VACCINATION STUDIES WITH THE MPER OF HIV-1 GP41 GRAFTED INTO THE TRANSMEMBRANE ENVELOPE PROTEIN OF A GAMMARETROVIRUS

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Wien, am _____

Nicola Strasz

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Abbreviations

Aa	amino acids
Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
ADCVI	antibody dependent cell mediated
AIDS	Acquired immunodeficiency syndrome
bNAb	broadly neutralizing antibody
bp	base pairs
CDR	complementary determining region
CCR	C-C chemokinereceptor
CHR	C-heptad repeat/C-terminal helix region
CROI	Conference on Retroviruses and Opportunistic Infections
CXCR	CXC-Chemokinereceptor
DC	dendritic cells
ddH_2O	double-distilled H2O
DNA	deoxyribonucleic acid
DREP	replicon DNA
E1	Epitope 1, equivalent with fusion peptide proximal region
E2	Epitope 2, equivalent with membrane proximal external region
E. coli	Escherichia coli
ELISA	enzyme linked immunosorbent assay
Env	envelope protein
ES	export signal
Fab	fragment antigen binding
Fc	fragment crystallizable
FCS	fetale calf serum
FeLV	feline leukaemia virus
FP	fusion peptide
FPPR	fusion peptide proximal region
Gag	group specific antigen
GM-CSF	granulocyte macrophage colony-stimulating factor
(H)AART	(Highly) active antiretroviral therapy
HIV	Human immunodeficiency virus
Ig	immunoglobulin
IN	integrase
kB	kilobases
kDa	kilodalton
KoRV	Koala retrovirus
LSM	Laser scanning microscopy
LTR	long terminal repeat

MA	matrix protein
MCS	multiple cloning site
MHC	major histocompatibility complex
MPER	membrane proximal external region
MSD	membrane spanning domain
NAb	neutralizing antibody
Nef	negative factor
NHR	N-terminal heptad repeat/N-terminal helix region
NIH	National Institute of Health
NK	natural killer cells
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PI	preimmune
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pol	polymerase
PR	protease
RKI	Robert Koch-Institute
RLU	relative light/luciferase units
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sufate
SFV	Semliki Forest Virus
SHB	six-helix-bundle
SHIV	SIV/HIV chimeric virus
SIV	Simian immunodeficiency virus
SPR	Surface plasmon resonance
Tat	transactivator
TE	translational enhancer
TM	transmembrane protein/transmembrane domain
UNAIDS	United Nations Department of AIDS
Vif	virion infectivity factor
WHO	World Health Organisation

Bases of nucleotide sequences:

A	Adenosine
C	Cytosine
G	Guanine
T	Thymine

1. Introduction

1.1 The discovery of HIV

The observation of an accumulated number of Pneumocystis carinii and Candida albicans infected individuals with additional appearance of Kaposi´s sarcoma, caused by Human herpesvirus 8, was first described in 1981 in two hospitals located in the US (Gottlieb et al., 1981). This cluster of syndroms, associated with immunosuppression was defined as acquired immunodeficiency syndrome (AIDS) by the CDC in 1982. A few years later, the retrovirus was isolated from samples derived from infected patients by Luc Montagnier and Françoise Barré-Sinoussi (Barre-Sinoussi et al., 1983) at the Pasteur Institute, France. This virus was primarily termed LAV (lymphadenophathy associated virus) which was later described as HIV (Human immunodeficiency virus) (Brown, 1986). The discovery of HIV was awarded the Nobel Prize in 2008 to Barré-Sinoussi and Montagnier. After the discovery of the virus and the link between AIDS in HIV infection three decades ago, a vaccine was predicted to be produced within the next 6 months, but until today, no vaccine tested in humans has been shown to have a significant effect on inhibiting virus infection.
Today, around 38 million people live globally with HIV infection. Despite intensive education and prevention programs or therapeutical opportunities, 2.5 million people became newly infected and 1.7 million died of AIDS-related causes worldwide in 2011 (UNAIDS World AIDS Day report 2012). Momentarily, 78.000 Germans and 9000 Austrians are living with an HIV infection (Country Progress Report for UNAIDS, Austria; Robert Koch-Institut, Epidemiologische Kurzinformation) and new infections per year increased in 2012.

