al., 2012). Since these strains replicated poorly in macaques and did not meet the requirements for appropriate hypothesis testing, better chimeric strains have been generated over years through cloning and serial blood passaging or adaptation. The new strains would finally replicate in multiple breeds, cause viremia and AIDS, show mucosal R5-tropic transmission and contain HIV-1 envelope glycoproteins – e.g. SHIV-1157ipd3N4 (Song et al., 2006). Figure 7 shows how this strain was initially cloned.

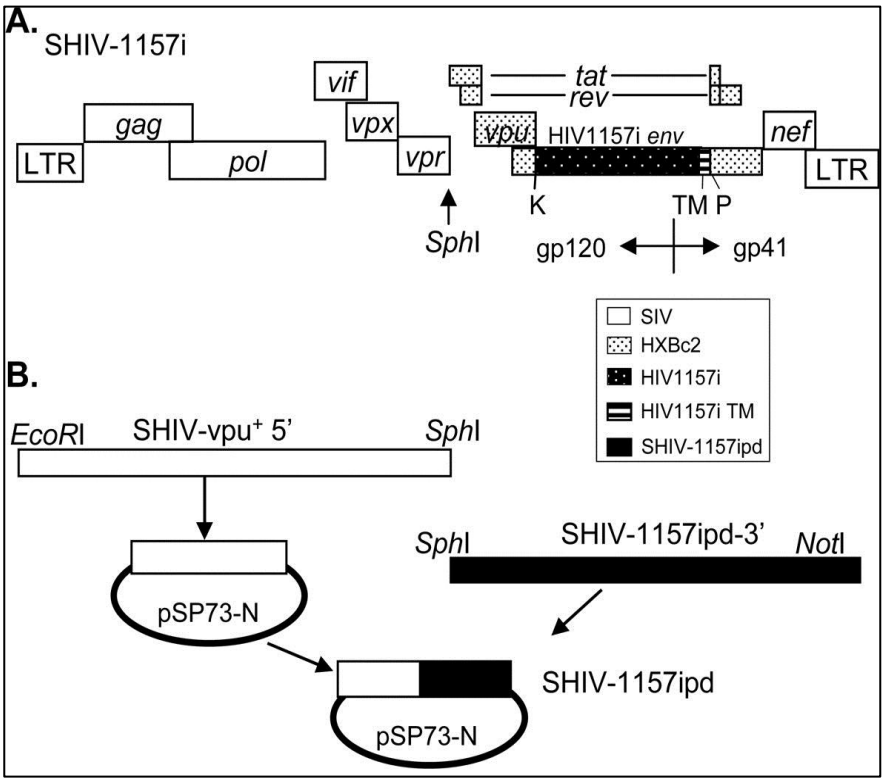


Fig. 7. Cloning of SHIV-1157ipd3N4 (modified after Song et al., 2006). A. SHIV-1157ipd3N4 is a combination of SIVmac239, HIV1157i and HIV-HXBc2. **B.** The new strain SHIV-1157i has then been cloned into the vector pSP73-N

Abbreviations: K, KpnI; TM, transmembrane region; P, PuvI

However, SHIV-1157ipd3N4 was not the only chimeric model that rose in the early 2000's. SHIV-1157ipd3N4 was a tier 2 virus and therefore a huge challenge for neutralizing antibodies. Although almost all epidemic HIV-1 strains in humans are declared tier 2, starting the research with tier 2 models was too big of a challenge at the very beginning. Consequently, a tier 1 SHIV strain was required. In 2010, the Ruprecht group developed the SHIV-1157ip3N4 derivate SHIV-1157ipEL. Figure 8 shows how it was initially cloned. In this virus the very well adapted *env* gene was replaced with an *env* gene from an early SHIV-infected macaque, which led to a decreased neutralization resistance in SHIV-1157ipEL. For optimized adaption and yielding, the virus was rapidly passaged in RMs. The final construct was designated SHIV-1157ipEL-p and served from now on as a reliable model for HIV-1 tier 1 vaccine development research.

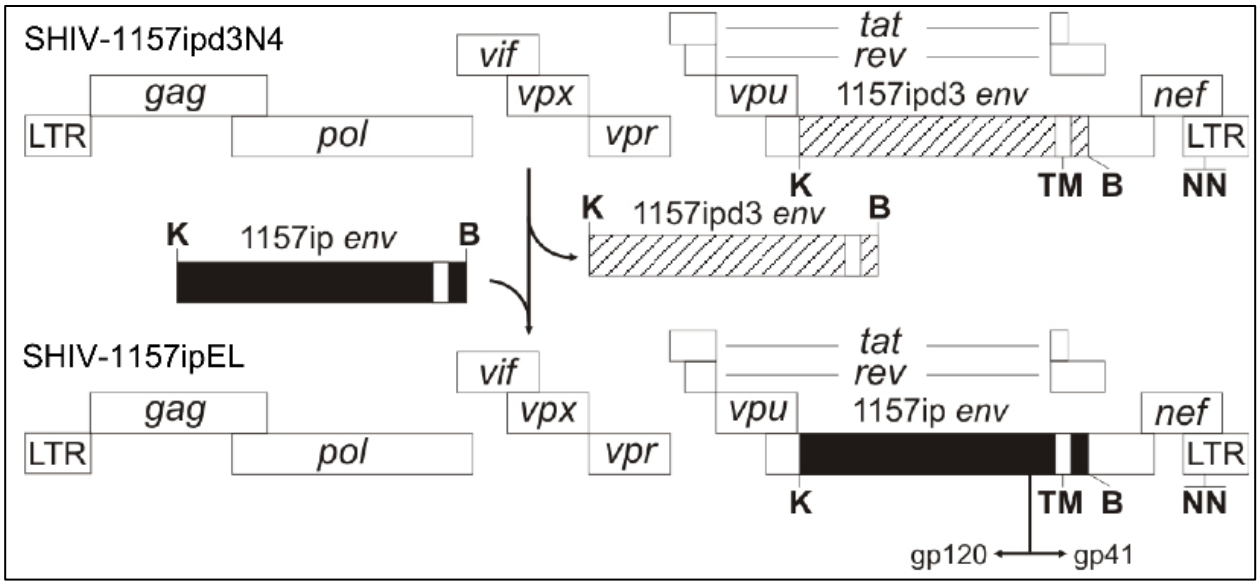


Fig. 8. Cloning of SHIV-1157ipEL (Siddappa et al., 2010). For the cloning of SHIV-1157ipEL the 'late' *env* gene of SHIV-1157ipd3N4 was replaced with a 'early' *env* gene, which led to a decreased neutralization resistance. These too genes diverge mainly in the V1 and V2 domain of the envelope glycoprotein gp120. Abbreviations: **K**, KpnI; **B**, BamHI; **TM**, transmembrane region; **NN**, 2 NF-κB sites

In 2004, Grisson and colleagues isolated a new infectious molecular HIV-1 clone from a Zambian infant. Interestingly, by that time only two infectious clade C proviral DNA clones have been described, although HIV-1 clade C is the dominating form of the pandemic. The new clone, designated HIV1084i, where the 'i' stands for 'infant', was the first evidence of intraclade recombination. HIV1084i contained Asian *gag* and African *pol* and *env* genes. It was then cloned into the pCR2.1-Topo Vector (Grisson et al., 2004). Finally, a complete HIV-1 clade C backbone was available as an infectious proviral molecular clone.

1.6. Simian-tropic HIV strains

Although the new SHIV strains are infectious in primates, SIV is significantly divergent from HIV-1. The reason for HIV-1 not being able to replicate in RMs is the presence of two genes – *TRIM5α* and *APOBEC3G*. These genes are highly active in these animals and make HIV-1 replication impossible. The tripartite motif-containing protein 5, encoded by *TRIM5α*, is an antiretroviral protein in primates that inhibits viral replication by inactivating incoming capsids. The human isoform of *TRIM5α* does not target HIV-1, but it is capable of inhibiting other retroviruses such as the murine leukemia virus (MLV) and the equine infectious anemia virus (EIAV). Additionally, the *APOBEC3G* gene, encoding the apolipoprotein B mRNA editing enzyme catalytic polypeptide 3, is responsible for antiretroviral inactivation by infiltrating virions during their assembly and blocking their genomes during subsequent reverse transcription. In pig-tailed macaques (PMs) *TRIM5α* encodes for the TRIMCyp isoform, which is less restrictive for HIV-1 replication. Nevertheless, the antiviral activity of *APOBEG3G* in PMs is crucial. Thus, HIV-1 is not able to overcome this inhibitor, whereas SIV's Vif is capable of degrading and inhibiting it. This explains why HIV-1 is not able to replicate in macaques (Hatziioannoua et al., 2009).

In order to overcome the antiretroviral activity of *APOBEC3G* in PMs, Hatziioannoua and colleagues cloned a new chimeric strain that contained only a minimum of SIV information. The SIVmac293 *vif* gene was supposed to inactivate the macaque's *APOBEC3G* activity. The gene was cloned into an HIV-1 backbone with macaque adapted glycoproteins from SHIV-KB9, a chimera with dual tropic HIV-1 clade B information (Etemad-Moghadam et al., 2000). The result was a new clade B simian-tropic human immunodeficiency virus (stHIV), designated stHIV$_{SV}$, able to replicate properly in macaques and containing as much HIV-1 infromation as possible (Hatziioannoua et al., 2009). Figure 9 shows how a similar clone was generated.

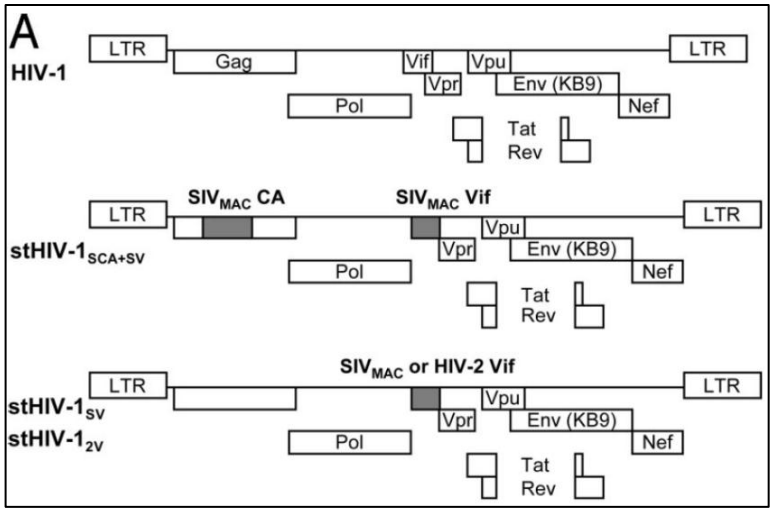


Fig. 9. Cloning of stHIV-1$_{SV}$ (modified after Hatziioannoua et al., 2009). The new simian-tropic (st) HIV-1 strain is composed of an HIV-1 backbone with macaque-adapted glycoproteins and a SIVmac293 *vif* gene. The dark boxes represent the SIV part in the new construct.

1.7. Background of the Thesis

1.7.1. stHIV-C8457

Although HIV-1 clade C is the most prevalent form of the pandemic, stHIV-1 clade C clones have not yet been published. Therefore, the Ruprecht lab generated a construct of a R5-tropic stHIV-1 clade C clone encoding the *env* gene and others of SHIV-1157ipd3N4 (Song et al., 2006), a SHIV strain that is able to replicate properly in PMs (Ho et al., 2009). For this purpose, the Ruprecht group cloned defined parts of SHIV-1157ipd3N4 into the HIV1084i backbone, a R5-tropic infectious molecular clone of a recently transmitted pediatric HIV-1 clade C isolate (Grisson et al., 2004). The cloning strategy used to generate a stHIV-1 clade C (termed stHIV-C for short) is depicted in Figure 10.

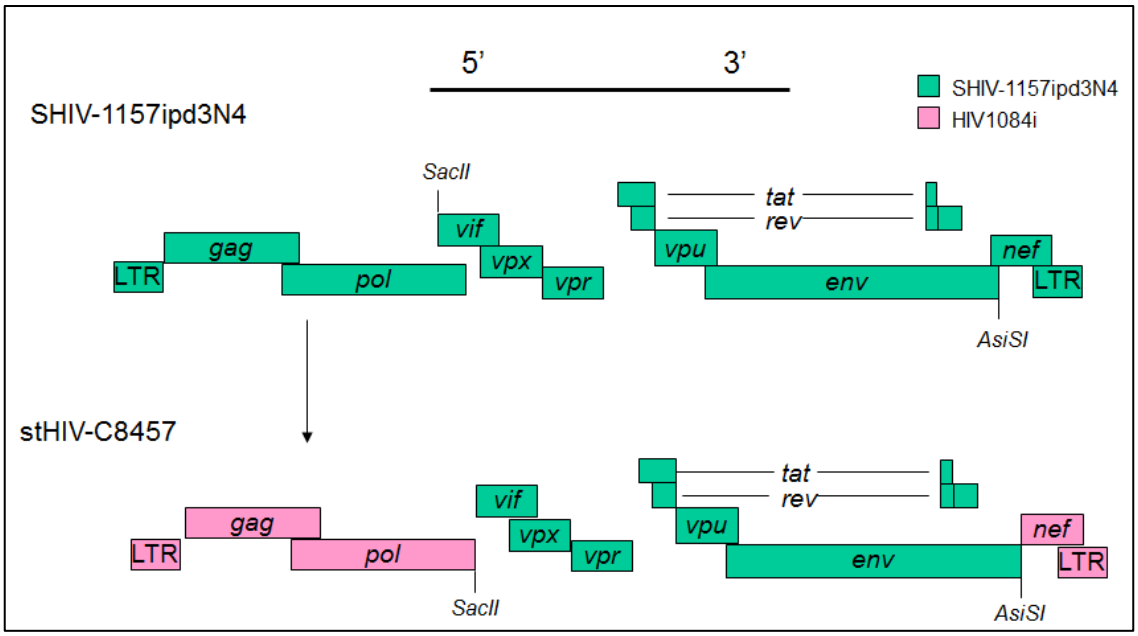


Fig. 10. Cloning of stHIV-C8457 (Thorat, Ruprecht Lab; unpublished data). Illustration of how Ruprecht and colleagues cloned *vif-env* of SHIV-1157ipd3N4 into the HIV1084i backbone within the pCR2.1-Topo vector.

The product was the R5-tropic strain designated stHIV-C8457. The new strain contains mostly HIV-1 information with *vif*, *vpx* and *vpr* SIV genes within the pCR2.1-Topo vector from HIV1084i. This construct would then be adapted to PMs for optimized reproduction. By rapid serial animal-to-animal passaging, the virus was adapted to PMs. The organization of the genome can be seen in Figure 11.

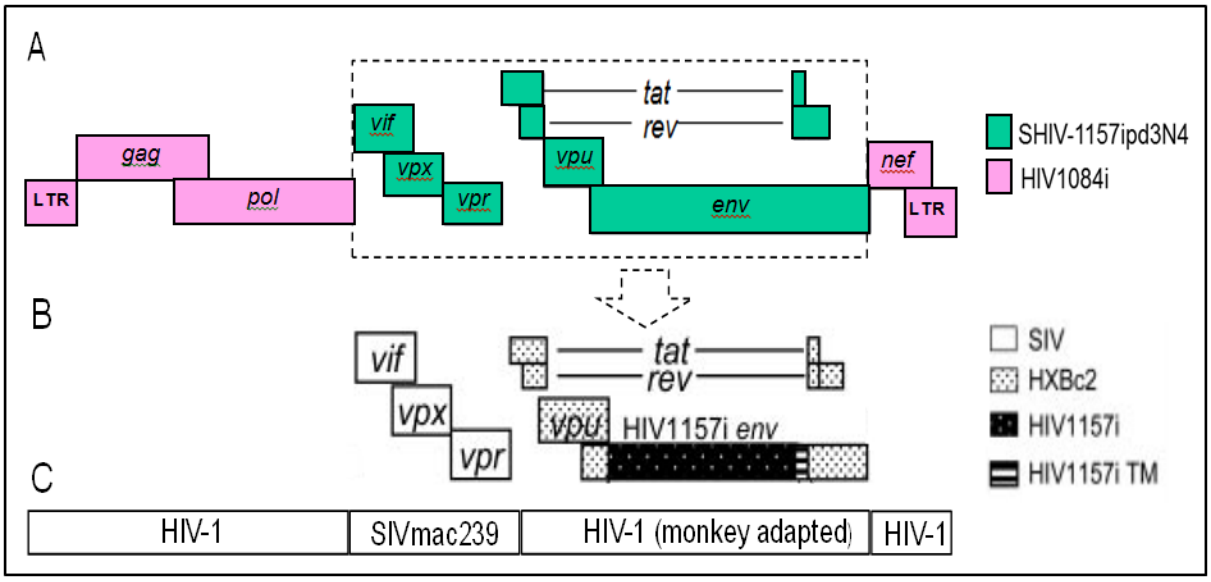


Fig. 11. Organization of stHIV-C8457 Genome (Thorat, Ruprecht Lab; unpublished data). The new stHIV-C8457 strain contains mostly HIV1084i information within the pCR2.1 Topo Vector with only as little as three SIV genes from SIVmac293. **A.** The genetic structure of stHIV-C8457 shows the distribution of SHIV and HIV1084i. **B.** The insert of Lei Wang's cloning is a defined part of SHIV-1157ipd3N4 which already contains HIV parts such as *env*, *tat*, *rev* and *vpu*. **C.** The result is a new construct that contains mostly HIV and only three genes from SIV.

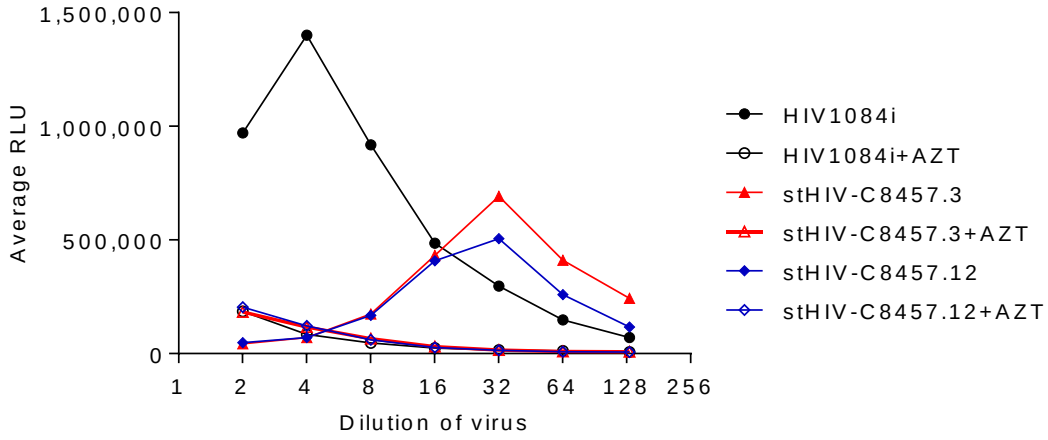


Fig. 12. TZM-bl Infectivity Assay of HIV1084i, stHIV-C8457.3 and stHIV-C8457.12 (Thorat, Ruprecht Lab; unpublished data). The TZM-bl infectivity assay is a virological method that evaluates the infectivity of a virus. The virus is serially diluted, which decreases the response of the cells and directly correlates with the infectivity of the clone. The graph shows that stHIV-C8457 clones 'stHIV-C8457.3' and 'stHIV-C8457.12' grow very in TZM-bl cells. The control was treated with the well-known AIDS drug AZT (3'-azido-3'-deoxythymidine).

1.7.2. In Vivo and Ex Vivo Adaptation of stHIV-C8457

The next step was to adapt the new stHIV-C strain to PMs. In order to prevent immediate virus elimination by the hosts, a new adaption strategy has been conducted. This novel adaption strategy was previously very successful at the adaption of SHIV-KNH1144p4, a novel clade A SHIV strain. During the adaption of this strain, the Ruprecht lab performed a rapid serial passage of the virus through six RMs. Re-isolated virus or infected blood was then transferred from one animal to the next. In order to avoid suppression of viral replication caused by the host's immune system, $CD8^+$ and $CD20^+$ T cells where marked with anti-CD8 (cM-T807) and anti-CD20 (Rituximab) monoclonal antibodies (mAbs), depleting all B and $CD8^+$ T cells during virus inoculation. The result was a RM adapted SHIV-KNH1144p4 progeny virus that was able to cause severe viremia (more than 10^7 copies/ml) in two naïve immunocompetent animals of the following passage. As both animals developed high viral RNA (vRNA) peaks, steady viremia and heavy $CD4^+$ cell depletion, confirmed the adaption of the virus to its new host. The adapted progeny virus replicated significantly better in peripheral blood mononuclear cells (PBMC) than its predecessor.

Since stHIV-C8457 needed to be adapted to PMs, the Ruprecht lab attempted to make use of the novel adaption strategy. The first immunosuppressed PM (PM1, A09163) was infected with proviral DNA of stHIV-C8457 by intramuscular injection (i.m.). B cells were depleted before virus administration using the anti-CD20 mAb Rituximab and $CD8^+$ T cells were depleted immediately after viral injection with use of the anti-CD8 mAb cM-T807. Although the viremia was controlled after only a few days, the animal became viremic again when the number $CD8^+$ T cells started to decline. Infected blood was then passaged to the immunodepleted PM2 (L08228). The strategy is depicted in Figure 13. Continuing the passaging to PM3 (T10067) and PM4 (K10108), the Ruprecht group observed that emergence of $CD8^+$ T cells was associated with control of the viremia.

In order to overcome the control of viral replication by $CD8^+$ T cells, caused by soluble virus inhibitory factors (reviewd in Levy, 2003), the Ruprecht lab attempted to obtain a progeny of stHIV-C8457 that is resistant to such factors. Therefore, the levels of $CD8^+$ T cells were increased during ex vivo virus growth. The result was an adapted progeny virus that showed an increased resistance to $CD8^+$ T-cell mediated antiviral activity.

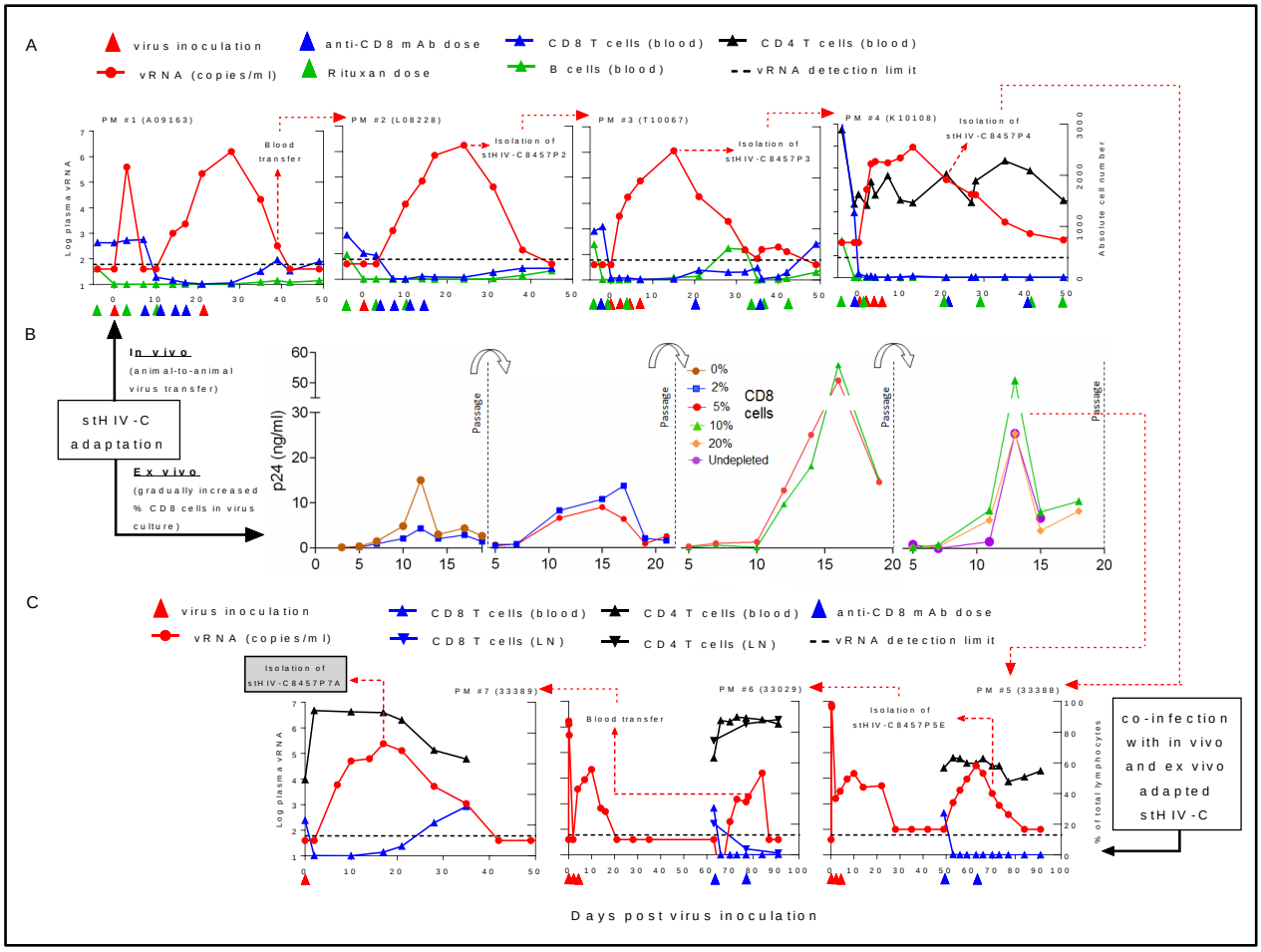
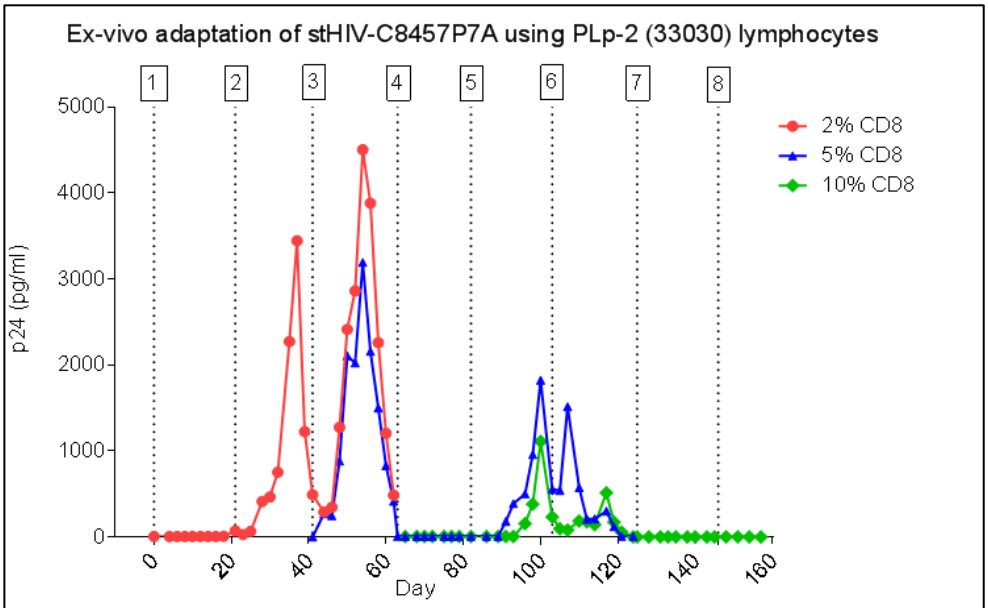


Fig. 13. Adaption of stHIV-C8457 from PM1 to PM7 (Ruprecht Lab; unpublished data). The stHIV-C8457 strain was adapted to pig-tailed macaques (PMs) through rapid serial animal to animal passaging. After low viremia, the virus was adapted in vitro in order to develop a $CD8^+$ T-cell resistance after the fourth passage. Both stHIV-C8457 stocks, the in vitro adapted progeny virus and its parental predecessor, were then co-inoculated into three naïve immunocompetent PM5 (33388), PM6 (33029) and PM7 (33389). Although the virus was able to replicate and form a reservoir, viral replication was controlled rapidly after the emergence of $CD8^+$ T cells. The virus was isolated from PM7 and co-cultured with naïve peripheral blood mononuclear cells (PBMC). The isolate from PM7 was designated stHIV-C8457P7A.

To overcome the in vivo repression by $CD8^+$ T cells, the virus was adapted ex vivo. Cell cultures with PBMC from animal 33030 (or PLp-2) were seeded and harvested. $CD8^+$ T cells were depleted in one part of the culture. The other part was used to purify $CD8^+$ T cells. The results were two cultures with $CD8^-$ PBMC only and highly purified $CD8^+$ T cells. The depleted culture was inoculated with stHIV-C8457P7A. Gradually 2%, 5% and 10% of $CD8^+$ T cells were added to the culture (Figure 14) so the virus can replicate and adapt to the presence of these cells. The adapted virus was then again adapted in vivo from PM8 to PM13 (Figure 15).



1. stHIV-C8457P7A exposed to 33030 lymphocytes containing 2% CD8 cells.
2. Fresh 33030 lymphocytes with 2% CD8 cells added.
3. Infected cells splitted; culture with 2% CD8 cells continued, new culture with 5% CD8 cells started.
4. Culture with 2% CD8 cells terminated, infected culture containing 5% CD8 cells splitted and culture with 5% CD8 cells continued, new culture with 10% CD8 cells started.
5. Fresh 33030 lymphocytes with 5% or 10% CD8 cells added to respective culture. The sup collected on day 54 of 5% culture used for infection.
6. Fresh 33030 lymphocytes with 5% or 10% CD8 cells added to respective culture.
7. Culture with 5% CD8 cells terminated, infected cells from 10% CD8 culture mixed with fresh 33030 lymphocytes and culture continued in the presence of 10% CD8 cells.
8. Fresh 33030 lymphocytes with 10% CD8 cells added to the culture.

Figure 14: Ex-Vivo Adaptation of stHIV-C8457P7A (Thorat, Ruprecht Lab; unpublished data). After the virus stHIV-C8457P7A was adapted to $CD8^+$ T cells, pig-tailed macaque (PM) 8 (36096) was inoculated with the virus, after immunodepletion of all $CD8^+$ T cells and administration of a type-I interferon antagonist (IFN-1ant). The results were promising as the viremia maintained for almost two months. The virus was passaged through the following animals PM9 (36226), PM10 (36224), PM11 (36095), and PM12 (36231). After PM12 the virus showed adaptation to the host. PM13 was then the first animal with no immunodepletion. It was immunocompetent and the new virus was tested under pressure of the host's immune system.

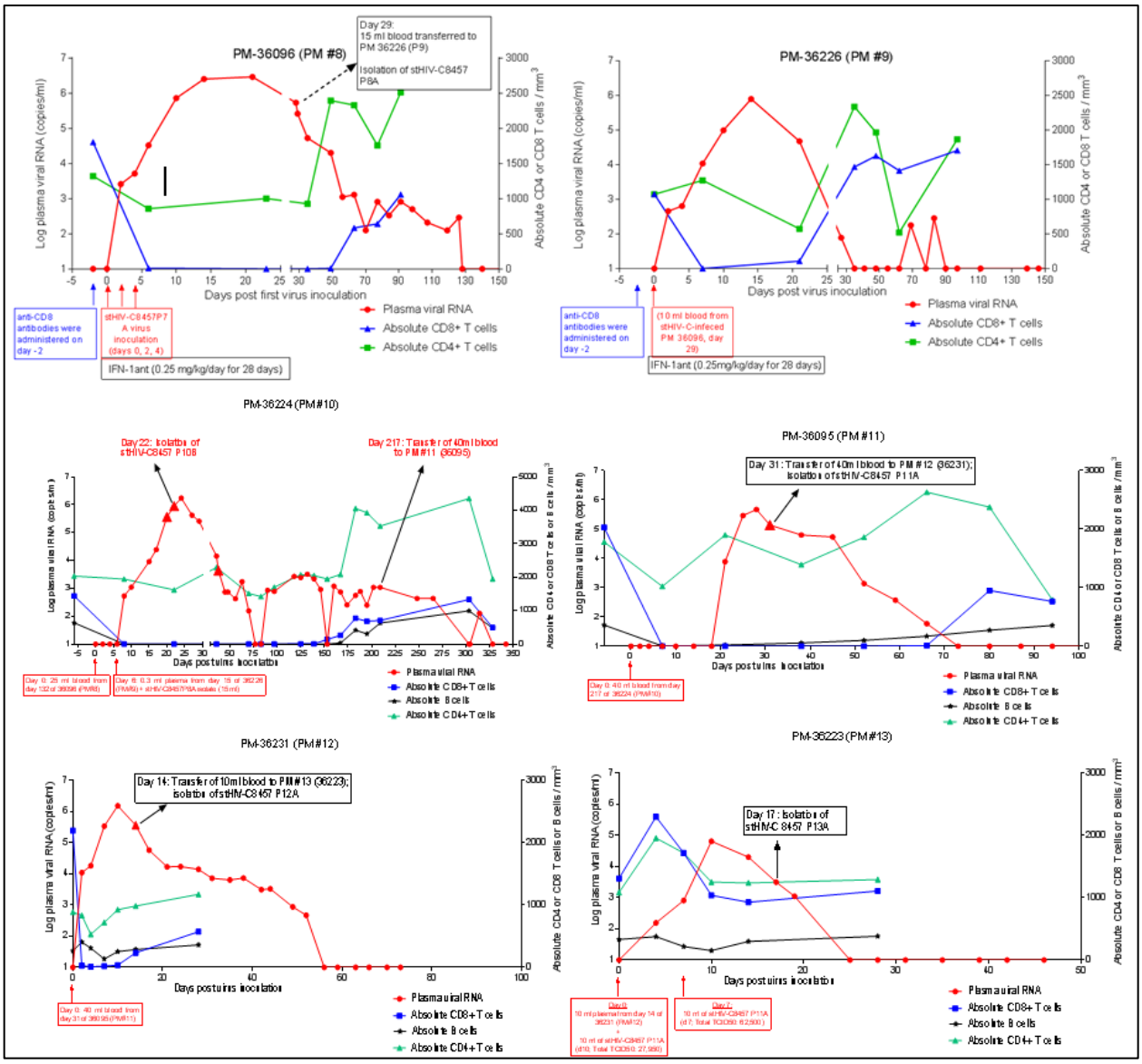


Fig 15. In Vivo Adaptation of stHIV-C8457P7A from PM8 to PM13 (Ruprecht Lab; unpublished data). stHIV-C457P7A was again inoculated in pig-tailed macaque (PM) number eight, after development of $CD8^+$ T cell resistance by ex vivo adaptation. The graphs show the viral load (red), the absolute number of $CD8^+$ cells (blue), the absolute number of $CD4^+$ cells (green) and the absolute number of B cells (black).

1.8. Aims and Goals of the Thesis

The aim of this thesis was to generate a new infectious R5-tropic stHIV-C clone, containing a PM- adapted genome, which is able to replicate in this unnatural host. The strain should be able to replicate without prior $CD8^+$ T-cell depletion and cause persistent viremia in its host. As the construct contains mostly HIV-1 and almost no SIV genetic information, it will serve as a better model for HIV-1 clade C infection in PMs for vaccine development. This new novel progeny virus will expand possibilities to test anti-HIV-1 Gag and Nef immune responses.

1.8.1. Serology of Infected Pig-tailed Macaques

In this thesis, we sought to demonstrate that stHIV-C8457 is capable of establishing long-term reservoirs in all PMs that were inoculated with the virus. Our results showed that the virus persisted and that the host's immune system was not able to eliminate the virus from the organism. Moreover, in the results we will present a possible case of seroreversion in PM5.

1.8.2. Ex Vivo Adaptation of stHIV-C8457P13A

During early passages in the PMs, the virus was inhibited by the soluble antiviral factors of $CD8^+$ cells. Therefore, we adapted the virus ex vivo by gradually increasing the amount of $CD8^+$ T cells in vitro. Our results showed that the virus significantly adapted to such factors and was able to increase the replication 5-fold.

1.8.3. stHIV-C8457P13A Cloning

We attempted several cloning strategies to generate the proviral plasmid of stHIV-C8457P13A. We concentrated the virus, extracted viral RNA and transcribed it into cDNA. We amplified the whole genome and made attempts cloned it into the pCR2.1-Topo vector. This vector was used during the isolation of HIV1084i and during the cloning of the initial stHIV-C8457 strain. A second approach included the amplification of the proviral genome in two long fragments and attempts to clone the amplicons into the same vector. Ongoing work includes different cloning approaches. We also confirmed the integrity and infectivity of the parental strain stHIV-C8457.3.

1.8.4. Summary

This thesis contributed to the development, adaptation and improvement of stHIV-C. We tried to develop a proviral plasmid of a PM-adapted stHIV-C progeny. We showed that the virus was able to establish reservoirs in all inoculated PMs. Our ex vivo adaptation increased the virus' resistance to $CD8^+$ T cell-derived antiviral factors. The novel virus may expand our opportunities to perform anti-HIV vaccine efficacy studies in a non-human primate model.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. List of Plasmids

- stHIV-C8457.3

The plasmid was cloned and transformed by the Ruprecht lab in 2011. The stHIV-C8457.3 strain is the third clone of the initial stHIV-C8457 and frozen in glycerol at -80°C. The plasmid is approximately (approx.) 14,000 base pairs (bp) long and contains stHIV-C8457.3 within the pCR2.1-Topo vector from HIV1084i. The vector contains ampicillin and kanamycin/neomycin resistance.

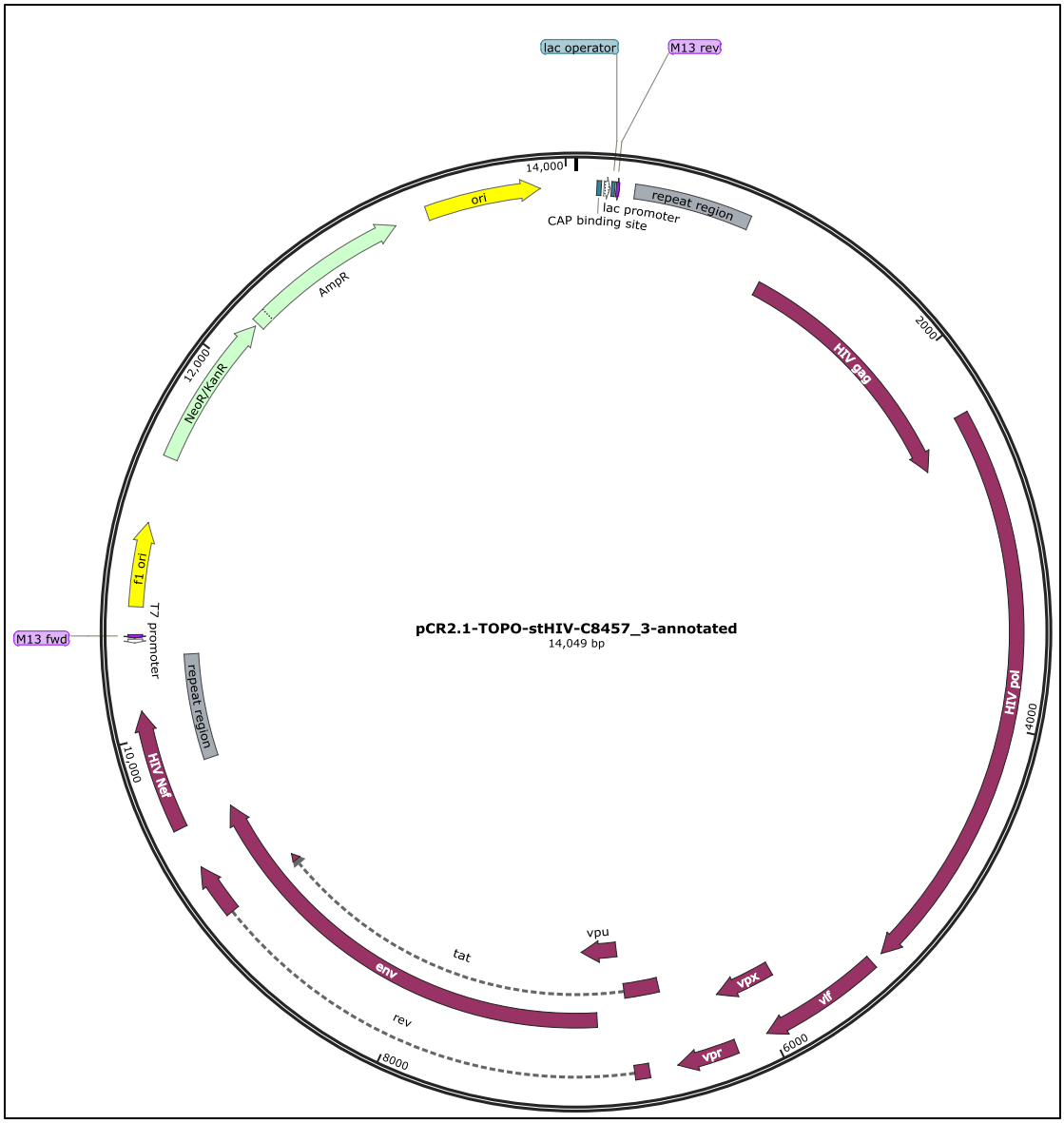


Fig. 16. Plasmid Map of stHIV-C8457.3 in pCR2.1-Topo

- pCR2.1-Topo

  The vector was used for cloning of HIV1084i and is therefore necessary for the molecular cloning of the new strain. The vector is 3,931 bp long and contains resistance genes for ampicillin and kanamycin/neomycin and was purchased from Invitrogen (Carlsbad, CA).