1.2 HIV transmission and pathogenesis

HIV can be transmitted via direct contact of contaminated body fluids containing a distinct number of viruses with mucosal surfaces or through direct blood to blood contact. The infection is mostly transmitted by sexual intercourse (vaginal, oral, anal) or directly from mother to child during birth or breastfeeding. Infection also occurs by blood transfusions or sharing of needles during drug abuse.
Pathogenesis of HIV infection can be divided into 3 phases (early, acute, late) and are determined by the plasma viral load (Figure 1.1). HIV infection occurs predominantly via the mucosal barrier in the gut-associated lymphoid tissue, infecting cells displaying the CD4 and CCR5 receptor. Other target cells for HIV-1 infection represent mucosal Langerhans´ cells, expressing c-type L-lectins by binding to these L-lectins via gp120. Mucosal Langerhans cells, dendritic cells or macrophages mediate “trans” infection via transport of bound virus across the mucosal barrier, which subsequently infects $CD4^+$ T-cells (Mascola et al., 2000a). Infected lymphocytes migrate to the local lymph nodes and persist for 2-6 weeks after infection. During this period, the infected individual may experience influenza-like symptoms with fever and joint pain (Cooper et al., 1985). Plasma viral loads peak around 10^4 – 10^8 particles per ml during the acute phase, which induces an extensive decrease in $CD4^+$ cell counts following HIV-1 infection. About three to six weeks after infection, specific antiviral humoral and cellular immune responses are detected and associated with reduction of plasma viremia. Numbers of $CD4^+$ cells slightly rebound after the acute phase, although never reach pre-infection levels (Clark et al., 1991; Graziosi and Pantaleo, 1998).

The chronic phase is characterized by fast turnover of $CD4^+$ T-cells (Mellors et al., 1996), and a stabilized viral load of 10^3 – 10^5 particles per ml, defined as set-point. This plasma viral load at the set-point determines declining of $CD4^+$ cells and the further progression towards AIDS (Mellors et al., 1996).

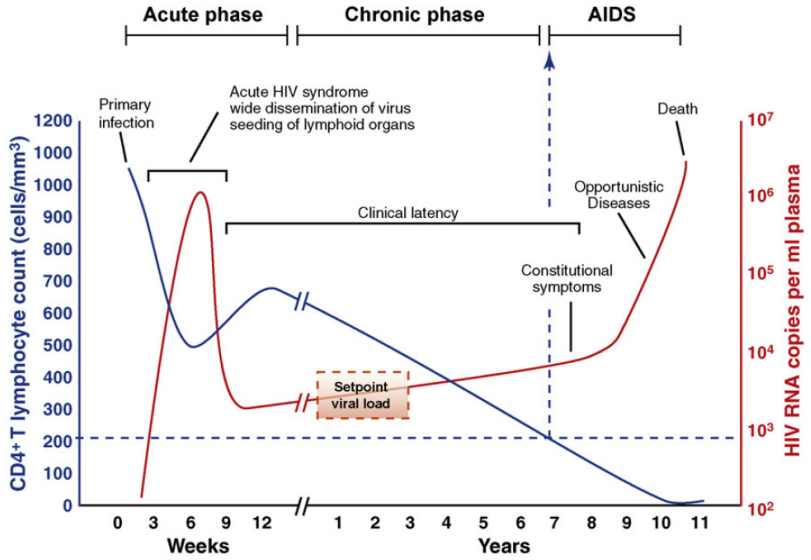


Figure 1.1: HIV course of infection. The pattern of CD4+ T-cell decline and virus load largely varies between infected individuals (An and Winkler, 2010). The set-point viral load is defined as steady-state viral load in the chronic phase of infection.