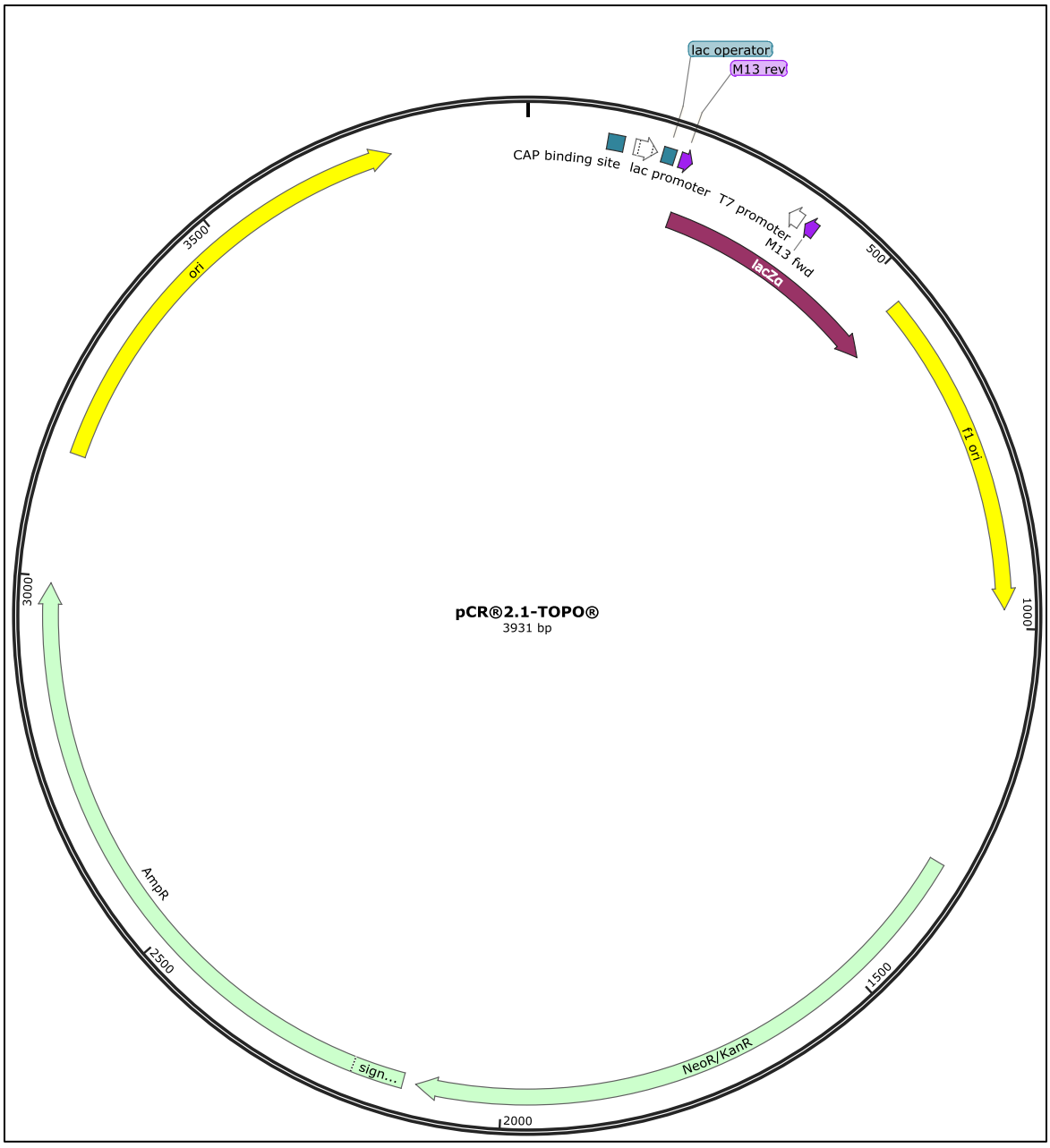


Fig. 17. Plasmid Map of pCR2.1 Topo

2.1.2. List of Primers

stHIV-C8457P13A amplification primers	primer name	fragment (Frg.)	
AAGCTTGGTACCGAGCTCGGATCCAtggatgggttaatttactccaagaaaag	Frg. A FWD	A	1
TGGCCCCCTACTTTTATTGAGACGAGgggtcgctg	Frg. A REV		
CTCGTCTCAATAAAAGTAGGGGGCCAaataaggg	Frg. B FWD	B	
CCAAATAGGGTCTCTGCTGTCTCTGTaataaacc	Frg. B REV		
ACAGAGACAGCAGAGACCCTATTTGGaaaggacc	Frg. C FWD	C	2
CTCCACCACTCTCCTTTTTGCCTTAGTGGgtgctattcc	Frg. C REV		
CCACTAAGGCAAAAAGGAGAGTGGTGGAGagagaaaaaag	Frg. D FWD	D	
TCGAGCGGCCGCCAGTGTGATGGAtgggtctgagggatctctag	Frg. D REV		

The stHIV-C8457P13A genome was amplified in four and two fragments. Fragment A was approximately 2,400 bp, including the 5'-LTR and *gag* (HIV-1). Fragment B was be approximately 2,600 bp long and included mostly the *pol* gene (HIV-1). Fragment C was the longest fragment with approx. 3,200 bp and included *pol* (HIV-1), *vpx* (SIV), *vpr* (SIV), *vif* (SIV), *vpu* (HIV-1) and parts of *tat* (HIV-1), *rev* (HIV-1) and *env* (HIV-1). Fragment D was the last part of the genome and included the rest of *env* (HIV-1), *tat* (HIV-1), *rev* (HIV-1), *nef* (HIV-1) and the 3'-LTR.

During the cloning from genomic DNA, the genome was amplified in two fragments. In this amplification, we fused Fragment A and Fragment B to Fragment 1, with 4,915 bp, and Fragment B and Fragment D to Fragment 2, with 5,229 bp length. The primers were designed with the NEBuilder Assembly Tool (v.1.12.17).

HIV-1 *gag* primers	primer name
CTCCAAGGGCAAATGGTACAC	HIV gag FWD
GCTGAAAGCCTTCTCCTCTATTACTT	HIV gag REV

The *gag* primers were used to verify the presence of HIV-1 and served as a tool to confirm successful cDNA synthesis. The primers were designed for RT-PCRs in the Ruprecht lab and bind at the highly conserved *gag* region of HIV-1.

2.1.3. List of Products

Reagent	Producer
6x DNA loading dye	Thermo-Fisher Scientific (Waltham, MA)
96-well flat bottom tissue culture plate	Thermo-Fisher Scientific (Waltham, MA)
96-well round bottom tissue culture plate	Thermo-Fisher Scientific (Waltham, MA)
Ambion® nuclease free water	Life Technologies (Grand Island, NY)
Anti-CD3 antibody (SP34-2)	Becton Dickinson (Franklin Lakes, NJ)
Anti-CD4 antibody (M-T477)	Becton Dickinson (Franklin Lakes, NJ)
Anti-CD45 antibody (D058-1283)	Becton Dickinson (Franklin Lakes, NJ)
Anti-CD8 antibody (DK25)	Merck Millipore (Burlington, MA)
Bright-Glo™ luciferase assay system	Promega (Madison, WI)
CD8 T-cell isolation kit	Miltenyi Biotech (Bergisch Gladbach, Germany)
Concanavalin A (Con A)	Sigma-Aldrich (St. Louis, MO)
Corning® T125 cell culture flask	Sigma-Aldrich (St. Louis, MO)
Corning® T25 cell culture flask	Sigma-Aldrich (St. Louis, MO)
Corning® T75 cell culture flask	Sigma-Aldrich (St. Louis, MO)
Cytofix™ fixation buffer	Becton Dickinson (Franklin Lakes, NJ)
Diethylaminoethyl cellulose (DEAE)-dextran hydrochloride (7,5 mg/ml)	Sigma-Aldrich (St. Louis, MO)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St. Louis, MO)
DNeasy blood & tissue kits	QUIAGEN (Valencia, CA)
dNTP grade PCR (10 mM each)	Invitrogen (Carlsbad, CA)
DreamTaq™ hot start DNA polymerase	Thermo-Fisher Scientific (Waltham, MA)
Dynabeads™ CD8 depletion	Thermo-Fisher Scientific (Waltham, MA)
Elution buffer	QUIAGEN (Valencia, CA)
Ethidium bromide (EtBr)	Invitrogen (Carlsbad, CA)
EDTA (0.5M)	Fisher Scientific (Hampton, NH)
Ficoll-Paque™ PLUS medium	GE-Healthcare (Little Chalfont, United Kingdom)
First strand cDNA synthesis kit (#E6300S)	New England Biolabs (Ipswich, MA)
Futura® safety scalpels	Merit Medical (South Jordan, UT)
GeneJet® gel extraction kit	Thermo-Fisher Scientific (Waltham, MA)
GeneRuler 1 kb plus DNA ladder	Thermo-Fisher Scientific (Waltham, MA)
Gibco® 0,25% trypsin/EDTA	Life Technologies (Grand Island, NY)
Gibco® Dulbecco's Modified Eagle Medium (DMEM+++)	Life Technologies (Grand Island, NY)
Gibco® fetal bovine serum (FBS)	Life Technologies (Grand Island, NY)
Gibco® gentamicin (50 mg/ml)	Life Technologies (Grand Island, NY)
Gibco® HEPES	Life Technologies (Grand Island, NY)
Gibco® L-glutamine	Life Technologies (Grand Island, NY)
Gibco® Opti-MEM	Life Technologies (Grand Island, NY)
Gibco® penicillin-streptomycin (Pen-Strep)	Life Technologies (Grand Island, NY)
Gibco® Roswell Park Memorial Institute Medium - 1640 (RPMI-1640)	Life Technologies (Grand Island, NY)
Gibco® β-mercaptoethanol	Life Technologies (Grand Island, NY)
GS HIV type 1 western blot kit	Bio Rad Laboratories Inc. (Hercules, CA)
HIV-1 p24 antigen capture assay	ABL Inc. (Rockville, MD)
Human Interleukin-2 (10 U/µl, IL-2)	Sigma-Aldrich (St. Louis, MO)

Human tumor necrosis factor-α (10 ng/µl, TNF-α)	Sigma-Aldrich (St. Louis, MO)
Max efficiency® DH10B competent cells	Invitrogen (Carlsbad, CA)
Max efficiency® Stbl 2 competent cells	Invitrogen (Carlsbad, CA)
Mr. Frosty freezing container	Thermo-Fisher Scientific (Waltham, MA)
NEBuilder® HiFi DNA assembly master mix	New England Biolabs (Ipswich, MA)
Plasmid DNA purification kit	Macherey-Nagel (Düren, Germany)
Q5® hot start high-fidelity 2x master mix	New England Biolabs (Ipswich, MA)
QIAamp viral RNA mini kit	QIAGEN (Valencia, CA)
QIAGEN®Plasmid maxi kit	QIAGEN (Valencia, CA)
QIAGEN®Plasmid midi kit	QIAGEN (Valencia, CA)
QIAGEN®Plasmid mini kit	QIAGEN (Valencia, CA)
QualiCode™ HIV-1/2 western blot kit	Immunetics (Boston, MA)
Stable competent *E. coli* (#C3040I)	New England Biolabs (Ipswich, MA)
SYBR™ green 10.000x in DMSO	Invitrogen (Carlsbad, CA)
Syringe filter unit, 0.45 µm PVDF	Merck Millipore (Burlington, MA)
Tris-acetate-EDTA buffer 50x	Thermo-Fisher Scientific (Waltham, MA)
Tween 20	Sigma-Aldrich (St. Louis, MO)
Ultra-Clear™ 13.2 ml ultracentrifuge tube	Beckman Coulter (Brea, CA)
UltraPure™ agarose	Invitrogen (Carlsbad, CA)
UltraPure™ distilled water	Invitrogen (Carlsbad, CA)
UltraPure™ Tris-HCl (1 M, pH 8.0)	Invitrogen (Carlsbad, CA)
X-tremeGENE™ 9 DNA transfection reagent	Sigma-Aldrich (St. Louis, MO)

2.1.4. List of Restriction Enzymes

Enzyme	Catalog Number	Editing Sequence	Manufacturer
EcoR V-HF	R0195S	GAT↑↓ATC	New England Biolabs (Ipswich, MA)
Spe I-HF	R0133S	A↓CTAG↑T	New England Biolabs (Ipswich, MA)

2.1.5. List of Media

Media	Ingredients/Producer
HEK293T/17 medium	5 ml of L-glutamine 5 ml of Pen-Strep 50 ml of FBS DMEM +++ to 500 ml
Freezing medium	10 ml of DMSO FBS to 100 ml
LB-agar-ampicillin^{+} medium	InvivoGen (San Diego, CA)
LB-liquid-ampicillin^{+} medium	InvivoGen (San Diego, CA)
TZM-bl medium	12.5 ml of HEPES (1M) 0.5 ml of gentamicin (50 mg/ml) 50 ml of FBS DMEM+++ to 500 ml
S.O.C. medium	Invitrogen (Carlsbad, CA)
R-15 medium	5 ml of L-glutamine 5 ml of Pen-Strep 0.5 ml of β-mercaptoethanol 75 ml of FBS RPMI-1640 to 500 ml
R-2 medium	10 ml of FBS RPMI-1640 to 500 ml

2.1.6. List of Buffers

Buffer	Ingredients/Producer
1x tris-acetate-EDTA (TAE) buffer	20 ml of 50x TAE concentrate UltraPure water to 100 ml
50x tris-acetate-EDTA (TAE) buffer	242 g of Tris free base 18.61 g disodium EDTA 57.1 ml of glacial acetic acid double distilled H_2O (ddH_2O) to 1 L
10x Taq buffer with $(NH_4)_2SO_4$ and 20 mM $MgCl_2$	Thermo-Fisher Scientific (Waltham, MA)
CutSmart buffer (#B7204S)	New England Biolabs (Ipswich, MA)
Depletion buffer	10 ml of 10x PBS pH 7.4 0.5 g of BSA 400 µl of 0.5 M EDTA ddH_2O to 100 ml
Gibco® 10x phosphate buffered saline (PBS) pH 7.4	Life Technologies (Grand Island, NY)
Gibco® 1x PBS pH 7.2	Life Technologies (Grand Island, NY)
Isolation buffer	10 ml of 10x PBS pH 7.4 0.1 g of BSA 400 µl of 0.5 M EDTA ddH_2O to 100 ml
PBS with Tween20 (PBST)	100 ml of Gibco® 10x PBS pH 7.4 500 µl Tween 20 (Sigma-Aldrich) UltraPure water to 1 L

2.2. Methods

2.2.1. Serology of PM5 to PM13

2.2.1.1. Western Blots

In order to prove that all PMs infected with stHIV-C8457 acquired a viral reservoir, we performed western blots on all animals that are still alive (PM5 to PM13). For the western blots, the clinical GS HIV Type 1 Western Blot Kit from Bio Rad and the QualiCode™ HIV-1/2 Western Blot Kit by Immunetics was used. The tests were performed following the manufacturer's instructions. The kits contain strips that with HIV-1 proteins separated by electrophoresis. Exposing these proteins to antibodies that can be found in plasma of infected animals leads to high affinity binding of these antibodies to their target protein (antigen). The antibodies will be marked with a secondary antibody containing a horseradish peroxidase (HRP) conjugate. This enzyme converts the latter added substrate into an indicator that changes color and shows were the antibodies did bind.

GS HIV Type 1 Western Blot Kit

First, the 5x wash specimen diluent was diluted to a 1x concentration. Then, 1 ml of the buffer was added into each well of the tray and the Strips placed into the wells. Next, 10 µl of heat inactivated (56°C for 1 hour) plasma was added to the well. The tray was placed on a rocking platform, shaking the strips at 50 rpm for 55-60 minutes. Then, the strips were washed three times with the washing diluent. After the third wash, 1 ml of the diluent was added into each well and the tray was again placed on the rocking platform for 4-6 minutes. After the wash, 1 ml of conjugate solution was added into each well and incubated on the rocking platform for another 45-50 minutes. After the conjugate addition, the strips were again washed as before and 1 ml of color development solution was added into each well for 3-5 minutes. The solution was removed and the strips washed with distilled water. The strips were air dried and stored in the dark.

QualiCode™ HIV-1/2 Western Blot Kit

The QualiCode Kit by Immunetics contains a 10x washing buffer. The latter was diluted to 1x concentration at the beginning and left at room temperature. The strips were placed in the tray and washed with the washing buffer for 1 minute. The buffer was aspirated and replaced with 1 ml of sample diluent. We added 10 µl of the heat-inactivated plasma as well as a positive and a negative control. The strips were incubated on a rocking platform for 2 hours. Then, we washed the strips four times with the washing buffer and added 1 ml of the anti-human IgG conjugate and incubated the tray on a rocking platform for 15 minutes. The strips were washed again four times and 1 ml of substrate solution was added. The strips were placed on the rocking platform for 8 minutes and then rinsed with the washing buffer. We transferred the strips on a paper towel and air-dried them.

SNPRC Id	Alternate ID 1	Alternate ID 2	Date (M/D/Y)	Days after infection
33388	94Z	P5	08/13/14	2
			04/30/15	262
			12/22/16	864
			11/16/17	1193
33029	PKp2	P6	01/14/14	Pre-infection
			02/15/18	1100
33389	25A	P7	04/21/15	0
			06/30/15	70
			04/12/16	328
			02/15/17	666
36096	Z14282	P8	08/24/16	0
			02/22/17	182
36226	Pt73C	P9	09/22/16	0
			02/22/17	153
36224	Pt54C	P10	01/03/17	0
			07/17/17	195
36095	Z13020	P11	09/08/17	0
			01/22/18	136
36231	Pt111C	P12	10/09/17	0
			01/29/18	112
36223	Pt36C	P13	11/03/17	0
			01/23/18	81

Table 1: Table of all samples tested for anti-HIV-1 antibodies

2.2.2. Preparation of pCR2.1-Topo Vector

As the pCR2.1-Topo plasmid was needed to clone the adapted stHIV-C8457P13A into the vector, it was necessary to produce a specific amount of the plasmid. Therefore, the vector was transformed into competent cells, amplified, and isolated.

2.2.2.1. Transformation

The transformation of the pCR2.1-Topo plasmid was performed using competent cells from the Max Efficiency® DH10 Bac Kit by Thermo Fisher Scientific (Waltham, MA) following the manufacturer's protocol. The cells were thawed on ice. Then 100 µl of them were transferred into a chilled 14 ml polypropylene tube together with 1 ng of the plasmid and left on ice for 30 minutes. The cells were transformed by a heat-shock treatment at 42°C for 45 seconds and then placed on ice again for 2 minutes. A volume of 0.9 ml S.O.C media at room temperature was added and placed in the shaker at 37°C at 225 rpm for 1 hour. The cells were then streaked out on prepared LB-agar-ampicillin^{+} plates and incubated overnight at 37°C.

2.2.2.2. Inoculation and Plasmid Isolation

After 24 hours of incubation single colonies were picked, inoculated in 2 ml LB-liquid-ampicillin^{+} media and incubated at 37°C. After 6 hours the culture was expanded to 100 ml and incubated overnight for another 16 hours. The plasmid isolation was performed using the QIAGEN®Plasmid Maxi Kit following the manufacturer's protocol and DNA was eluted in 500 µl elution buffer (EB).

2.2.2.3. DNA Measurement

The purified DNA was then measured with the NanoDrop1000 Spectrophotometer using the Nucleic Acid Application module. After adding 2 µl of the purified sample, the photometer determines the sample concentration by measuring the absorbance at 260 nm wavelength.

2.2.2.4. Plasmid Preparation

In order to prepare the vector for the insert, we performed a restriction digestion with the restriction enzymes Spe I-HF and EcoR V-HF. Both enzymes are high-fidelity enzymes that have a 100% activity in CutSmart Buffer at 37°C. The reactions were incubated at 37°C for 1 hour in the Incu Block by Denville Scientific.

	Spe I-HF (µl)	EcoR V-HF (µl)	Spe I-HF & EcoR V-HF (µl)	DNA only (µl)
enzyme	1	1	5 (each)	No enzyme
CutSmart	5	5	10	No buffer
DNA 100 ng/µl 1400 ng/µl	5	5	3.6	5
H_2O	39	39	76.4	45
total	50	50	100	50

Table 2: Restriction Digestion Reactions of pCR2.1 Topo

After the restriction digestion, an agarose-gel-electrophoresis was performed. A 6x loading dye was added to the reactions and loaded on a 1% agarose gel, containing 400 ng/ml of ethidium bromide (EtBr). The gel was run in 1x TAE buffer for approximately 45 minutes at 125V. After the run, a picture was taken and the fragments were analyzed referring to the GeneRuler 1 kb Plus. The digest with Spe I-HF and EcoR V-HF removed an insert of approx. 150 bp. The remaining vector is linearized and ready for a new insert. The appropriate bands were extracted and stored at 4°C. All gel pictures were taken under UV light using the Molecular Imager® Gel Doc EQ System by Bio-Rad.

2.2.2.5. Gel Purification

The gel purification as performed with the Thermo Fisher GeneJet Gel Extraction Kit. The required band were cut from the gel with a scalpel under UV and transferred into a microcentrifuge tube. The band was weighed and an equal amount of Binding Buffer was added to the tube (mg = µl). After 15 minutes of incubation at 55°C, the suspension was transferred to a spin column and centrifuged for 1 minute. The column was washed with 700 µl Washing buffer and eluted in 30 µl. The elution was measured with the NanoDrop and stored at 4°C.

2.2.3. stHIV-C8457.3

stHIV-C8457.3 is the parental strain of stHIV-C8457P13A and was used for the very first inoculation of PM1 back in 2011. The plasmid will be used to compare stHIV-C8457P13A with its parental predecessor.

2.2.3.1. Inoculation and Plasmid Isolation

Since the plasmid was stored in a -80°C glycerol stock, it was necessary to streak the bacteria on a LB-agar ampicillin$^+$ plate. A loop-tip with transformed bacteria was used for the inoculation and streaked on the warmed up agar plate. From that agar plate, we picked five clones and inoculated them in 5 ml of LB ampicillin$^+$ liquid medium. The cultures were incubated at 30°C and 225 rpm for 16 hours. After positive verification via restriction digest, the best three clones were expanded into 100 ml of LB liquid ampicillin$^+$ medium. After another 16 hours of incubation, the plasmid was extracted using the QIAGEN®Plasmid Maxi Kit following the manufacturer's instructions. The plasmid was eluted in 500 µl EB and the concentration was measured with the NanoDrop1000 Spectrophotometer.

2.2.3.2. Transfection and Virus Production

For transfection of the plasmid, the HEK293T/17 cell line was used to transfect the proviral DNA. Five wells of a 6-well plate were used containing three different plasmid preps, one positive and one negative control. As a positive control a stHIV-C8457.3 clone was chosen, which was already tested positively before. First, 10^6 cells were seeded into each well together with 2 ml of 293T cell media. The cells were incubated for one day to give them time to grow. After 24 hours, the cells should have reached a confluency of approx. 70%. One half of the media was removed and 100 µl of the following mixture with approx. 2 µg DNA was added.

	conc. (ng/µl)	X-treme gene reagent (µl)	DNA (µl)	Opti-MeM (µl)
stHIV-C8457.3 (#1)	1100	6	2	89.1
stHIV-C8457.3 (#2)	500	6	4	89.1
stHIV-C8457.3 (#3)	600	6	3.5	89.1
positive control	50	6	40	89.1
negative control	No DNA	6	No DNA	89.1

Table 3: Transfection Mix of stHIV-C8457.3

The transfection mix was added very slowly, drop by drop, for optimal efficiency. After an incubation of 3 hours at 37°C, the other half of the 293T cell media was added. The cells were left for 24 hours. After one day, the media was aspirated and replaced. After two more days, the supernatant was collected (day 3 after inoculation) and stored at -80°C.

2.2.3.3. Screening for Virus Production

In order to test the capability of the infectious molecular clone (IMC) to grow actual virus, we performed a p24 ELISA assay, using the pre-coated HIV-1 p24 Antigen Capture ELISA Assay by ABL Inc. (Rockville, MA). This ELISA assay screens for the presence and concentration of p24, the HIV capsid protein. First, 25 µl of disruption buffer was added into each well of the pre-coated 96-well plate. The standards were serially diluted 1:2 with an antigen concentration ranging from 100 pg/ml to 3.1 pg/ml. Then, 100 µl of the standards as well as 100 µl of neat viral supernatant was added into each well. Two negative controls with R-15 medium were used. The plates were incubated at 37°C for 1 hour and then washed three times with 300 µl p24 Washing Buffer per well with the 405 Microplate Washer by BioTek. After the plate washing step, 100 µl of conjugate solution was added per well, and the plates were incubated at 37°C for another hour, followed by another washing step. Next, 100 µl of peroxidase substrate (substrate solution) was added into each well and the plates were incubated at room temperature in the dark for 30 minutes. Finally, the reaction was stopped by adding 100 µl of stop solution into each well. The plates were then placed in the Mithras LB 940 Microplate Reader by Berthold Technologies. The microplate reader is measuring the absorbance at 450 nm wavelength. By interpolating the data from the standard curve, the concentration of the samples can be determined.

2.2.3.4. Assay to Assess the 50% Tissue Culture Infectious Dose ($TCID_{50}$)

The $TCID_{50}$ assay, or in this case more specifically the TZM-bl assay, is a common tool to test the infectivity of immunodeficiency viruses. TZM-bl cells are exposed to the virus to be tested – in our case, the stHIV-C8457.3 strain. The TZM-bl cells carry a luciferase reporter gene under the control of the HIV-1 LTR. Without Tat, no luciferase is generated in TZM-bl cells. Upon infection with primate immunodeficiency viruses, Tat is generated and triggers the transcription of the luciferase gene.

First, 100 µl of TZM-bl media was added into each well of three 96-well flat bottom plates followed by 25 µl of virus supernatant in 4 wells of the first row. In order to detect the limit of infectivity a 5-fold serial dilution was performed until row 11 and the last 25 µl were discarded. The last row served as a background luminescence control. Then, 100 µl of cells (10.000 cells) with DEAE-dextran hydrochloride was added into each with a final concentration of 10 µg/ml. After 48 hours incubation of the plates at 37°C, 100 µl of the supernatant was discarded and replaced with 100 µl of luciferase substrate (Bright-Glo™). The plates were incubated in the dark for at least two minutes and 150 µl of each well was transferred to a Corning™ 96-Well

Clear Flat Bottom Black Microplate by Thermo-Fisher Scientific. The luminescence (relative light units, RLU) was measured with the Centro Pro LB 962 Luminescence Microplate Reader by Berthold Technologies (Bad Wildbad, Germany).

2.2.4. Cloning of stHIV-C8457P13A from Viral RNA

2.2.4.1. PBMC Isolation from Blood

Previously, PBMC were isolated from blood from PM 36227. A volume of 40 ml of blood was drawn in a sodium heparin blood-collection tube. The blood was centrifuged on top of an equal amount of Ficoll media at 400 rpm for 30 minutes with slow deceleration, creating a gradient with plasma on top, then a thin PBMC layer and red blood cells on the bottom of the tube. The plasma was completely removed and stored in liquid nitrogen. The layer of PBMC was isolated and washed three times with R-2 medium. Afterwards, the cells were counted, aliquoted and stored at -80°C in freezing media in a Mr. Frosty Box.

2.2.4.2. Growth of PBMC, $CD8^+$ T-cell Depletion and Inoculation

PBMC from PM 36227 had been isolated from sodium heparin blood. The cells were thawed, seeded, and grown in a T25 Corning® Cell Culture flask with R-15 medium. The cells were activated with Con A (70 U/µl) for two days, followed by stimulation with human IL-2 (40 U/ml medium) for another three days. After the IL-2 incubation, $CD8^+$ T cells had to be removed from the culture in order to guarantee efficient virus growth. For CD8 depletion, the Dynabeads CD8 Kit from Thermo Fisher was used, following the manufacturer's instructions. First, 50 µl of beads per 1 x 10^7 cells were washed in depletion buffer. After the wash, 4.5 x 10^7 cells were incubated with 225 µl beads at 4°C for 30 minutes. After the incubation, the beads were attached to the cells and could be separated in a magnetic field. After the depletion, 2 x 10^7 cells were left and used for the production of the virus

The cells were inoculated with a previously prepared solution containing concentrated stHIV-C8457P13A. The cells were kept in R-15 medium containing IL-2 and TNF-α (10 U/ml) and the supernatant was collected for 15 – 21 days.

2.2.4.3. Virus Screening

The collected supernatants from day 2, 3, 6, 8, 10, 13, 15 were screened for the presence and concentration of p24, the capsid protein of HIV-1, using the pre-coated HIV-1 p24 Antigen Capture ELISA Assay by ABL Inc. (Rockville, MA). The supernatants were measured in three different dilutions: neat, 1:20 and 1:200. The standards range from 3.1 pg/ml to 100 pg/ml. Under these conditions, we performed the p24 Assay.

2.2.4.4. Virus Concentration with Ultracentrifugation

In order to get highly purified RNA from the virus, 10 ml of supernatant from the culture was used for purification. First, 2 ml of 20% sucrose was added into an Ultra-Clear™ tube by Beckman Coulter. Then, 10 ml of supernatant was added with another 2 ml of PBS on top. The tube was centrifuged at 100,000 x g for 2 hours. After the centrifugation, the supernatant was removed very slowly and the tube dried out. After most of the liquid was removed from the tube, 160 µl of PBS pH 7.2 was added to the viral pellet, and the tube was incubated at 4°C for three days. On the third day, the viral pellet was resuspended gently 30 times until the entire concentrated virus was dissolved from the tube.

2.2.4.5. RNA Extraction

For RNA extraction, the QIAamp viral RNA (vRNA) extraction kit was used following the manufacturer's instructions. First, three 1.5 ml tubes with 560 µl of AVL (lysis buffer) were prepared with 5.6 µl of carrier RNA-AVE. Then, the virus was distributed among these tubes. The tubes were left for 5 minutes at room temperature before 560 µl of 100% ethanol was added. The product was transferred and spun at 8,000 rpm for 2 minutes one by one into one spin column until the entire viral RNA was collected in one column. The tube was washed with 500 µl AW1, 500 µl AW2 and finally eluted into two tubes with 30 µl AVE (elution buffer) each. The NanoDrop confirmed an RNA concentration of 380 ng/µl and 115 ng/µl.

2.2.4.6. cDNA Synthesis from vRNA

The RNA was transcribed into complementary DNA (cDNA) with the Proto Script First Strand cDNA Synthesis Kit by New England Biolabs. The reverse transcription was conducted under the manufacturer's instructions. First, 2,280 ng of RNA (6 µl) and 2 µl the Oligo d(T)23VN primers were mixed in an 8 µl reaction. The secondary structure of the RNA was denatured at 70°C for 5 minutes, followed by short spinning and incubation on ice. Then, 10 µl of the M-MuLV reaction mix (dNTPs and buffer) and 2 µl of the M-MuLV enzyme mix (RT, etc.) were added. The 20 µl reaction was then incubated at 42°C for one hour. After the reverse transcription, the enzymes were inactivated by incubating them at 80°C for 5 minutes. The viral cDNA was diluted to 50 µl and stored at -20°C. For this PCR, the GeneAmp™ PCR System 9700 Thermo Cycler by Applied Biosystems was used.

	viral cDNA (µl)	stHIV-C8457.3 (µl)
Q5 master mix	12.5	12.5
HIV gag FWD primer	1.25	1.25
HIV gag REV primer	1.25	1.25
DNA	2.5	2
UltraPure H_2O	7.5	8
total volume	25	25

Table 4: *gag* PCR Reactions for the viral cDNA

In order to prove that the cDNA synthesis worked, we performed a PCR that amplifies an 84 bp amplicon of the highly conserved *gag* region. As a positive control, a stHIV-C8457.3 clone was chosen. The PCR product was analyzed on a 2% agarose gel, which was run for 1.5 hours at 135V.

	Initial Denaturation	35 Cycles			Final Extension	Hold
Temperature	98°C	98°C	60°C	72°C	72°C	4°C
Time	30 sec	15 sec	30 sec	1 min	2 min	Hold

Table 5: PCR Conditions

2.2.4.7. PCR Amplification

The long fragment amplification PCR was conducted with a Thermo Scientific 10x Taq Buffer, containing $(NH_4)_2SO_4$, KCl and 20 mM $MgCl_2$. The polymerization was performed by the Thermo Scientific Dream Taq Polymerase (5 U/µl). The amplification will be performed in two steps. First, the whole genome will be amplified in four fragments, designated A, B, C and D. The fragments were amplified in a larger scale as the positive and negative control as they were extracted from the gel and used for the following cloning. The PCR product was then be loaded onto a 0.8% agarose gel with 400 ng/ml EtBr; the expected band was extracted and used for the assembly.

	viral cDNA (µl)	positive control (µl)	negative control (µl)
10x Taq buffer	5	2.5	2.5
dNTPs	5	2.5	2.5
FWD primer	2.5	1.25	1.25
REV primer	2.5	1.25	1.25
Dream Taq	0.5	0.25	0.25
DNA	4	2	No DNA
Ultrapure H_2O	30.5	15.25	17.25
total volume	50	25	25

Table 6: PCR Reactions for viral cDNA amplification conditions

	Initial Denaturation	35 Cycles			Final Extension	Hold
Temperature	95°C	95°C	65°C	72°C	72°C	4°C
Time	3 min	30 sec	30 sec	3 min	5 min	Hold

Table 7: PCR Conditions first Amplification

2.2.4.8. Gel Purification

In order to extract the desired fragments from the gel, the Thermo Fisher GeneJet Gel Extraction Kit was used, following the manufacturer's instructions. First, the bands were cut out of the agarose gel under UV. The bands were weighed and an equal amount of Binding Buffer was added to the tube (mg = µl). The mix was incubated at 55°C for 15 minutes until the gel was completely dissolved. The solution was added to a gel extraction purification column. The tubes were spun for one minute and 700 µl washing buffer was added into each tube. The columns were spun twice to remove the entire residual buffer. In the end, the fragments were eluted in 30 µl of EB (QUIAGEN) to maximize the concentration. After the first elution, the suspension was re-inserted into the column and eluted a second time.

2.2.4.9. Gibson Assembly

The assembly was designed with the NEBuilder Assembly Tool. For the assembly of the five fragments (vector, A, B, C and D) an equal ratio of pmol was used in the reaction.

	size (bp)	0.05 pmol (ng)
pCR2.1-Topo	3876	128
Fragment A	2347	77
Fragment B	2642	87
Fragment C	3204	106
Fragment D	2054	68

Table 8: Amount of DNA of all Fragments Used for the Gibson Assembly. For the cloning, the molar weight of the DNA fragments was calculated with the following formula:

$$pmol\ DNA = \mu g\ \mathrm{DNA} * \frac{pmol}{660\ pg} * \frac{10^6\ pg}{1\ \mu g} * \frac{1}{bp}$$

	conc. (ng/µl)	amount (µl)	0.05 pmol (ng)
pCR2.1-Topo	71	1.9	128
Fragment A	28	2.8	77
Fragment B	61	1.5	87
Fragment C	56	1.9	106
Fragment D	36.5	1.9	68
2x Master Mix	2x	10	No DNA

Table 9: Gibson Assembly Reaction Mix. For the required amount of DNA was calculated for a total reaction volume of 20 µl and a total molarity of 0.25 pmol.

For the Gibson Assembly, the NEBuilder HiFi Assembly Master Mix was used following the manufacturer's instructions. No less than 0.05 pmol of each fragment was used, adding up to a total amount of 0.25 pmol of DNA. First, the 2x Assembly Master Mix was added into a microcentrifuge tube as well as the required amount of each fragment (see Table 8). The reaction mix was incubated in a thermocycler at 50°C for 4 hours. The product was kept on ice for the following transformation of s*tbl2* chemically competent *E. coli*.

2.2.4.10. Transformation, Plating and Colony PCR

In order to guarantee proper growth of the newly cloned plasmid, the *stbl2* competent cells were chosen for transformation. At the beginning, 100 µl of thawed cells were transferred to a 5 ml polypropylene tub. Then, 1 µl of the suspension was added to the cells. As a positive control, the pUC19 control plasmid was used. The mixture was gently suspended and placed on ice for 30 minutes, followed by a heat-shock phase of accurately 25 seconds at 42°C. The tube was placed on ice again for two more minutes. Together with 900 µl of S.O.C. medium, the tube was placed in a shaker at 30°C for 60 minutes at 225 rpm. During the incubation an LB-agar, the ampicillin$^+$ plate was warmed to 30°C. After shaking, the cells were centrifuged at 8,000 rpm on a tabletop centrifuge for 3 minutes. The supernatant was discarded, and the cell pellet was resuspended in 100 µl of S.O.C. medium. This way all cells were spread on the agar plate since the efficiency of the ligation was unclear. The plates were incubated overnight at 30°C. The positive control plates confirmed the positive transformation. Since the growth of this bacterial strain is very slow and the replication of this large plasmid happens slowly, the plate needed to be incubated for at 24 hours. After 24 hours, a colony PCR with 20 separate colonies was performed to confirm the presence of HIV-1 *gag* DNA. We prepared the PCR reaction and inoculated the mix reaction with a loop tip of the colony. As a positive control, stHIV-C8457.3 was used.

	Colony 1-20 (µl)	positive control (µl)	negative control (µl)
10x Taq buffer	2.5	2.5	2.5
dNTPs	2.5	2.5	2.5
FWD primer	1.25	1.25	1.25
REV primer	1.25	1.25	1.25
Dream Taq	0.25	0.25	0.25
DNA or colony	colony	2	No DNA
Ultrapure H_2O	17.25	15.25	17.25
total volume	25	25	25

Table 10: Colony PCR Reactions

	Initial Denaturation	35 Cycles			Final Extension	Hold
Temperature	95°C	95°C	65°C	72°C	72°C	4°C
Time	3 min	30 sec	30 sec	3 min	5 min	Hold

Table 11: Colony PCR Conditions

2.2.4.11. Inoculation and Plasmid Preparation

After 36 hours of incubation, 10 single colonies were selected and inoculated into 2 ml of LB liquid ampicillin^{+} medium and incubated for 6 hours. After six hours, the culture was expanded to 12 ml and incubated overnight. The next day, 10 ml of the culture were used for a plasmid quality control and isolation, whereas the other 2 ml were used for expansion to 100 ml media. The 10 ml cell suspension was divided into two separate Mini Plasmid Preparations and was collected in one spin column for maximized yield. First, two times 5 ml of culture was spun on a tabletop centrifuge for 3 minutes. The supernatant was discarded and the pellet was resuspended in 250 µl P1 (suspension buffer) buffer containing LyseBlue. After suspension of the cell pellet, 250 µl P2 (lysis buffer) was added and the tube inverted until the suspension turned blue. After adding 350 µl N3 (neutralization buffer), the suspension lost the blue color. The mixture was transferred into a spin column and centrifuged. After washing the spin column with ETR (endotoxin removal buffer) and PE (wash buffer), the DNA was eluted in 30 µl EB, re-inserted to the column and eluted again. Before expanding the culture to 100 ml, the quality of the plasmids was verified via restriction digestion.

2.2.4.12. Plasmid Purification from Vector Contamination

The isolated plasmid was loaded on a 0.8% agarose gel containing 400 ng EtBr per ml. In order to purify the ligated plasmid from vector contamination, the DNA was run on the gel with a stHIV-C8457.3 control at 125V for 45 minutes. The required bands representing the linearized and circular form of stHIV-C8457P13A were cut out and purified from the gel. We re-ligated the linearized plasmid and re-transformed both into the *stable* Competent *E. coli* by New England Biolabs. The transformed cells were spread on a LB agar ampicillin^{+} plate and incubated at 30°C for 24 hours. After incubation overnight, 10 colonies were inoculated in 2 ml LB liquid ampicillin^{+} medium in the morning and placed in the shaker at 250 rpm at 30°C for 8 hours. We expanded the medium volume to 7 ml after 8 hours and incubated the culture at the same conditions for another 16 hours. The plasmid was verified a second time and expanded.

2.2.5. Cloning of stHIV-C8457P13A from Genomic DNA

2.2.5.1. Extraction of Genomic DNA

For the extraction of the genomic DNA of the cells, we used the QUIAGEN DNeasy Blood and Tissue Kit, following the manufacturer's instructions. First, we washed 2 ml with approx. 9 x 10^6 cells in 10 ml PBS. The solution was split into two tubes with 5 ml each. The following steps were conducted for both tubes separately since the maximal amount of cells for the kit is 5 x 10^6. The tubes were centrifuged at 400 x g for 10 minutes. The PBS supernatant was discarded and aspirated completely. We added 200 µl of PBS, 20 µl Proteinase K and 200 µl of buffer AL. The tubes were mixed thoroughly and vortexed. After incubation at 56°C for 10 minutes, we added 200 µl of 100% ethanol and mixed the solution thoroughly by vortexing. The solution was transferred to the QUIAGEN spin column and centrifuged at 6,000 x g for 1 minute. We added 500 µl of AW1 and centrifuged the column again at 6,000 x g for 1 minute, followed by another washing step with 500 µl of AW2 and centrifugation at 20,000 x g for 3 minutes. The spin column was transferred into a new collection tube and 150 µl of AE was added. The columns were incubated at room temperature for 2 minutes prior centrifugation at 6,000 x g for 1 minute. After the first elution, this step was repeated once, yielding a final elution volume of 300 µl.

2.2.5.2. Long Fragment Amplification

For the cloning of the proviral DNA, we amplified the whole proviral genome of stHIV-C8457P13A in two fragments that include the 5' and 3' LTR. We used the Thermo Fisher Scientific 10x Taq Buffer, containing Taq optimized $(NH_4)_2SO_4$, KCl and 20 mM $MgCl_2$. The reaction also contained the Dream Taq High-Fidelity Polymerase (5 U/µl) and dNTPs by Thermo Fisher Scientific. For the amplification of Fragment 1, we used the Fragment A FWD and the Fragment B REV primers. Fragment 2 was amplified with the Fragment C FWD and Fragment D REV primers. The PCR products were loaded on a 0.8% agarose gel with 350 ng/µl of EtBr. The gel was run at 125V or approx. 45 minutes.

	genomic DNA (µl)	positive control (µl)	negative control (µl)
10x Taq buffer	2.5	2.5	2.5
dNTPs	2.5	2.5	2.5
FWD primer	1.25	1.25	1.25
REV primer	1.25	1.25	1.25
Dream Taq	0.25	0.25	0.25
DNA	2	2	No DNA
Ultrapure H_2O	15.25	15.25	17.25
total volume	25	25	25

Table 12: PCR Reaction for Long Fragment Amplification

Fragment 1	Initial Denat.	10 Cycles			25 Cycles			Final Ext.	Hold
Temp.	95°C	95°C	60°C	72°C	95°C	60°C	72°C	72°C	4°C
Time	30 sec	15 sec	30 sec	5 min	15 sec	30 sec	5 min	5 min	Hold

Fragment 2	Initial Denaturation	35 Cycles			Final Extension	Hold
Temperature	95°C	98°C	60°C	72°C	72°C	4°C
Time	30 sec	15 sec	30 sec	5 min	5 min	Hold

Table 13: PCR Conditions for Fragment 1 and 2

2.2.5.3. Gel Purification

In order to extract the desired fragments from the gel, the Thermo Fisher GeneJet Gel Extraction Kit was used, following the manufacturer's instructions. First, the bands were cut out of the agarose gel under UV. The bands were weighed and an equal amount of binding buffer was added to the tube (mg = µl). The mix was incubated at 60°C for 15 minutes until the gel was completely dissolved. The solution was added to a gel extraction purification column. The tubes were spun for one minute and 700 µl washing buffer was added into each tube. The columns were spun twice to remove the entire residual buffer. In the end, the fragments were eluted in 20 µl of EB (QUIAGEN), which was previously warmed up to 60°C to maximize the final yield. After the first elution, the suspension was re-inserted into the column and eluted a second time.

	volume (µl)	concentration (ng/µl)
Fragment 1	18	8.8
Fragment 2	18	27.8

Table 14: Elution from Gel Extraction of Fragment 1 and 2

2.2.5.4. Re-Amplification

In order to gain a higher concentrated product, we re-amplified the fragments from the extracted band of the first PCR. The PCR was conducted under the exact same conditions in a larger scale with twice the volume.

	extracted Bands (µl)	positive control (µl)	negative control (µl)
10x Taq buffer	5	2.5	2.5
dNTPs	5	2.5	2.5
FWD primer	2.5	1.25	1.25
REV primer	2.5	1.25	1.25
Dream Taq	0.5	0.25	0.25
DNA	10	2	No DNA
Ultrapure H_2O	24.5	15.25	17.25
total volume	50	25	25

Table 15: PCR Reactions for Re-Amplification of Fragment 1 and 2

After successful re-amplification of the fragments, we extracted them again from the agarose gel and measured their concentration with the NanoDrop.