Continuous decline of $CD4^+$ cells down to 200 $CD4^+$ cells/µl defines the start of the late phase of the HIV-1 infection course. Reason for this extensive decrease is the cytolytic activity of the virus or apoptotic processes caused by production of antiviral cytokines and chemokines of infected monocyte and macrophages. This correlates furthermore with the change of coreceptor usage from CCR5 to CXCR4 in about 50% of HIV-1 infected individuals, termed as R5- or X4-tropism and is furthermore associated with a worsened prognosis (Kamp, 2009) of disease progression.

Two types of HIV with 55% (Guyader et al., 1987) sequence homology were identified until today and are further differentiated into subclasses. HIV-2 is found predominantly in West-Africa and India, described as less virulent compared to HIV-1. HIV-1 is globally prevalent and can be divided into the subtypes of group M (main), O (outlier) and N (non-M, non-O). HIV-1 group M viruses are classified into nine clades A, B, C, D, F, G, H, J and K. clade B viruses are mostly prevalent in Northern America, Europe and Australia, causing 12% of all HIV infections. Viruses of clade C predominantly circulate in South Africa and are responsible for greater than 50% of HIV infections worldwide.

The whole genomic spectrum of HIV subclasses is further expanded by mosaic viruses, evolving by co-infection of a single cell by different subtypes (Carr et al., 1996; Robertson et al., 2000). The diversity of viruses is also increased due to the lack of proof reading activity of the viral reverse transcriptase (Roberts et al., 1988), host selective immune pressure and the high production rates of 10^9 – 10^{10} viral particles per day.

1.3 Genome structure of HIV-1

HIV belongs to the family of *Retroviridae*, genus of lentiviruses, each carrying two identical copies of positive and single stranded RNAs as genome. During the replication cycle, the RNA is transcribed into DNA which is subsequently integrated into the host´s genome.

The 9800 bp genome of HIV-1 (Figure 1.2) and the 9000 bp genome of PERV encode the major viral enzymes (*pol*), the group-specific antigens (*gag*) and the envelope proteins (*env*), which are present in all retroviruses.

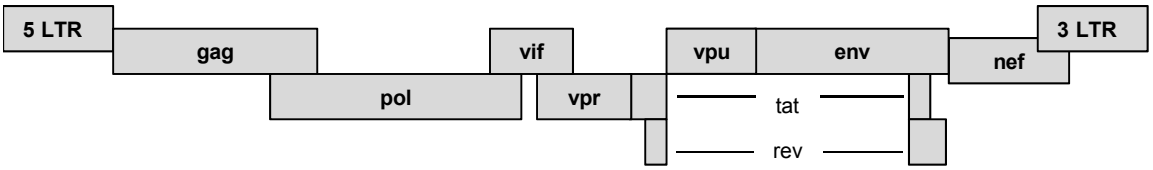


Figure 1.2: Schema of the HIV-1 proviral genome. The genome is composed of 9 open reading frames *env, gag, pol, tat, rev, vpu, vif, vpr and nef*. (LTR) long terminal repeats (Weiss, 2006).

The genus of lentiviruses encodes in contrast to other retroviruses additional regulatory and accessory proteins. These proteins are described in the following table:

Gene	Encoding protein	Function
nef *(HIV-1 negative factor)*	Nef	promotion of viral replication downregulation of CD4 receptor downregulation of MHC class II molecules modulation of cellular protein expression
tat *(transactivator of transcription)*	p16, p14	transcriptional transactivators for the LTR promoter indirect phosphorylation and stimulation of RNA-polymerase II
rev *(regulator of expression of virion proteins)*	p16, p14	transport of unspliced viral mRNA from nucleus to cytoplasm activates transcription of viral genes
vif *(viral infectivity factor)*	Vif	enhancing infectivity by inhibition of APOBEC3G
vpr *(virus protein rapid)*	Vpr	transporting the pre-integration complex into the nucleus
vpu *(viral protein U)*	Vpu	ubiquitination of CD4 enhances budding of the virus

Table 1.1: Regulatory and accessory proteins of HIV-1

1.4 Morphology of HIV-1

In addition to the two copies of viral RNA, the viral enzymes reverse transcriptase (RT), integrase (IN) and protease (PR) are packaged into the capsid. Reverse transcriptase transcribes the RNA into DNA with one error in 10^3 - 10^4 basepairs. This error-prone RT of HIV is responsible for the high genomic variability of the HIV-1 genome, since an ordinary DNA polymerase statistically produces one mutation in 10^{-7} to 10^{-9} basepairs (Preston et al., 1988; Roberts et al., 1988).
The capsid is composed of the capsid protein p24, which is enveloped by the outer membrane (envelope) originated from the cellular membrane of the host cell (Gelderblom, 1991). The inner

surface of the lipid membrane faces the matrix protein p17 and envelopes the inner virion with its viral enzymes, RNA, the RNA-associated nucleocapsid protein (p7) and the linker protein (p6) between p17 and the capsid.

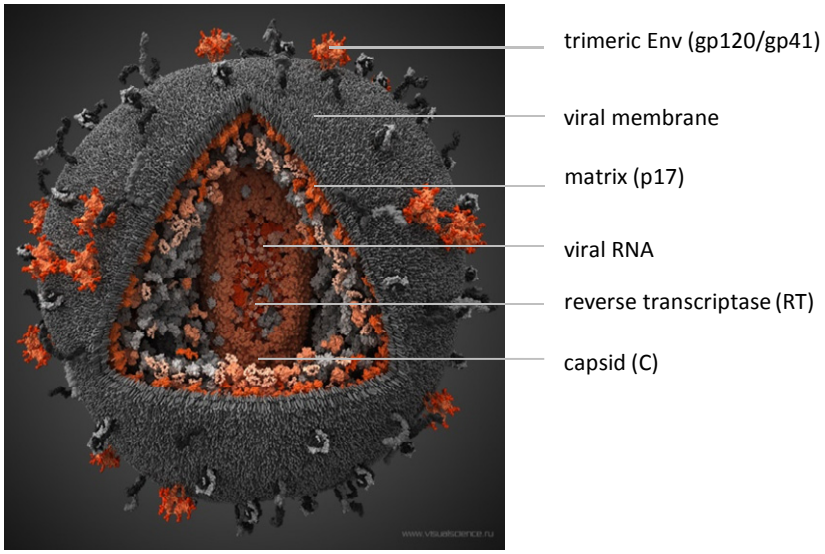


Figure 1.3: Schematic illustration of the HIV-1 virion structure (www.visualscience.ru).

The envelope proteins (Env) are non-covalently linked heterodimers, composed of the transmembrane glycoprotein gp41 (TM) and the surface protein gp120 (SU), which are transcribed from the *env* gene as a 160 kDa precursor protein. This precursor protein is transported to the Golgi apparatus for oligomerization and extensive glycosylation which is required for stable folding (Fenouillet et al., 1994). After the glycans are modified by a number of enzymes (Wyatt and Sodroski, 1998), the precursor protein is digested into gp120 and gp41 by the furinprotease This is followed by the transport to the target cell membrane and inserted as trimer via the transmembrane domain in gp41. Although trimeric and correctly folded spikes are essential for effective infection, a number of non-functional glycoproteins (monomer, dimers, tetramers) are likely to be expressed at the viral membrane due to ineffective trimerization at the Golgi apparatus or incomplete digestion of SU and TM (Pantophlet and Burton, 2006; Parren et al., 1997).

1.5 The replication cycle of HIV-1

The replication cycle of HIV-1 can be divided into two phases: early and late. The early phase of virus infection is characterized by membrane fusion and integration of the transcribed viral DNA into the host´s genome as provirus. The virus binds via the surface protein gp120 to the CD4 receptor of the host cell. Subsequent conformational changes in the spike proteins and binding to the coreceptor induce shedding of gp120 from gp41. After insertion of gp41 into the host cell´s membrane, gp41 folds into a six-helix bundle formation, mediating membrane fusion of the host cell and viral envelope with subsequent release of the capsid into the cytoplasm. After „uncoating“ of the viral RNA and enzymes, the reverse transcriptase transcribes the viral RNA into DNA, resulting in a RNA/DNA hybrid complex (Morrow et al., 1994). The transcription product is subsequently accumulated together with the co-transcriptional enzymes IN, MA, RT, the accessory protein Vpr and the cellular HMG-I(Y) (high-mobility group protein) for the pre-integration complex.