2.2.5.5. Gibson Assembly

We ligated the products with the Gibson Assembly strategy. The primers have a 25-30 bp overhand that aligns with the following fragments. For the ligation reaction, we used the 2x High-Fidelity NEBuilder DNA Assembly Master Mix. For the reaction of the three fragments (Fragment 1, Fragment 2 and pCR2.1-Topo) a vector:insert ratio of 1:2 was recommended. Therefore, we ligated 0.06 pmol of both inserts with 0.03 pmol of the vector in the mix summing up to a total DNA amount of 0.15 pmol (recommended maximum: 0.2 pmol). The mix was incubated at 50°C for 15 minutes in a thermocycler.

	size (bp)	ng	pmol
pCR2.1-Topo	3876	77	0.03
Fragment 1	4915	195	0.06
Fragment 2	5529	207	0.06

Table 16: Required DNA Amount for Gibson Assembly of Fragment 1 & 2 and pCR2.1-Topo. For the cloning, the molar weight of the DNA fragments was calculated with the following formula:

$$pmol\ DNA = \mu g\ \mathrm{DNA} * \frac{pmol}{660\ pg} * \frac{10^6\ pg}{1\ \mu g} * \frac{1}{bp}$$

	conc. (ng/µl)	amount (µl)	DNA (ng)
pCR2.1-Topo	71	1.08	128
Fragment 1	42	4.64	77
Fragment 2	67	3.08	87
dH_2O	No DNA	1.2	No DNA
2x Master Mix	2x	10	No DNA

Table 17: Gibson Assembly Reaction Mix of Fragment 1 & 2 and pCR2.1-Topo

2.2.5.6. Transformation and Plating

We transformed the final ligation product into the NEB *stable* competent *E. coli*, cells that grow at 30°C and support the replication of large plasmids. First, we thawed the cells on ice and transferred 50 µl of the cells into a 5 ml polypropylene tube. We added 2 µl of the ligation product to the cells and gently mixed them. As a positive control, the pUC19 plasmid was used. The tubes were left on ice for 30 minutes, followed by heat shock at exactly 42°C for 25 seconds. After the heat shock, the cells were placed on ice again for 5 minutes. We added 950 µl of Stable Outgrowth Medium to the tube and placed the cells in a shaker at 30°C at 250 rpm for 60 minutes. After shaking, we transferred the cells to a 1.5 ml Eppendorf tube and centrifuged the cells to a pellet at 8,000 rpm on a tabletop centrifuge for 3 minutes. The supernatant was discarded, and the cell pellet was resuspended in 100 µl of the same medium. We spread all 100 µl of the concentrated cells on a LB agar ampicillin$^+$ plate and incubated the agar plates for 24 hours at 30°C

2.2.5.7. Inoculation and Plasmid Isolation

After 24 hours of incubation at 30°C, we picked ten colonies from the plate and inoculated them in 5 ml of LB liquid with ampicillin in a 15 ml polypropylene tube. The tubes were placed in a shaker at 250 rpm for 16-18 hours at 30°C. After approx. 17 hours, we isolated the plasmid with the QUIAGEN Plasmid Mini Kit. We eluted the DNA in 30 µl EB and loaded 300-400 ng of the plasmid on a 0.8% agarose gel containing 350 ng/µl of EtBr. The gel was run at 140V for 30 minutes and analyzed under UV.

2.2.6. Ex Vivo Adaptation

The stHIV-C8457 virus was already adapted ex vivo once after animal passage number seven. The isolated virus was designated stHIV-C8457P7A and was adapted for increased resistance to $CD8^+$ T cells in order to create higher and permanent viremia in PMs. The following passages showed that the virus needed additional ex vivo adaptation. After passage 13, the virus was isolated from PBMC after a maximal blood collection. The PBMC were co-cultivated with naïve PBMC from PM 36227. The cells produced virus and the virus was isolated from the supernatant. The adaptation was continued by increasing the percentage of $CD8^+$ T cells in the culture over time and passaging the virus at peak production from culture to culture. The cultures started with 2% CD8+ T cells and after 21 days of supernatant collection, the percentage was increased to 5%; after another 22 days to 10%; and then to 25%.

2.2.6.1. Screening of Naïve PBMC

At the very beginning of the experiment, it was necessary to choose PBMC from an animal that failed to support the growth of the virus ex vivo in the presence of CD8+ T cells. This way a viral growth after $CD8^+$ T-cell depletion could show that these cells really do inhibit the viral replication and furthermore a slow increase of those cells was thought to allow the virus to adapt gradually to the antiviral factors of the cells. Therefore, PBMC from several PMs were isolated, cultivated and exposed to stHIV-C8457P13A, which was previously isolated from animal PM13 on Day 16. The virus-exposed cells were co-cultivated with naïve non-infected cells from the same animal. The viral supernatant was collected over 14 days. After 14 days, a p24 assay of all supernatants from all animals was conducted. After the assay, animal 33030 (short PLp-2) was chosen since it supported viral growth only after complete depletion of $CD8^+$ T cells.

For the start of the P13A ex vivo adaptation, PBMC from animal 33030 were revived from -80°C. The cells were washed with R-2 medium two times to purify them from the freezing medium and seeded in R-15 medium at a concentration of 2×10^6 per ml. The cells were activated with Con A (70 U/ml) for two days, followed by activation with human IL-2 (40 U/ml) for another three days. After incubation in IL-2 medium, the cells were ready to undergo the $CD8^+$ T-cell depletion as well as purification. The purified $CD8^+$ T cells were set aside to be added at the required amount to the depleted culture.

2.2.6.2. $CD8^+$ T-cell Depletion

The CD8 depletion was performed with the Dynabeads™ CD8 Cell Depletion Kit. Both protocols were performed following the manufacturer's instructions. First, 50 µl beads per 10 million cells were washed with PBS two times and suspended in 1 ml of Depletion Buffer. The washed beads were then added to the cells and incubated at 4°C for at least 30 minutes. After cold incubation, the cells were separated in a magnetic field. The beads attached to all CD8 cells were now separated from the supernatant containing all the remaining cells. The aspirated supernatant was diluted in 10 ml of R-15 medium and the cells counted. The remaining cells were stored in R-15 medium with IL-2 (40 U/ml) at a concentration of 2×10^6 cells per ml.

2.2.6.3. $CD8^+$ T-cell Purification

For the purification, the $CD8^+$ T cell Isolation Kit (non-human primates) from MACS was used. The cells were suspended in 40 µl isolation buffer and 10 µl of $CD8^+$ T-cell Biotin-Antibody Cocktail per 10 million cells. The suspension was mixed well and stored at 4°C for 10 minutes. Next, the cells were washed in 1-2 ml buffer per 10 million cells and again resuspended in 80 µl buffer and 20 µl of Anti-Biotin Beads per 10 million cells. After incubation for another 15 minutes at 4°C, the cells were washed in buffer again and suspended in 500 µl buffer. The $CD8^+$ T cells were the only cells that are not marked with the beads at this point. Next, the cells needed to be separated in a magnetic field. Therefore, the MS Magnetic Separation Column by MACS was used. The column was rinsed with buffer once before the cells were applied to the column. The cells were eluted in 2 ml buffer, counted and incubated at a concentration of 2×10^6 cells per ml.

2.2.6.4. FACS Staining

After purification and depletion, the cells needed to be analyzed with fluorescence-assisted cell sorting (FACS) to evaluate the purity of the cultures. On base of the purity of the purified $CD8^+$ T cells and after verification that almost all CD8 cells were depleted in the second culture, the required number of $CD8^+$ T cells was added to the depleted culture (2%, 5%, 10% or 25%). First, 10^6 cells of both isolated $CD8^+$ T cells and CD8-depleted cells were divided into three 5 ml polypropylene tubes. One tube was to be used as negative culture ('unstained'), one tube served as a stained culture ('stained'), and one tube served as fluorescence minus one sample ('FMO'). Then 10 µl of anti-CD45, 10 µl of anti-CD3, 10 µl of anti-CD8 and 10 µl of anti-CD4 was added to the 'stained' sample. The 'FMO' did not contain anti-CD8 antibodies. The 'unstained' sample did not contain any antibodies. The tubes were incubated for 30 minutes in the dark, followed by washing with PBS and final resuspension in 150 µl of PBS and 150 µl of Cytofix™ Fixation Buffer. The tubes were kept at 4°C for 30 minutes and sent to Vida Hodora for FACS reading.

2.2.6.5. Virus Inoculation of Cell Cultures

The very first cultures were inoculated with stHIV-C8457P13A isolated from cultured PBMC. The cells to be infected were suspended in a very small volume of R-15 medium. The virus was stored in liquid nitrogen in R-15 medium collected from supernatant. The virus was thawed and added to the cells at a high dose. The cells were incubated at 37°C for 4 hours and the tube was shaken and mixed thoroughly every 30 minutes. After 4 hours, the cells were washed and suspended in R-15 media, containing IL-2 (40 U/ml) and TNF-α (10 U/ml), at a concentration of 2×10^6 cells per ml. This day is designated day 0.

2.2.6.6. Supernatant Collection

The cells were kept in Corning T-75 flasks at 37°C with 5% CO_2. Every second or third day, 75% of the cell suspension was collected. The suspension was centrifuged at 400 x g for 10 minutes. The supernatant was transferred into a syringe, filtered through a 0.45 µm filter, and stored in liquid nitrogen. The cell pellet was resuspended in the required volume of R-15 medium for a concentration of 2×10^6 cells per ml.

2.2.6.7. Screening for Virus Production

At 21 days post-inoculation, the amount of virus released into the supernatant was measured with the p24 ELISA assay using the p24 Antigen Capture ELISA Assay kit by ABL Inc. (Rockville, MA) and following the manufacturer's instructions. The supernatants were applied undiluted or at dilutions of 1:20 and 1:200. The supernatant with the highest concentration was used for inoculation of fresh PBMC for $CD8^+$ T cell increase.

3. RESULTS

3.1. Serology of Infected Animals PM5 to PM13

For the in vivo adaptation of stHIV-C8457, 13 PMs were serially inoculated with the virus. The strain was passaged through the macaques by rapid animal-to-animal blood transfer. After passage 13, the virus was re-isolated from PM 36223 for analytical purposes. In order to guarantee that the virus is capable of establishing a persistent reservoir in the new host species, we performed western blots on plasma samples from all PMs that are still alive.

3.1.1. Western Blots

The western blot assays were performed with the GS HIV Type 1 Western Blot Kit from Bio Rad and the QualiCode™ Western Blot Kit by Immunetics. Due to limited availability of the Immunetics kit, we had to switch to the kit by Bio Rad after performing the first assay with PM7, PM8, PM9 and PM10. Consequently, the strips from the different companies cannot be compared. Still, the two assays show seroconversion and the development of anti-HIV antibody development in the animals.

The assay was performed with at least two samples that contain plasma from day 0 of inoculation, pre-infection or shortly after inoculation and the most recent plasma sample available. For some selected animals, we decided to include two more samples in between in order to observe the development over time. PM5 showed a potential case of seroreversion. In order to confirm the decreasing band strength from strip number 4 to 7 in Figure 18 A, we repeated the assay on these samples (Figure 19).

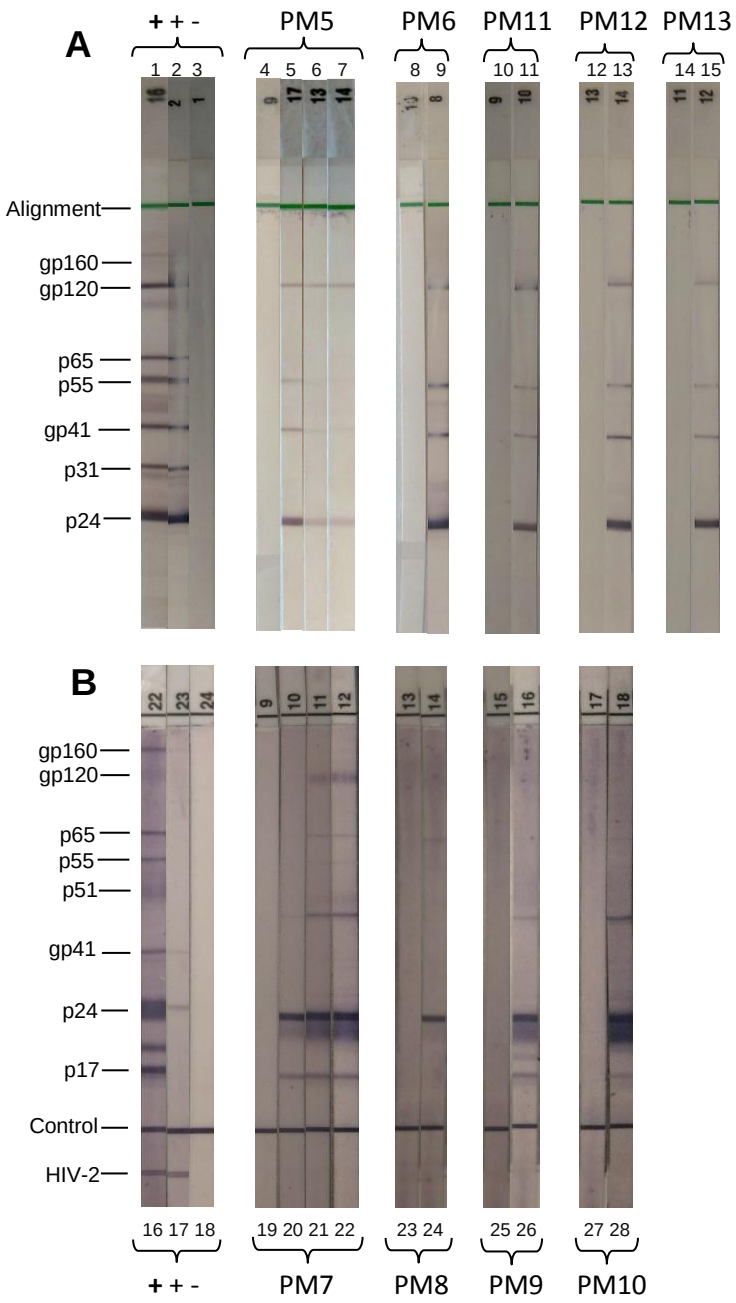


Fig. 18 A

GS Western Blot Kit by Bio Rad

1. Strong positive control
2. Weak positive control
3. Negative control
4. PM5 – Day 2 after inoculation
5. PM5 – Day 262
6. PM5 – Day 864
7. PM5 – Day 1193
8. PM6 – Pre-Infection
9. PM6 – Day 1100
10. PM11 – Day 0
11. PM11 – Day 136
12. PM12 – Day 0
13. PM12 – Day 112
14. PM13 – Day 0
15. PM13 – Day 81

Fig. 18 B

QualiCode™ Kit by Immunetics

16. Strong positive control
17. Weak positive control
18. Negative control
19. PM7 – Day 0 of inoculation
20. PM7 – Day 70 after inoculation
21. PM7 – Day 328
22. PM7 – Day 666
23. PM8 – Day 0
24. PM8 – Day 182
25. PM9 – Day 0
26. PM9 – Day 153
27. PM10 – Day 0
28. PM10 – Day 195

The results show that all animals produce anti-HIV antibodies, which clinically proves that all animals are infected even years after inoculation. All animals show a clear seroconversion. Interestingly, animal 5 shows a potential case of seroreversion since the production of antibodies is decreasing over the years. However, since the western blot procedure is a qualitative and not a quantitative method, we performed an end-point titration ELISA to determine the exact concentration of those antibodies over time. Animal 7 represents a classic case of seroconversion with increasing antibody production over time (Baba et al., 1995). In order to confirm our finding in PM5, we repeated the assay with the same samples.

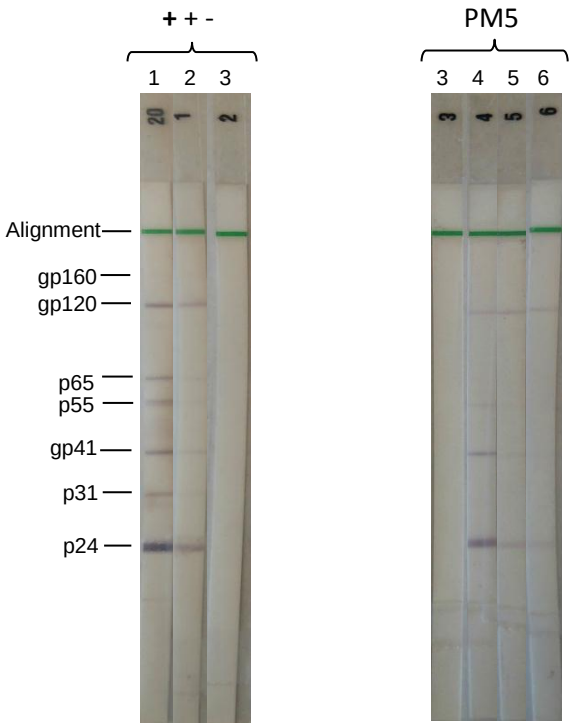


Fig. 19

GS Western Blot Kit by Bio Rad

1. Strong positive control
2. Weak positive control
3. Negative control
4. PM5 – Day 2 after inoculation
5. PM5 – Day 262
6. PM5 – Day 864
7. PM5 – Day 1193

This western blot repeat, confirms our finding that PM5 has a decreasing anti-p24, anti-gp41, and anti-p55 antibody production. The anti-gp120 bands are steady.

3.2. Infectivity of stHIV-C8457.3

The stHIV-C8457.3 plasmid is the parental predecessor of stHIV-C8457P13A. In this thesis, we used it as a positive control for several assays. It served as a template for the primers and as a positive control during PCRs. It was also grown and analyzed for comparative purposes. We multiplied the plasmid and performed a p24 and a $TCID_{50}$ assay. In the following the chapter, we will present the results of the analysis.

3.2.1. Viral Growth of stHIV-C8457.3

The p24 assay of the parental strain stHIV-C8457.3 was performed on three different clones from the same proviral stock. The assay was performed in order to prove that the plasmid is capable of producing a virus that is not only intact but also able to infect cells and multiply.

We transfected the proviral plasmid into HEK293T cells on day 0. The cells were incubated for three days until the supernatant was collected, which served as a template for the p24 assay. Since the concentration of the virus was not important the supernatant from day 3 after transfection was added neat only and no dilutions were made. Figure 20 represents the standard curve from our assay, and consequently its accuracy.

standard (pg/ml)	absorbance
100	1.667
50	0.8
25	0.436
12.5	0.254
6.3	0.129
3.1	0.076

Table 18: Values of the Standards

sample	absorbance
positive control	9.947
negative control	0.028
Clone #1	9.958
Clone #2	9.956
Clone #3	9.951

Table 19: p24 Results of

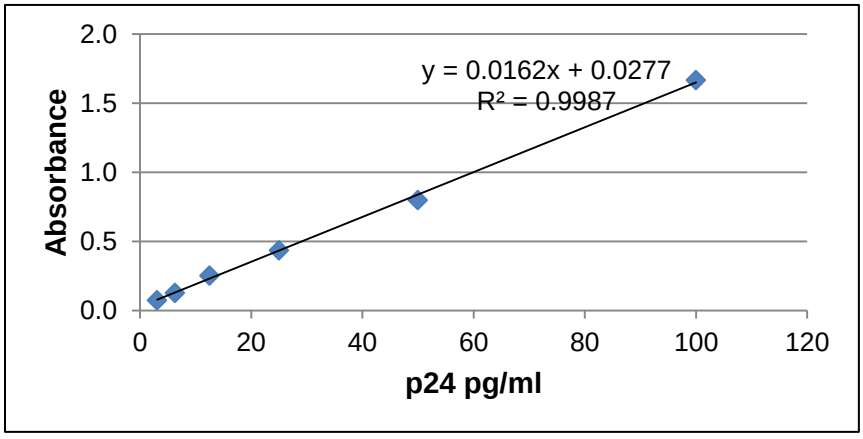


Fig. 20. Standard Curve of stHIV-C8457.3 p24 assay

3.2.2. $TCID_{50}$ of stHIV-C8457.3

The $TCID_{50}$ value is the amount or the dilution of the virus that infects 50% of all cells in the culture. Therefore, a very high $TCID_{50}$ value represents a virus that is highly infective and can infect 50% of all cells at a very high dilution. All $TCID_{50}$ measurements are performed in quadruplicates. The table below represents a $TCID_{50}$ measurement of stHIV-C8457.3, the parental virus of stHIV-C8457P13A.