Vpr transports the complex into the cellular nucleus, followed by integration of the DNA into the host cell´s genome. The viral DNA integrates into DNA regions with predominantly high transcription frequency (Schroder et al., 2002), promoted by the viral integrase.
The structure of integrated provirus includes flanking long terminal repeats (LTR), composed of U3, R and U5. LTRs are arranged in the same direction at the 5´ and 3´ end of the provirus. Transcription of provirus is driven by the promoter located at the 5´ LTR. Cellular transcription factors can additionally bind to the U3 region in order to induce transcription of provirus by the cellular RNA polymerase II (Pereira et al., 2000)

The late phase in the HIV-1 replication cycle is characterized by expression of accessory proteins: Rev, Nef and Tat. Rev has its main role in transport of viral mRNAs, whereas Tat´s function relies in stabilization of viral gene transcription. The HIV-1 Env is synthesized as the precursor protein gp160 and co-translationally glycosylated at the endoplasmatic reticulum (Blay et al., 2007). After trimerization gp160 is cleaved into gp120 and gp41 by cellular proteases in the trans-Golgi network (Moulard and Decroly, 2000). Both envelope proteins are then transported to the cellular surface and inserted in the membrane via the transmembrane anchor of gp41. *Gag* and *pol* are transcribed as a single unspliced mRNA with different translational reading frames. The assembly of the capsid is regulated by local concentrations of Gag protein, starting from approximately 1500 copies. Two identical copies of unspliced viral mRNA containing the viral genome are assembled with Gag-Pol precursor proteins and transported to the cellular membrane. The immature particle buds subsequently from the host cell. Maturation of the viral particle is completed after Gag-Pol precursor proteins are cleaved into the structural proteins (MA, CA and NC) and viral enzymes (IN, RT and PR).

1.6 The envelope proteins

The surface unit gp120 of the viral envelope protein is composed of five conserved (C1-C5) and five variable (V1-V5) segments, organized to an inner and outer domain, connected by a bridging sheet. The first four variable segments V1-V4 form loops, which are predominantly exposed to the immune system. The conserved segments are responsible for gp41 interaction and receptor binding on the surface of the host cell (Kwong et al., 1998). Clusters of non-neutralizing antibody epitopes were detected in the inner domain, which is thus termed as non-neutralizing site of gp120 (Moore et al., 1995; Parren et al., 1999). The main contact to the TM unit was described to be attributed to C1 and C5, whereas large parts of C2, C3 and C4 form a hydrophobic core at the center of gp120 (Moore et al., 1994; Pollard et al., 1992). The inner domain is rarely covered with carbohydrates (Wyatt et al., 1998), supporting its proximity and contact to gp41. The outer domain is largely covered by glycans, which in turn lowers the immunogenicity of the viral protein (Kwong et al., 2000).

Highly immunogenic regions of the protein are detected in the V-domains and especially within the variable V1/V2 and V3-region (Hwang et al., 1991), which are suggested to be exposed to the

surface of gp120 and target of several neutralizing antibodies. Thus, the V1-V3 regions are subject to selective pressure, which induces a large number of escape mutants. Neutralizing antibodies directed against this region are mainly able to neutralize only the autologous virus. Due to the potential large part of the gp120´s outer domain, the protein is highly glycosylated with 26 glycosylation sites (Cutalo et al., 2004).

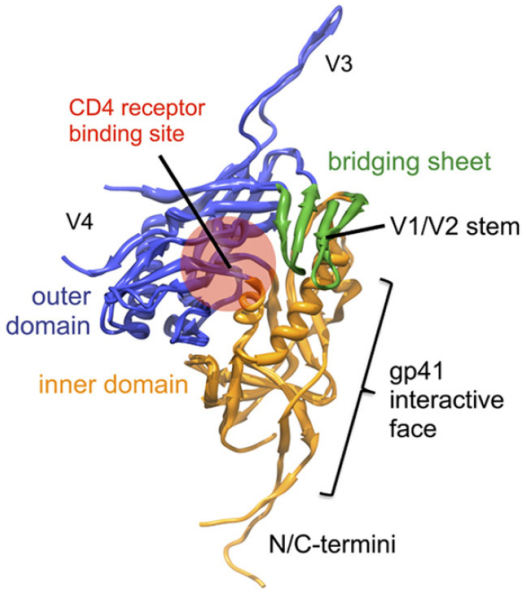


Figure 1.4: Organization of gp120 structure into inner domain (indicated in yellow) and outer domain (indicated in blue). The four-stranded bridging sheet is composed of two strands from the outer domain and the V1/V2 stem from the inner domain. CD4 binding site is highlighted as red circle (Guttman et al., 2012).

The 41 kDa TM protein (Figure 1.5) is organized into 3 sections (ectodomain, membrane spanning domain, endodomain) and this structural organization of the TM protein is common among retroviruses (gp36 of HIV-2 or p15E of PERV). The extraviral section or ectodomain is located at the N-terminal part of the protein, followed by the membrane spanning domain and the intraviral section at the C-terminal end. The ectodomain can further be divided into 6 subdomains, including the fusion peptide (FP), fusion peptide proximal region (FPPR), two heptad repeats (N-heptad repeat (NHR), C-heptad repeat (CHR)), the cysteine loop (C-C), immunodominant epitope (IDO) and the membrane proximal external region (MPER). The fusion peptide is responsible for insertion of gp41 into the host cell membrane with its hydrophobic amino acids (Freed et al., 1990; Pereira et al., 1997) and the subsequent membrane fusion.

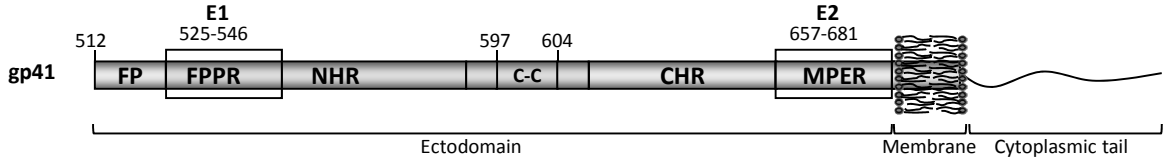


Figure 1.5: Schematic illustration of the TM protein gp41 (HIV-1), anchored in the lipid membrane. Main structure components represent the fusion peptide (FP), fusion peptide proximal region (FPPR), N-heptad repeat (NHR), cysteine loop (C-C); C-heptad repeat (CHR) and the membrane proximal external region (MPER). Definition of epitope region one and two (E1/E2) are equivalent with corresponding FPPR and MPER domains (Fiebig et al., 2009).

1.6.1 The role of the envelope proteins gp120 and gp41 in the attachment and membrane fusion process

Between 14 and 72 Env trimers are located on the surface of one HIV-1 particle (Zhu et al., 2006), which mediates binding to the host´s CD4 target cells (Liu et al., 2008). The CD4 receptor is inserted into the cellular membrane of monocytes, macrophages and dendritic cells (DC), working as a coreceptor for the T-cell receptor and thus part of the adaptive immune system. HIV-1 infection starts with the interaction of the conserved region C3 of gp120 with the CD4 receptor. Conformational changes enable accessibility of the V3 domain, which binds, together with V1/V2 domains, to either one of the coreceptors, predominantly CXCR4 or CCR5 (Myszka et al., 2000). The sequence of the V3 region of the envelopes protein´s surface unit determines the tropism of the virus, infecting T-cells (X4 or T-tropism) or monocytes/macrophages (R5 or M-tropism).

The coreceptor CCR5 is expressed on macrophages, monocytes, memory T-cells, activated $CD4^+$ cells and dendritic cells, interacting usually with the chemokines RANTES, MIP-1α or MIP-1β (Samson et al., 1996a). X5-tropic viruses are predominantly transmitted and dominate during HIV-1 infection (Weinberger and Perelson, 2011). It was previously demonstrated that individuals who are homozygous for a distinct deletion of 32 base pairs within the CCR5 gene (CCR5-Δ32) were not susceptible for infection by R5-tropic viruses (Liu et al., 1996; Samson et al., 1996b). Although heterozygous individuals are not resistant to CCR5-using HIV-1 variants, progression of disease is delayed in these individuals (Dean et al., 1996; Huang et al., 1996; Michael et al., 1997).