# pos	4	4	4	4	4	4	0	0	0	0	0	c ctrl.
Mean	98419	148972	271981	95452	12801	3520	1366	936	894	868	869	915
A	91,728	145,929	315,994	83,467	14,318	4,011	1,393	982	990	956	1,024	998
B	106,952	166,501	372,460	115,238	13,453	4,237	1,267	998	915	931	889	1066
C	97,220	138,881	223,827	93,480	9,860	2,836	1,208	923	822	747	680	831
D	97,776	144,578	175,644	89,623	13,571	2,995	1,594	839	847	839	881	764

Table 20. $TCID_{50}$ Measurement Results of stHIV-C8457.3. The relative light units (RLU) measurement is performed in quadruplicates and 5-fold dilutions. All four rows A-D are the same virus and all rows 1-11 are the same dilutions starting with 1:5 in row 1. The last row was kept blank with R-15 instead of a viral dilutions and serves as a blank and as a negative control.

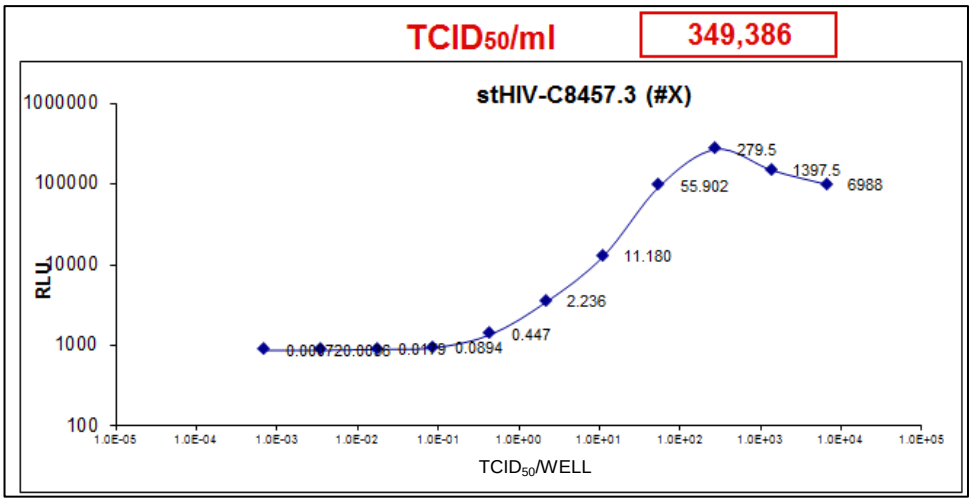


Fig. 21. $TCID_{50}$ Graph of stHIV-C8457.3. The graph shows the 5-fold dilutions of the sample and the measured relative light units (RLU). For the calculation of the $TCID_{50}$/ml value, the 'Montefiori Lab $TCID_{50}$ Calculation Tool' was used (Montefiori, 2006). The value determines the required dilution of a virus that is necessary to9 infect 50% of all cultures. In this case it means that the virus needs to be diluted 350,000 fold to infect 50% of tissue cultures.

3.3. Ex Vivo Adaptation of stHIV-C8457P13A

During early passages in PMs, stHIV-C8457 was easily inhibited by the soluble antiviral factors secreted by $CD8^+$ T cells. Therefore, all PMs needed to be immunodepleted prior inoculation for proper viral infection. In order to overcome this issue, we adapted the virus to an increased $CD8^+$ T cell amount in vitro. We selected an animal that did not support the viral replication under naïve conditions. We depleted all $CD8^+$ T cells and gradually increased the percentage over time. This way the virus was selected for resistance to the antiviral factors.

3.3.1. Screening PBMC from Different Donors

At the very beginning of the project, eight PMs were chosen for the screening. PBMC were isolated from their blood and seeded. They were infected with stHIV-C8457P13A and supernatant was collected for 14 days. After two weeks a p24 assay was conducted screening for viral growth.

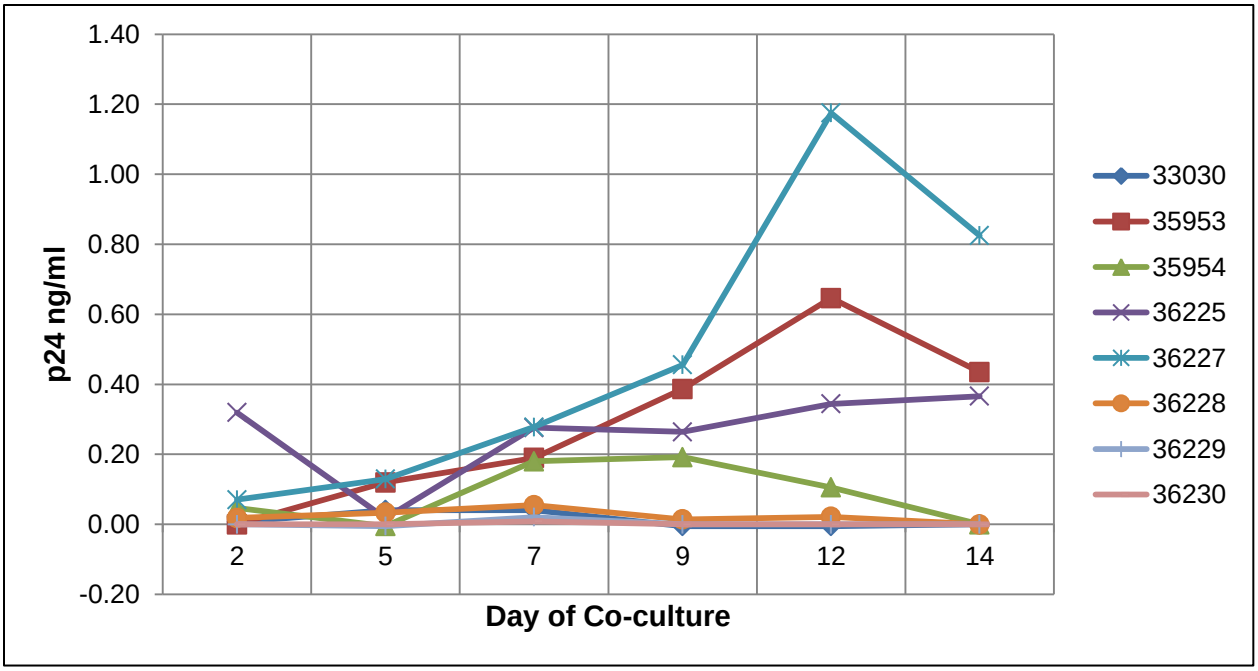


Fig. 22. Screening of Naïve PBMC for Viral Replication. The replication of the unadapted stHIV-C8457P13A isolate was poor in animals 33030, 36229, 36228 and 36230. Animals 35954, 36225, 35953 and 36227 seemed to support the growth of the virus without CD8 depletion. Therefore, PM 33030 (PLp-2) was chosen for the ex vivo adaptation. After CD8 depletion the culture should already show a significant increase of the viral growth. The amount of $CD8^+$ T cells would then be increased over time to adapt the virus to the antiviral soluble factors of $CD8^+$ T cells.

3.3.2. Selection for Virus Resistant to CD8$^+$ T cells

At first, two cultures with CD8 depleted and 2% CD8$^+$ T cells were started. The cell free supernatant was collected every second to third day. After 21 days all supernatants were analyzed for p24 presence. The amount of p24 particles increases proportionally with the number of virions. In this assay supernatants from both cultures, 0% CD8 cells (designated A1) and 2% CD8 cells (designated A2), were used as templates.

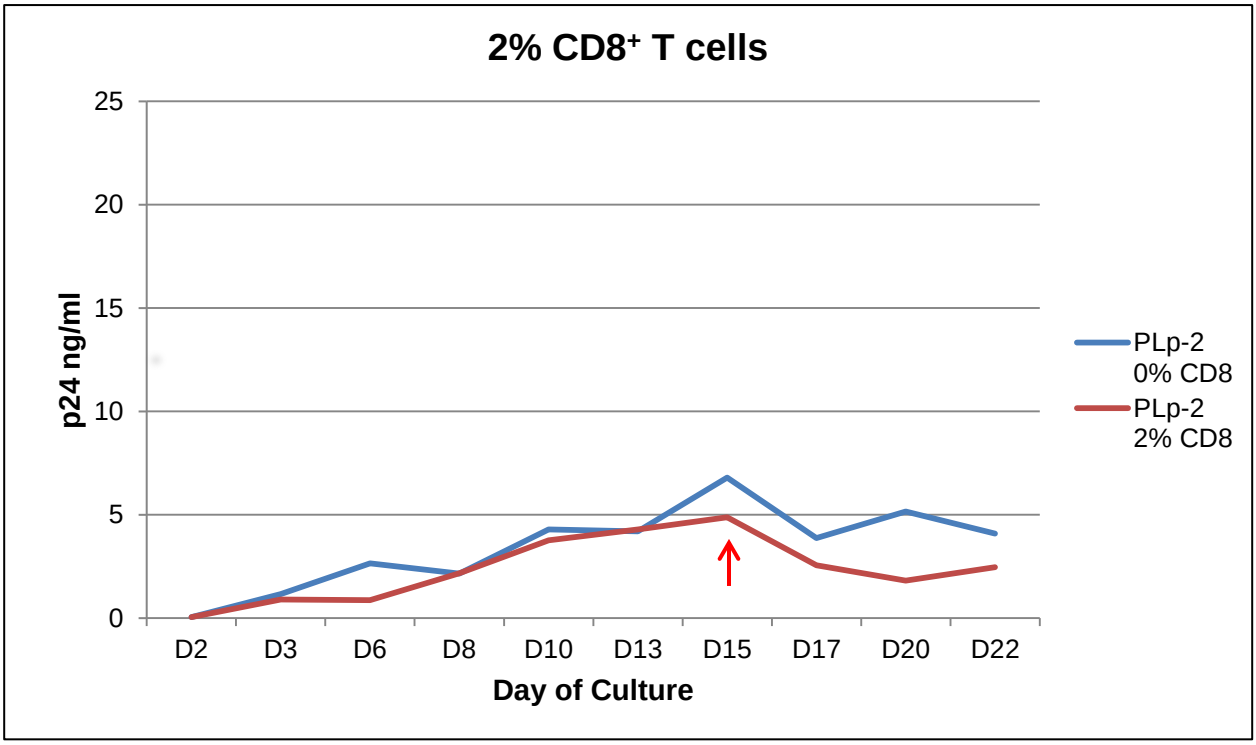


Fig. 23. Ex Vivo Adaptation with 2% CD8$^+$ T Cells. The viral growth curve in this graph shows the amount of p24 particles of both, the CD8 depleted and the 2% CD8$^+$ T cell culture. Not surprisingly, the CD8 depleted culture (red) has a slightly better growth curve of the virus than the culture with 2% CD8 cells (blue) among the peripheral blood mononuclear cells (PBMC). For infection of the next culture, we used the sup with the highest viral concentration (red arrow).

After performing the first p24 assay of cultures 0% and 2%, the supernatant with the highest viral concentration was used to infect the next cultures. We revived PBMC from animal PLp-2 and created two sub-cultures. Again, one culture was $CD8^+$ T cell depleted and other culture was purified $CD8^+$ T cells only. We started two cultures with 2% and 5% $CD8^+$ T cells. For infection of the new culture with 2% (designated B1) and 5% CD8 T cells (designated B2) day 15 of the culture A1 was used as an inoculum.

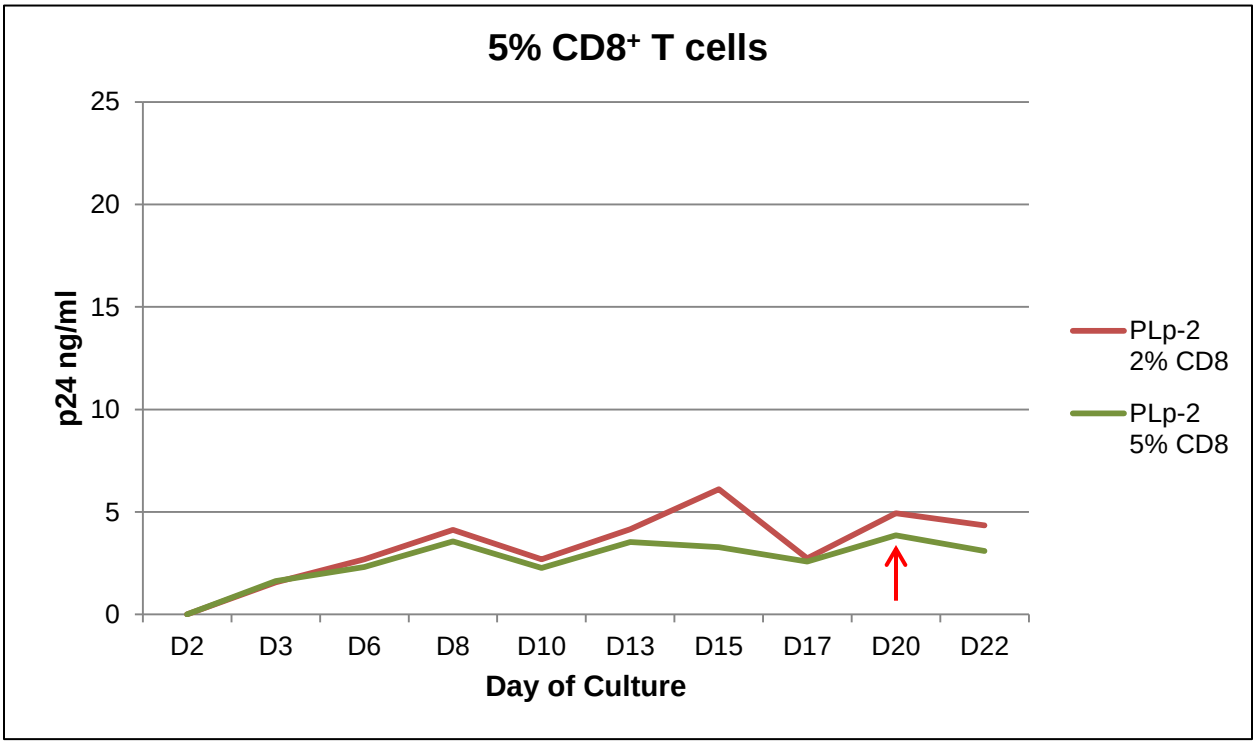


Fig. 24. Ex Vivo Adaptation with 5% CD8 cells. The graph above shows the virus growth curve over 21 days. Clearly the culture with the lower amount of CD8 cells (B1 - blue) grows more virus than the culture with the increased percentage of CD8 cells (B2 - green). Nonetheless, the red curve indicates that the virus is still able to replicate properly even under the increased immune pressure. Again, the day with the highest viral load was chosen for infection of the next cultures (red arrow).

Since the last p24 assay of cultures B1 (2%) and B2 (5%) showed that the virus is capable of growing in the presence of 5% CD8 cells, it was necessary to increase the percentage of the T cells up to 10%. We revived fresh PBMC from animal PLp-2, depleted $CD8^+$ T cells and purified $CD8^+$ T cells. We started two more cultures with 5% (designated C1) and 10% (designated C2) $CD8^+$ T cells. The graph below shows the p24 values after 22 days of viral replication.

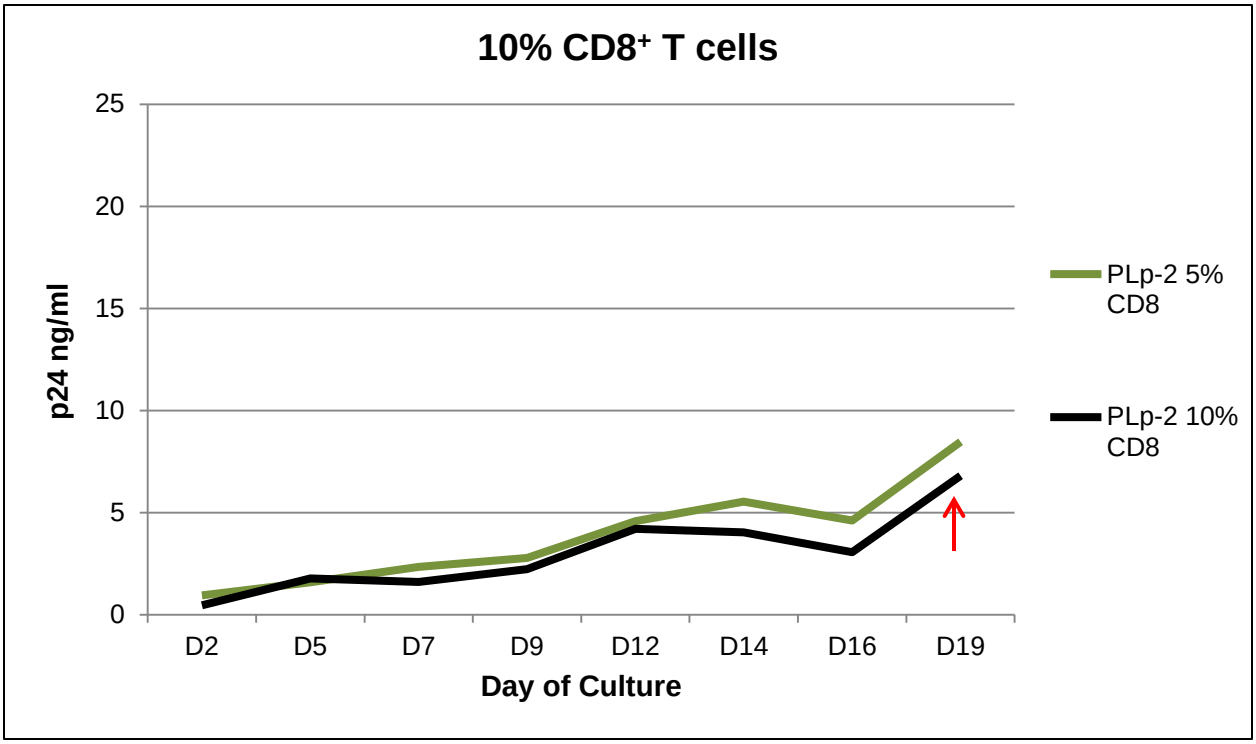


Fig. 25. Ex Vivo Adaptation with 10% $CD8^+$ T cells. The graph above shows the virus growth curve over 21 days. Clearly the culture with the lower amount of $CD8^+$ T cells (C1 - green) grows more virus than the culture with the increased percentage of $CD8^+$ T cells (C2 - black). However, the rising tendency of the growth curves shows that the virus started to adapt and overcome the antiviral activity of the $CD8^+$ T cells. The supernatant with the highest viral concentration was used for infection of the next cultures (red arrow).

The last culture of this assay was performed under 25% $CD8^+$ T-cell pressure. For this culture, we had to isolate fresh PBMC from PM PLp-2 from blood. We depleted $CD8^+$ T cells, purified $CD8^+$ T cells and started two new cultures with 10% (designated D1) and 25% (designated D2) $CD8^+$ T cells. We took supernatant from day 19 of culture B2 (10% $CD8^+$ T cells) and inoculated the fresh cultures. We collected supernatant and performed a p24 assay after D22.

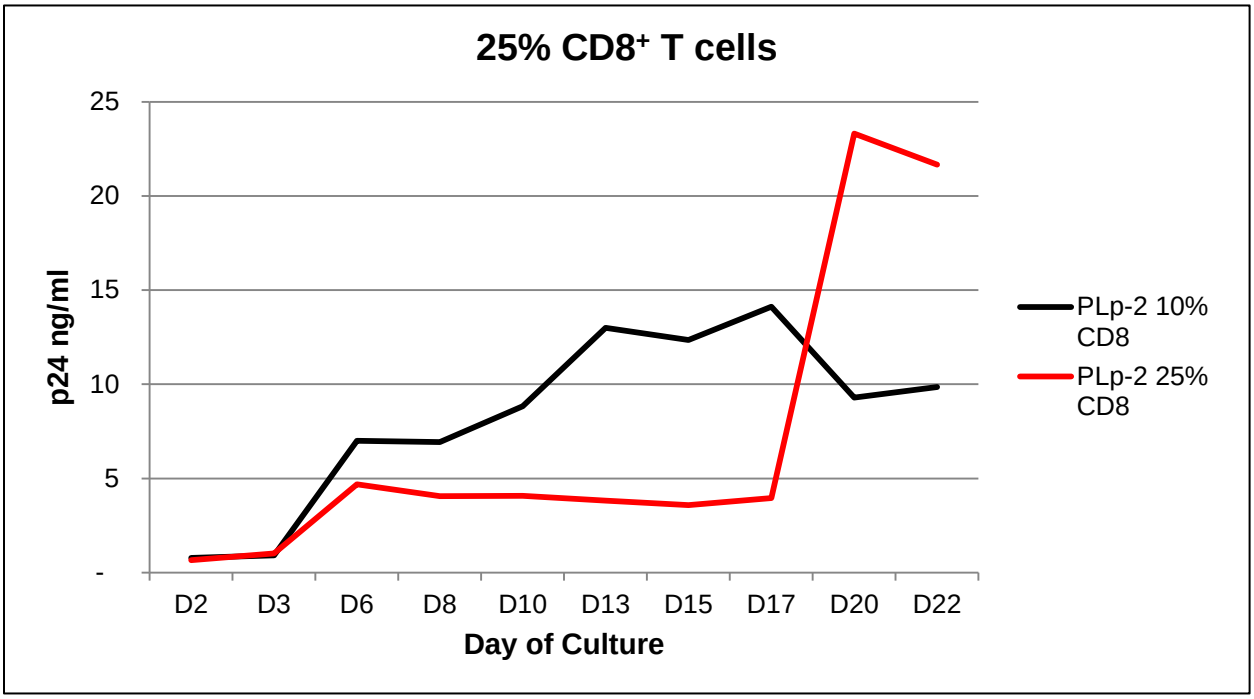


Fig. 26. Ex Vivo Adaptation with 25% $CD8^+$ T cells. The graph shows that the virus already had an increased replication in the backup culture D1 with 10% $CD8^+$ T cells. Surprisingly, the virus seemed to overcome antiviral activity after day 17. Culture D2 with 25% $CD8^+$ T cells has an almost 5-fold increased viral replication after this day. Also, the viral growth in our 10% backup culture (D1) is increased 3-fold compared to our first 10% culture (C2). This culture shows the best p24 data of all cultures although it contains the highest percentage of $CD8^+$ T cells.

3.3.3. Summary of All Cultures

The adaptation of stHIV-C8457P13A caused significant adaptation of the virus to $CD8^+$ T cells. The virus was able to replicate properly even in the presence of 25% $CD8^+$ T cells in the culture. Although the viral growth curve declines with increasing CD8 percentage, it was still able to replicate and future in vivo experiments will show the virus' ability to replicate even without immunodepletion in the next PMs of the series.

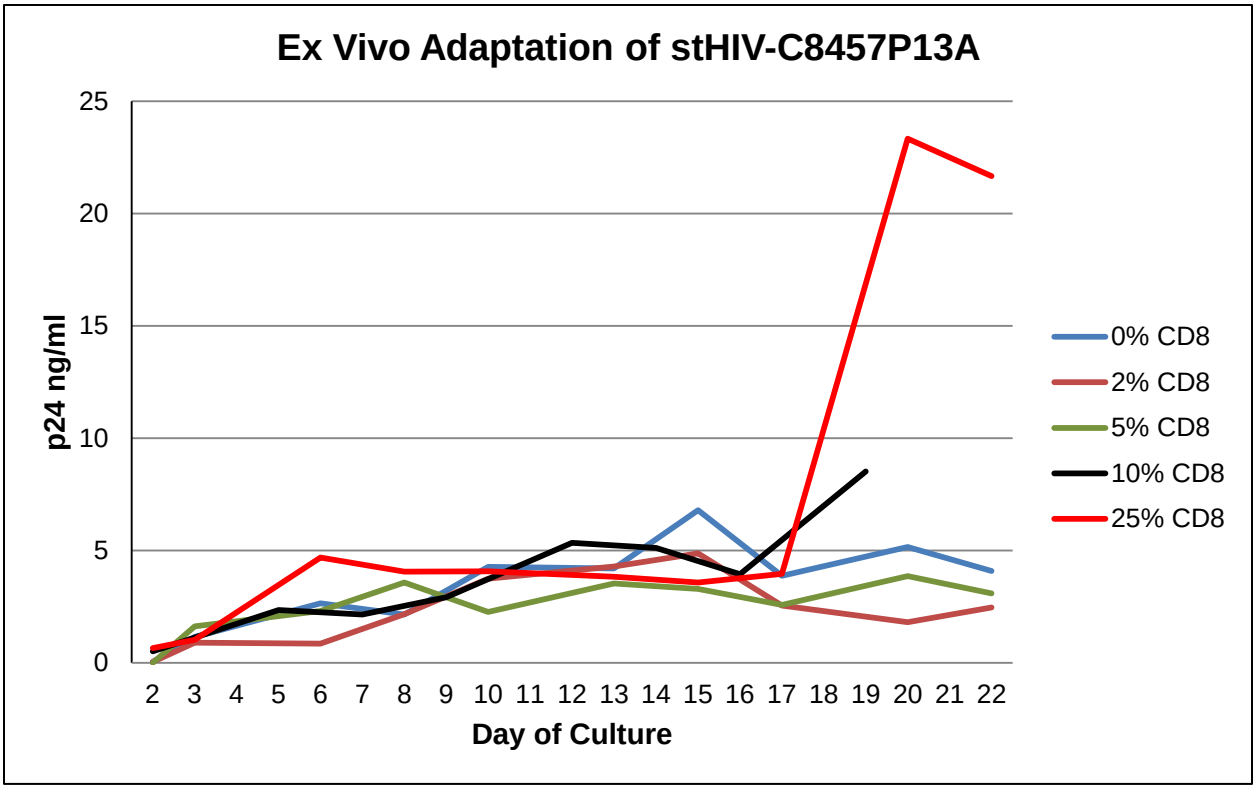


Fig. 27. p24 Values of P13A Ex Vivo Adaptation. However, the curves show that the virus did not entirely overcome the antiviral activity of the $CD8^+$ T cells' effector molecules. With increased CD8 percentage the virus was controlled better and better in culture, which indicates that the virus was not capable of resisting $CD8^+$ T cells and grow uninhibitedly. On the other hand, the virus was nonetheless capable to replicate itself which shows that the virus was not entirely controlled by the T cells. Surprisingly, it seems like the virus made an evolutionary jump on day 17. The viral growth increased by 5-fold.

3.4. Molecular Cloning of stHIV-C8457P13A from Viral RNA

After evaluating several approaches, we decided to clone stHIV-C8457P13A from viral RNA. For this purpose, we infected PBMC from PM 36227 with the isolate from PM #13 (36223). The cells produced virus over 15 days. We collected the supernatant every second or third day. The p24 assay showed the viral growth curve and the amount of p24 particles per ml in pg. The supernatant from one day was concentrated per ultracentrifugation. The concentrated virus was suspended in PBS. We extracted the viral RNA and transcribed it into cDNA, which we designated concentrated cDNA. We amplified the whole genome in four fragments. After isolating a proper amount of all fragments, we ligated them into the prepared pCR2.1-Topo vector. The assembled product was transformed into the *Stable* competent cells. We spread the cells on an agar plate and grew the colonies at 30°C. After 24 hours, we inoculated the colonies into liquid cultures and grew them in the shaker overnight. The plasmids were isolated from the cultures and analyzed. We verified the integrity of the plasmid and expanded the cultures. We transfected HEK293T/17 cells with the plasmid and verified that the plasmid is capable of producing virions.

3.4.1. Virus Production in PBMC

In order to gain a proper amount of the initial isolate of stHIV-C8457P13A, we infected PBMC from PM 36227 with the isolate. In order to guarantee the support of viral growth, we depleted CD8 cells prior inoculation. We collected supernatant for 15 days and conducted a p24 ELISA assay. This assay showed the concentration of virus over time. On base of these results, we chose the day with the highest amount of p24 particles for the concentration of the virus via ultracentrifugation.

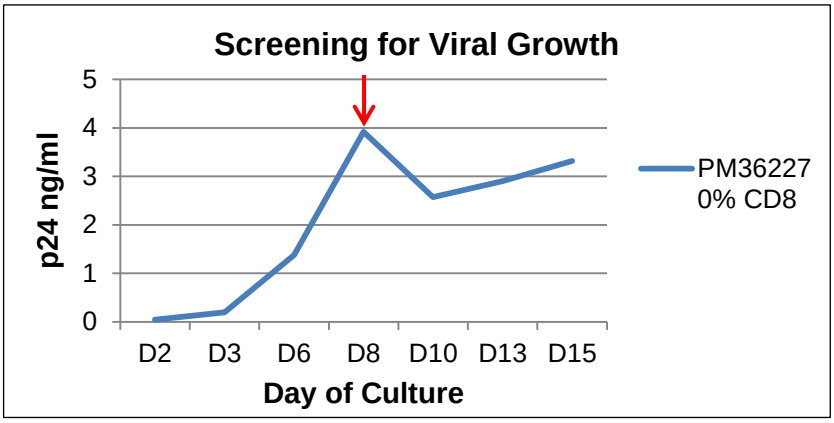


Fig. 28. Screening of Viral Growth. Peak viral replication in CD8-depleted PBMC was on day 8 (red arrow) by p24 assay. Virus from 10 ml of day 8 supernatant was concentrated by ultracentrifugation for RNA extraction and cDNA synthesis.

3.4.2. Verification of cDNA Synthesis

After ultracentrifugation of the supernatant from day 8, we extracted the viral RNA and transformed it into cDNA. In order to prove that the reverse transcription worked, we performed a PCR on the product. The PCR amplified a conserved sequence in the *gag* gene of HIV-1. A positive result proves the presence of HIV-1 DNA in the template. The PCR product was loaded on a 0.8% agarose gel with 400 ng/ml EtBr.

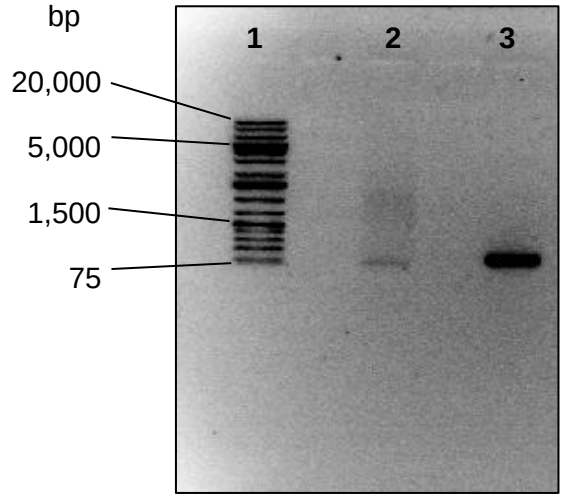
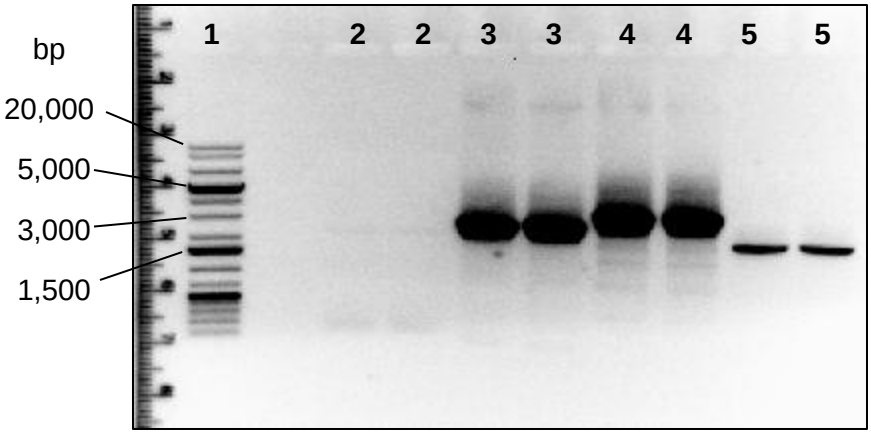


Fig. 29. *gag* PCR of concentrated cDNA. The amplicon of accurately 84 bp confirms the presence of HIV-1 DNA in the template and verifies the cDNA synthesis.

Lane
1. GeneRuler 1 kb Plus
2. 10 µl of the PCR product that had concentrated cDNA as a template
3. Positive Control

3.4.3. Fragment Amplification

After the cDNA synthesis and positive verification, we amplified all fragments from the concentrated cDNA as a template. All PCRs were performed with stHIV-C8457.3 as a positive control and H_2O as a negative control. The amplification was performed in four fragments – A, B, C and D.

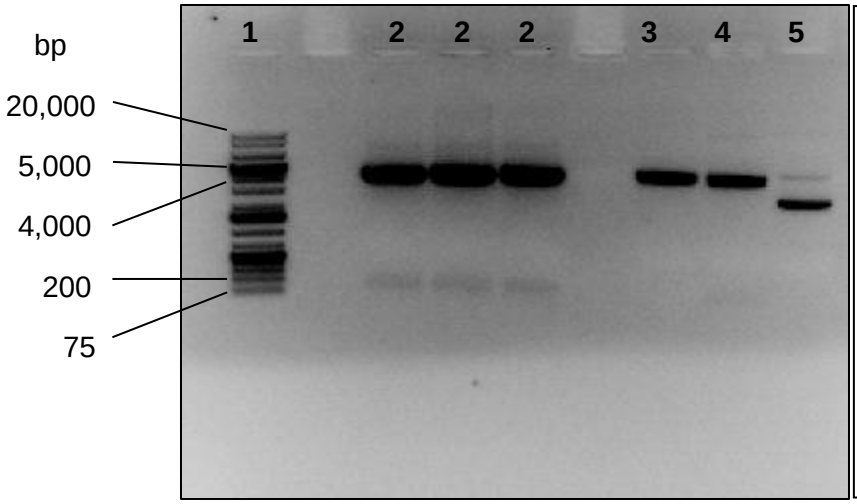


Lane
1. GeneRuler 1 kb Plus
2. Fragment A (2347 bp)
3. Fragment B (2642 bp)
4. Fragment C (3204 bp)
5. Fragment D (2054 bp)

Fig. 30. Amplification of All Fragments. All fragments were amplified in a large scale and the required bands were cut out of the gel. After this PCR, fragment A was amplified again for a better yield after gel extraction.

3.4.4. Preparation of pCR2.1-Topo

In order to transform the whole genome of stHIV-C8457P13A into the pCR2.1-Topo vector, the vector plasmid needed to be linearized. Therefore we digested the vector with the restriction enzymes Spe I-HF and EcoR V-HF. These enzymes cut out a fragment of approximately 150 bp. The remaining plasmid is linearized and has a size of 3,876 bp. We extracted the linearized plasmid from the gel and saved it for the ligation.



Lane
1. GeneRuler 1 kb Plus
2. pCR2.1-Topo digested with Spe I-HF and EcoR V-HF
3. pCR2.1-Topo digested with Spe I-HF
4. pCR2.1-Topo digested with EcoR V-HF
5. pCR2.1-Topo uncut

Fig. 31. Amplification of All Fragments. The results of the digest show that both enzymes alone linearize the plasmid. Lane number six shows that a certain amount of the plasmid is already linearized due to nicks. The lower band is he circular form of the vector and runs slightly but significantly lower than the linearized form. The large reaction with both enzymes was divided among three lanes due to the high volume. It clearly shows the ejected insert of approx. 150 bp and the linearized remaining vector with roughly 3,900 bp. The three bands were extracted from the gel and saved for the ligation.

3.4.5. Colony PCR

After amplification of all fragments and their extraction from the gel, we ligated them per Gibson Assembly. All amplicons contain an overlap of at least 25 bp on both ends. The overlapping sequences align with the surrounding fragments. We ligated the fragments with the NEBuilder HiFi DNA Assembly Master Mix. The assembled product was transformed into *stable* competent *E. coli* by New England Biolabs. The cells were spread on an LB agar plate with ampicillin. After 24 hours a colony PCR was performed to verify the presence of HIV *gag* DNA.

Lane
1. GeneRuler 1 kb plus
2-21. colonies 1-20
22. Positive control
23. Negative control

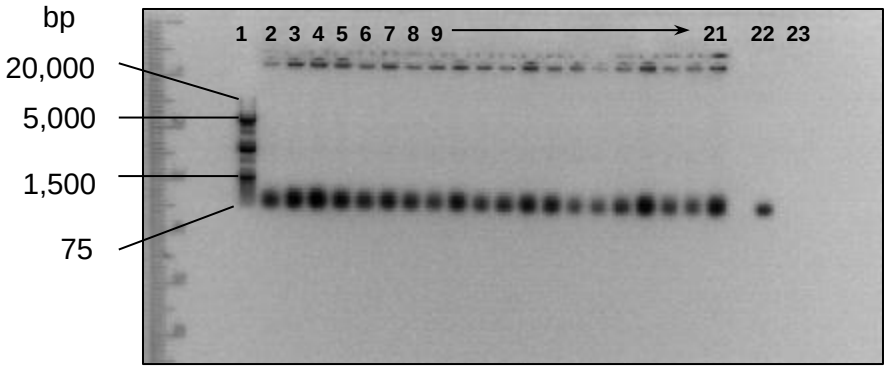


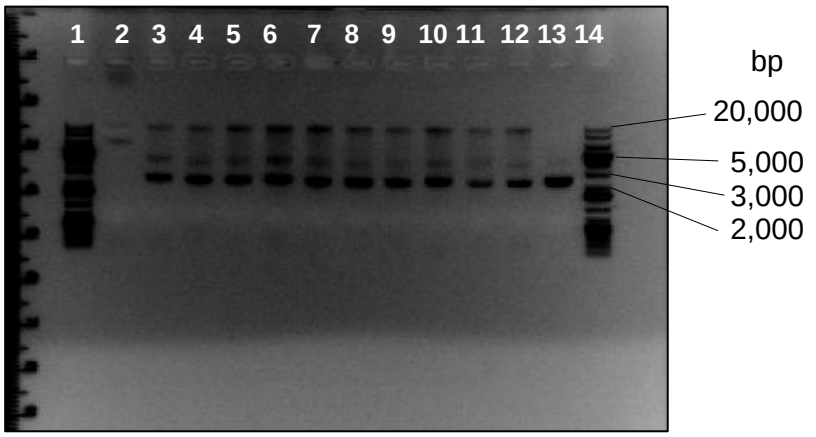
Fig. 32. Colony PCR. The result of this PCR proves the presence of HIV-1 *gag* DNA since the amplicon has the correct size of approximately 84 base pairs. This confirmed that the ligation of at least fragment A and the vector ligated since the cells were able to grow in the presence of ampicillin.

3.4.6. Plasmid Isolation and Gel Analysis

After the positive colony PCR, we inoculated ten colonies in 2 ml and expanded the culture after 8 hours of incubation in the shaker 7 ml. After incubation of at least 16 hours, we performed a plasmid-isolation from all ten clones and loaded 5 µl of the elution on a 0.8% agarose gel with 400 ng/ml of EtBr.

Fig. 33. Plasmid Isolation. The gel analysis showed that the ligation was not very efficient.

Lane
1. GeneRuler 1 kb plus
2. stHIV-C8457.3
3-12. colony 1-10
13. pCR2.1-Topo
14. GeneRuler 1 kb plus



3.5. Molecular Cloning of stHIV-C8457P13A from Genomic DNA

3.5.1. Long Fragment Amplification from Genomic DNA

For the cloning of stHIV-C8457P13A, we extracted genomic DNA from PBMC, which we infected with highly concentrated virus. The cells were maintained for 15 days and supernatant was collected for cloning from viral RNA. After two weeks, we terminated the culture and used almost 10 million cells for the extraction of the cellular genome. Infected cells carry a proviral copy of the viral genome in their own genome. We made use of this integration and amplified the whole proviral genome in two large fragments.

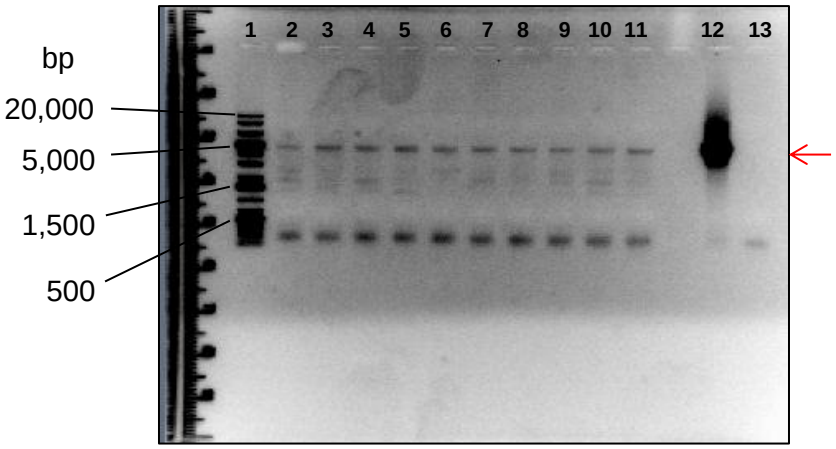


Fig. 34. Amplification of Fragment 1. The red arrow shows the band of the successfully amplified fragment (4,915 bp)

Lane

1. GeneRuler 1kb Plus
2.-11. Reactions 1-10 with genomic DNA from 36227 as template
12. Positive control
13. Negative control

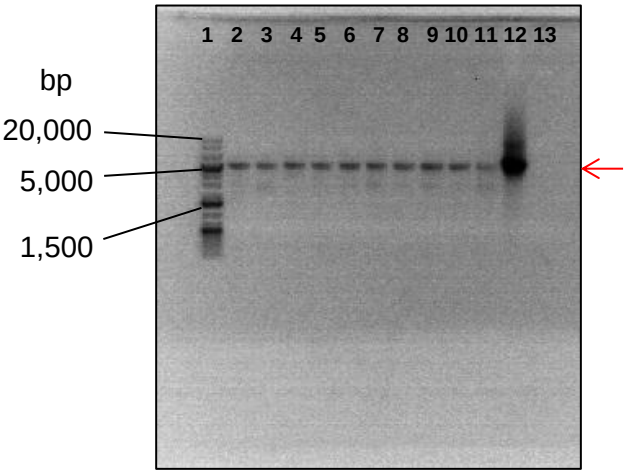


Fig. 35. Amplification of Fragment 2. The red arrow shows the band with the successfully amplified fragment (5,229 bp)

Lane

1. GeneRuler 1kb Plus
2.-11. Reactions 1-10 with genomic DNA from 36227 as template
12. Positive control
13. Negative control

3.5.2. Plasmid Isolation Gel Analysis

We successfully repeatedly amplified Fragment 1 and 2 from genetically integrated proviral DNA. The amplicons have an 25-30 bp overlap with the next fragment and the pCR2.1-Topo vector. After ligation of Fragment 1, Fragment 2 and pCR2.1-Topo, we transformed the ligation product into the *stable* competent *E. coli* by New England Biolabs. The cells were incubated on an LB agar plate with Ampicillin for 24 hours. We picked ten colonies and inoculated them in liquid culture, which led to a multiplication of the plasmid. After plasmid isolation from the cells, we loaded 300-400 ng of the product on a 0.8% agarose gel with 350 ng/µl of EtBr.