The natural ligand of CXCR4 was identified as SDF-1α (stromal cell-derived factor) which was previously described as Fusin (Bleul et al., 1996). Later in the course of infection, virus tropism can switch from CCR5 to CXCR4, which occurs in about 50% of infected individuals (Berger et al., 1999). Viruses infecting via the CXCR4 receptor induce syncytium formation (Johnston et al., 2003) and are controversially discussed to be more sensitive to neutralization (Bunnik et al., 2007; Cecilia et al., 1998).

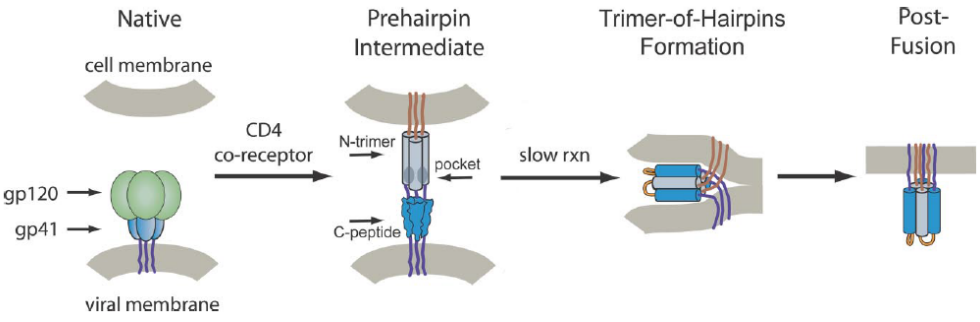


Figure 1.6: Fusion process of viral and host cell´s membrane for HIV-1 entry mediated by envelope proteins gp120 and gp41 of HIV-1. NHR is depicted in grey, CHR in blue, respectively (Welch et al., 2007).

Binding of the CD4 receptor to gp120 induces a shift within the C1 and C4/5 region, which were suggested to interact with gp41 (Ivey-Hoyle et al., 1991; Moore et al., 1997). Interaction of the coreceptors with V1-3 of gp120 and subsequent conformational changes within Env induce attenuation of the non-covalent bond between SU and TM and allows subsequent shedding of gp120 and exposure of gp41.

The fusion peptide of gp41 inserts into the host cell membrane after shedding of gp120, which induces an intermediate pre-hairpin conformation of gp41 (Eckert and Kim, 2001; Finnegan et al., 2002; Jones et al., 1998), bridging the viral and cellular membrane (Figure 1.6). This conformation is metastable, and is followed by a hydrophobic interaction of amino acids in the gp41 CHR and NHR in an anti-parallel manner (Figure 1.6), forming a six-helix bundle (SHB). The six-helix bundle is composed of three gp41 monomers, in which three NHR subunits form a coiled-coil formation and the corresponding CHR is packed around. Formation of the SHB is essential for driving membrane fusion, bringing the viral and cellular membrane to close proximity (Buzon et al., 2010; Melikyan, 2008; Weissenhorn et al., 1997).

1.7 Blocking the infection process by neutralizing antibodies against envelope proteins

For the majority of viral infections, neutralizing antibodies provide the best correlate for vaccine efficacy to prevent primary infection. Although other immune defence mechanisms can contribute to total virus clearance from the system, genome integrating viruses need one cell to infect and establish a permanent infection (Keele et al., 2008) in the host organism. Thus, the mechanism to neutralize the virus before its entrance and to prohibit subsequent integration is believed to be efficient for prevention of HIV infection.