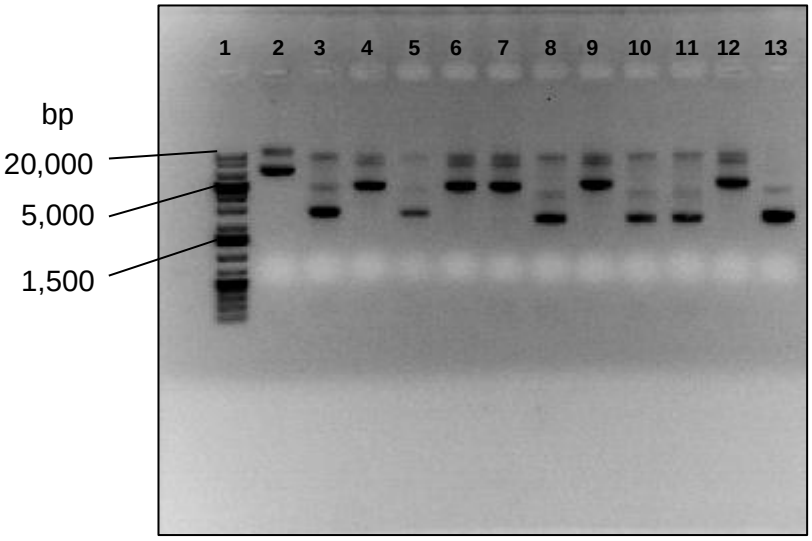


Fig. 36. Amplification of Fragment 2. The gel analysis shows a that he ligation was not very efficient either, although the bands are different from the ligation with four fragments from viral RNA (vRNA).

Lane

1. GeneRuler 1kb Plus
2. stHIV-C8457.3
3-12. Colonies 1-10
13. pCR2.1-Topo

4. DISCUSSION

This thesis is contribution towards the development of an alternative model for HIV-1 clade C infection in PMs. The stHIV-C8457 clone was initially designed to replace or at least complement the popular SHIV strains. The chimeric SHIVs have been very useful in anti-HIV Env vaccine studies in macaques. However, anti-Gag antibodies and therefore important cell-mediated immune responses are not covered in such studies. It was necessary to generate a clone that covers more HIV-1 properties during in vivo studies than the SHIV models used most often. The cloning of stHIV-C8457 was the first step for potentially replacing the standard SHIV strains in vaccine research. Although the parental stHIV-C was able to replicate in the first PM, the virus needed adaptation. The in vivo adaptation conducted by the Ruprecht lab was an important contribution to adjust the novel virus to its new host species. Nevertheless, the virus had issues replicating in the presence of $CD8^+$ cells and causing persistent viremia in its host. Consequently, it was necessary to deplete $CD8^+$ cells in the host species prior inoculation to give the virus a chance to establish a persistent systemic infection (termed PSI) in the organism. Serial animal-to-animal blood transfer at peak viremia improved the viruses' capability to replicate. Moreover, the ex vivo adaptation of isolate stHIV-C8457P7A by Ruprecht and colleagues slightly improved the viruses' resistance to $CD8^+$ T cells. The subsequent virus passages through PM8 and PM9 showed high viremia after inoculation. The improved viral growth led to the first macaque inoculations without immunodepletion. PM10, PM11, PM12 and PM13 confirmed that the virus is finally able to replicate without immunodepletion, although it still needs improvements in adaptation. This thesis continued the adaptation of the virus.

4.1. Serology of Infected Animals PM5 to PM13

Since the animal PM5 (or 33388) was inoculated with stHIV-C8457 progeny, four years have passed (PM1 to PM4 used earlier have been euthanized). Viremia is monitored regularly by the Ruprecht lab. We used the stored plasma samples to assess anti-stHIV-C antibody responses over time. The analysis of the antiviral antibody responses was performed on all PMs that are still alive – PM5 to PM13 – using HIV-1 western blot strips. For example, a classic HIV-1 and SIV infections show first an increase of anti-p24/p27 antibodies over time. Later during infection, there is also an increase of anti-Env responses (Baba et al., 1995).

All stHIV-C-inoculated PMs seroconverted. The virus has successfully established a reservoir in all animals. PM7 shows a classic case of chronic HIV-1 infection. It is not viremic but the western blots clearly show that the animal is constantly facing the antigen. The p24 antibody response is very strong and even increases over time. The strips also show that the second phase of infection comes with increased anti-Env response. In this case, anti-gp120 antibodies have developed over the years.

PM5 showed an unusual pattern of the western blot strips. As mentioned, a usual case of HIV-1 infection comes with an increased anti-p24 antibody response over time. The strips of PM5 showed the direct opposite. The anti-p24/p51/gp41 responses seem to decrease over time. Consequently, the intriguing western blot results of PM5 together with the undetectable plasma viral loads led us to the hypothesis that this animal has low antigen exposure, which may lead to a decreased antibody production. We postulate that this animal may eventually clear the stHIV-C reservoir – in the absence of ART.

In order to confirm our hypothesis, further experiments need to be conducted. A precise end-point titration ELISA should give the exact antibody titers over time. The animal seems to undergo the viral reservoir clearance process. PM5 is still antibody positive, an indirect indication of persistent viral antigens.

It will be of interest to examine the anti-stHIV-C cell-mediated immune responses in PM5. Such assays are planned for the near future. Since we know that T cells play a major role in antiviral activity, we postulate that PM5's T cells might differ from the others. It may be possible that as soon as the virus emerges from reservoir cells harboring dormant stHIV-C provirus, systemic spread could to be controlled by T-cell activity that kills virus-producing cells. Consequently, it is possible that T cell-mediated immune responses suppress the replication of the virus within the reservoir and maybe in future even lead to its elimination.

Another interesting experiment would be to deplete $CD8^+$ cells in PM5 as soon as the animal becomes anti-HIV-1 antibody negative and monitor the viral load in plasma. If the animal stays aviremic, this would indicate that PM5 has cleared the stHIV-C reservoir and is no longer HIV-1 positive. Furthermore, lymph node biopsies would give us more insights about the HIV reservoir in this animal.

In summary, all PMs that were inoculated with stHIV-C have seroconverted and PM5 may be a potential case of seroreversion.

4.2. Infectivity of Parental Infectious Molecular Clone - stHIV-C8457.3

For reproducibility purposes, we decided to prove that stHIV-C8457.3, the parental clone of stHIV-C8458P13A, is infectious and stable. We produced virus from plasmid, proved the virus' capability to replicate and determined the precise infectivity of the strain. In our assay, we generated three clones from the initial glycerol stock. We expanded the plasmid and transfected it into eukaryotic cells. Our results show that the virus harvested as cell-free supernatant was able to replicate to high levels. Moreover, we determined the infectivity of the clones. The $TCID_{50}$ assay gave us an insight about how infectious the clones are. Our results showed that the virus has a high $TCID_{50}$ value and thus is highly infectious.

4.3. Ex Vivo Adaptation of stHIV-C8457P13A

The ex vivo adaptation of stHIV-C8457P13A was an important step to improve the ability of the virus to replicate in the presence of an ever higher percentage of $CD8^+$ T cells. Based upon the results of this "Darwinian" experiment, the final virus selected over time might be able to replicate in its new host species without immunodepletion and at high levels of viremia. As the percentage of the $CD8^+$ T cells was increased in the cultures, we noted a significant adaptation of the virus to the changing environment. The virus was able to replicate in all cultures – even when the percent of $CD8^+$ T cells reached 25%. Interestingly, the virus seemed to have made an extraordinary evolutionary jump in this last culture. The virus increased its replication 5-fold in only two days. The reason for this fast and intense adaptation might be explained by a rare mutation that helped the virus to overcome the antiviral activity of the $CD8^+$ T cells. Sequence analysis is planned to confirm this finding.

Although the experiment was not designed for sudden adaptations, we significantly changed the virus ability to replicate in the presence of a very high percentage of $CD8^+$ T cells. Nonetheless, we are facing many limitations in the interpretation of this result. Firstly, the virus is only adapted to the immune cells of PM 33030. The virus may not be adapted to all PMs. Whether it will be able to replicate in other PBMC cultures in the presence of $CD8^+$ T cells is not known currently.

An important first step would be to inoculate a naïve PBMC culture of animal 33030 without any $CD8^+$ T-cell depletion. If the virus is able to grow better compared to our initial screening of naïve PBMCs, the adaptation was successful. Second, it is necessary to investigate whether the adaptation is limited to PM 33030. An inoculation of PBMC culture from different PMs, for example PM 36227, would give us an insight about the specificity of the adaptation. An

adaptation that is not restricted to animal 33030 would be important for the establishment of stHIV-C as a novel model for HIV-1 clade C infection in PMs.

As mentioned, the genomic adaptation of the virus requires investigation. Deep sequencing will give us more insight about the specific genetic changes that occurred in the provirus. The genetic adaptations could give us an idea about the changes that are necessary for an adaptation of this type.

4.4. Cloning of the Proviral Plasmid of stHIV-C8457P13A

The cloning of the proviral plasmid of stHIV-C8457P13A was another focus of this thesis. The virus has been circulating in PMs for over four years now. However, stHIV-C still needs improvement and adaptation, and strain stHIV-C8457P13A is a promising progeny. At this point, a proviral plasmid is crucial. The plasmid not only serves as a source for a precise copy of the virus, it is also very important for future cloning adaptations. Possibly, the proviral genome needs specific changes just like other proviruses did before (e.g. SHIV-1157ipEl-p). However, in this thesis, the cloning was challenging.

Our two cloning approaches are both based on the Gibson assembly. We used primers with an overlapping sequence of 25-30 bp to the next fragments. The amplification of all fragments was critical. Also the generation of a proper amount of all fragments was crucial. We assumed that the long overlapping sequence would lead to a specific assembly of all fragments, including the vector pCR2.1-Topo. However, our results showed that the assembly was not successful. Both cloning approaches, with two and four fragments from genomic DNA and viral cDNA, failed. At this point, it is hard to interpret the bands in Figures 32 and 35.

An alternative cloning strategy includes the amplification of two fragments with an overlapping sequence of approx. 150 bp around the Sac II restriction site. This way, both fragments can be cloned into the vector with the traditional cloning technique.

In parallel, we are currently trying to amplify the whole proviral genome from genomic DNA in one large fragment – a strategy used successfully by Hiener et al. (2017). The 9.8 kb amplicon will then be cloned into the vector by T.A. cloning. The new plasmid will be tested for infectivity by transfecting the proviral DNA into HEK293T/17 cells.

5. ZUSAMMENFASSUNG

Das Humane Immundefizienz Virus (HIV) ist der Krankheitserreger einer der weltweit herausforderndsten Epidemien aller Zeiten. Impfungen sind noch immer nicht existent und obwohl die Hochaktive Anti Retrovirale Therapie (kurz HAART) eine Entwicklung zu AIDS in HIV-Infizierten verhindern kann, kommt die Epidemie nach wie vor zu keinem Ende. Aus diesem Grund wird die Entwicklung einer Impfung immer wichtiger.

Bis zum heutigen Tag ist der Rhesusaffe (*Macaca mulatta*) eines der am häufigsten gewählten Tiermodelle. Der Rhesusaffe is natürlicher Wirt für das dem HIV verwandte SIV (Simianes Immunschwäche Virus). Seit geraumer Zeit werden Fusionsstämme kloniert, die man Simiane-Humane Immunschwäche Viren (oder kurz SHIV) nannte. Diese Viren enthalten das Grundgerüst von SIV mit Oberflächenproteinen von HIV. Bei Studien, die nicht nur Oberflächenproteine zum Ziel haben, ist der Erkenntnisgewinn jedoch nur beschränkt. Zu diesem Zweck hat Dr. Ruprecht's Gruppe einen simian-tropischen HIV (stHIV) Stamm entwickelt. Dieses Konstrukt besteht zu >90% aus HIV; nur drei Gene stammen noch von SIV – *vif*, *vpr* und *vpx*. Das neue Virus kann sich in südlichen Schweinsaffen (*Macaca nemestrina*) reproduzieren. Es enthält ein Subtyp C Grundgerüst, welche die prävalenteste Form von HIV ist. Der Klon wurde stHIV-C8457 genannt. Dr. Ruprecht's Gruppe haben es sich zur Aufgabe gemacht dieses Virus so gut wie möglich an Schweinsaffen anzupassen. Das Virus wurde über 13 Schweinsaffen passagiert. Das Virus konnte sich von Tier zu Tier immer besser an seine neue Wirtsspezies anpassen. Nach Affe #13 wurde das Virus isoliert und stHIV-C8457P13A genannt. In dieser Arbeit haben wir folgende Versuche durchgeführt:

i. Wir haben die Serologie der infizierten Schweinsaffen auf anti-HIV Antikörper mittels Western Blot untersucht. Die Ergebnisse haben gezeigt, dass sich in allen Tieren auch Jahre nach der Inokulation nach wie vor Antikörper gegen HIV befinden.
ii. Da frühe Resultate zeigten, dass das Virus durch lösliche antivirale Faktoren der $CD8^+$ T Zellen inhibiert wird, haben wir es ex vivo adaptiert. Dabei haben wir das Virus in Kulturen herangezüchtet und den Anteil an $CD8^+$ T Zellen in den Kulturen langsam erhöht. Mit den Resultaten konnten wir zeigen, dass sich das Virus nach bestimmter Zeit immer besser an den gesteigerten Gehalt anpassen konnte.
iii. Wir haben stHIV-C8457P13A in einen Vektor kloniert. Durch die Amplifizierung des Genoms und dessen integration in einen Vektor, versuchten wir das Virus als provirales Plasmid zur Verfügung zu stellen. Dieses Projekt ist noch nicht abgeschlossen.

6. SUMMARY

The human immunodeficiency virus (HIV) is the causative agent of one of the most challenging epidemics humanity ever had to face. To the date, prevention by a vaccine is still not in sight. Although highly active antiretroviral therapy (HAART) is potent and prevents disease progression to AIDS in HIV-infected people, the epidemic is still ongoing. Therefore, the development of a vaccine gains more and more importance.

The rhesus macaque (RM, *Macaca mulatta*) has been a popular model for preclinical vaccine studies. RMs are natural hosts for the simian immunodeficiency virus (SIV), a close relative of HIV. Over the years, chimeric strains have been developed, designated simian-human immunodeficiency viruses (SHIVs). These strains contain the HIV *env* (envelope) gene within a SIV backbone. However, immune prevention studies that target other HIV gene products than *env* are impossible. For this purpose, a new strain has been developed by the Ruprecht lab, containing up to 90% HIV and 10% SIV information in its genome. The novel virus was designated simian-tropic HIV (stHIV); this virus contains only three genes from SIV – *vif*, *vpr* and *vpx*. The initial stHIV clone was designated stHIV-C8457; it can replicate in pig-tailed macaques (PMs, *Macaca nemestrina*). The new stHIV strain was adapted to PMs that are no natural hosts for HIV. In order to adapt the virus to its new host species stHIV-C8457 was adapted to the PMs by rapid animal-to-animal blood transfer by the Ruprecht lab. By now, 13 animals have been infected with the virus. After the 13^{th} passage, the virus was re-isolated and designated stHIV-C8457P13A. In this thesis we performed the following experiments:

i. We investigated the serology of all infected PMs for anti-HIV-1 antibodies via western blot. We showed that all animals produced antibodies against HIV-1 – even years after inoculation.
ii. Early passages of the stHIV-C have shown that viral replication can be inhibited by soluble antiviral factors of $CD8^+$ cells. Therefore, we adapted stHIV-C8457P13A ex vivo to make it resistant to such factors by gradually increasing the amount of $CD8^+$ T cells in vitro. Our results showed that the virus became significantly better adapted to the increased fraction of $CD8^+$ T cells.
iii. We cloned stHIV-C8457P13A into a vector. With the amplification of the whole genome of the virus and the integration of the genome into a vector, we made efforts to create a proviral plasmid of stHIV-C8457P13A. This work is still ongoing.

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10. INDEX OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
APOBEC3G	apolipoprotein B mRNA editing enzyme catalytic polypeptide 3G
approx.	approximately
ART	antiretroviral therapy
AZT	3'-azido-3'-deoxythymidine
BIV	bovine immunodeficiency virus
bp	base pair
CAEV	caprine arthritis encephalitis virus
CCR5	chemokine co-receptor 5
CD	cluster of differentiation
CDC	Center of Disease Control
cDNA	complementary DNA
Con A	concanavalin A
conc.	concentration
CRF	circulating recombinant form
CXCR4	CXC-motive-chemokine-receptor 4
ddH_2O	double distilled water
DEAE	diethylaminoethyl cellulose
dH_2O	distilled water
dNTP	deoxyribunucleoside triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
E. coli	Escherichia coli
EB	elution buffer
EIAV	equine infectious anemia virus
Env	envelope
EtBr	ethidium bromide
ETR	endotoxin removal buffer
FACS	fluorescence-assisted-cell-sorting
FBS	fetal bovine serum
FIV	feline immunodeficiency virus
FMO	fluorescence-minus-one
Frg	fragment
FWD	forward (primer)
Gag	group specific antigen
GPR15	G-protein-coupled receptor 15
HAART	highly active antiretroviral therapy
HEK	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	high-fidelity
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
IMC	infectious molecular clone
kb	kilobase
LB	lysogeny broth
LTR	long terminal repeats
M	molar
mAb	monoclonal antibody
min	minutes
Nef	negative factor
NHP	non-human primate
OD	optical density

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCP	*Pneumocystis carinii* pneumonia
Pen-Strep	penicillin streptomycin
PLV	puma lentivirus
PM	pig-tailed macaque
pmol	picomol
Pol	polymerase
Rev	regulator of expression of the virion
REV	reverse (primer)
RLU	relative light units
RM	rhesus macaque
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
sec	seconds
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus
SIV_{cpz}	chimpanzee SIV
SIV_{mac}	macaque SIV
SIV_{smm}	sooty mangabey SIV
S.O.C.	super-optimal broth with catabolite repression
stHIV	simian-tropic human immunodeficiency virus
TAE	tris-acetat-EDTA buffer
Tat	transactivator of transcription
$TCID_{50}$	tissue culture infectivity $dose_{50}$
TMB	tetramethyl benzidine
TNF	tumor necrosis factor
TRIM5α	tripartite motif-containing protein 5
U	unit
URF	unique recombinant form
UV	ultraviolet
V	volt
V/MV	visna/maedi virus
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U
Vpx	viral protein X
vRNA	viral RNA

11. INDEX OF COMPANIES

Company	Head Office
Abcam	Camebridge – United Kingdom
Applied Bioscience Laboratories, Inc.	Rockville, MD – USA
Applied Biosystems	Foster City, CA – USA
Beckman Coulter	Brea, CA – USA
Becton Dickinson	Franklin Lakes, NJ – USA
Berthold Technologies	Bad Wildbad – Germany
Bio Rad Laboratories, Inc.	Hercules, CA – USA
BioTek Instruments, Inc.	Winooski, VT – USA
Corning, Inc.	Corning, NY – USA
Denville Scientific, Inc.	South Plainfield, NJ – USA
Eppendorf	Hamburg – Germany
Fisher Scientific	Hampton, NH – USA
GE-Healthcare	Little Chalfont – United Kingdom
GraphPad Software, Inc.	La Jolla, CA – USA
GSL Biotech, LLC.	Chicago, IL –USA
Immunetics, Inc.	Boston, MA – USA
Invitrogen	Carlsbad, CA – USA
InvivoGen	San Diego, CA – USA
Life Technologies	Grand Island, NY – USA
Macherey-Nagel	Düren – Germany
Merck Millipore	Billerica, MA – USA
Merit Medical	South Jordan, UT – USA
Miltenyi Biotech	Bergisch Gladbach – Germany
New England Biolabs	Ipswich, MA – USA
Promega	Madison, WI – USA
QUIAGEN	Valencia, CA – USA
Rockland Immunochemicals Inc.	Pottstown, PA – USA
Sigma-Aldrich	St. Louis, MO – USA
Thermo-Fisher Scientific	Waltham, MA – USA