Enveloped viruses bind to the host cell receptors via the viral spikes, which are essential targets for an effective antibody response. Therefore, the spike proteins contain one or more relatively conserved sites for host cell receptor binding, most likely to be exposed for a short time window during infection and represent different targets for immune defence mechanisms.
Stimulation of non-neutralizing antibody responses against irrelevant viral structures were shown to occur relatively early (Figure 1.7) in infection (Richman et al., 2003). All infected individuals develop weakly neutralizing antibodies against autologous virus and about 20% of chronically infected establish an antibody response with moderate neutralizing capacity against 70-80% of HIV-1 variants at later stages of infection (Doria-Rose and Connors, 2009; Sather et al., 2008; Walker and Burton, 2010). Only 2% of infected patients develop neutralizing antibodies that broadly neutralize all HIV-1 clades (Doria-Rose et al., 2008; Stamatatos et al., 2009).
The development of antibody responses after infection with HIV-1 follows a distinct time course for certain HIV-1 Env-specific antibody populations (Tomaras and Haynes, 2009). Primary IgM antibody responses after initial HIV-1 infection are anti-gp41 specific, followed by detection of anti-V3 antibodies (Figure 1.7). Antibodies capable of neutralizing autologous virus are detected 2-39 months after infection and predominantly directed against non-conserved epitopes on Env. This first wave of NAb responses is followed by extensive viral escape due to these autologous virus-specific NAb, enabling continuous viral replication (Deeks et al., 2006; Wei et al., 2003; Wrin et al., 1994).

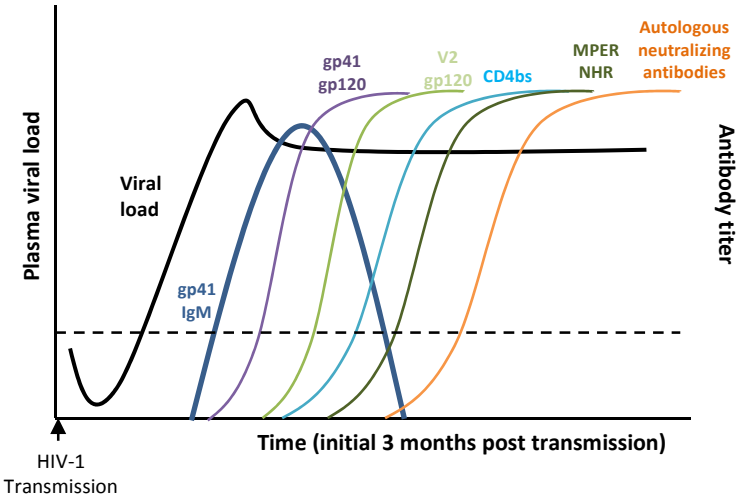


Figure 1.7: Time course of anti-Env antibodies within initial three months after infection. Primary antibody responses are anti-gp41 IgM antibodies, predominantly binding to the immunodominant loop, followed by anti-V3 antibodies. The first, weakly neutralizing anti-V3 antibodies are observed about six weeks post-infection. The dotted line represents the detection limit of either plasma viremia or Env-specific antibodies (Figure was modified from (Tomaras and Haynes, 2009)).

HIV-1 has developed several mechanisms to circumvent the generation of antibodies capable of virus neutralization. These conserved regions within Env are relatively small and shielded by carbohydrates (Pantophlet and Burton, 2006; Wyatt et al., 1998) or the protein itself during the fusion process to prevent generation of neutralizing antibodies. Although the glycan shield of Env is relatively conserved, there is some evidence that glycosylation directly correlates with the establishment of the virus in the new host and selection pressure of NAbs (Mascola and Montefiori, 2010; Scanlan et al., 2007). Selection of virus escape mutants and genetic variants is also caused by the error-prone reverse transcriptase and viral RNA recombination (Kwong et al., 2011; Roberts et al., 1988). Low density of spike proteins on the surface (Zhu et al., 2006) and flexible conformations of gp120 and gp41 likewise hamper the development of antibodies capable to prevent virus infection.

Since it was controversially discussed that high titers of NAb in serum of infected patients might delay the progression toward AIDS (Balla-Jhagjhoorsingh et al., 2011; Cecilia et al., 1999; Pilgrim et al., 1997; Scarlatti et al., 1996), isolation of these antibodies and monoclonalization for further characterization of distinct functions, structure, epitopes and neutralizing capacity was performed. The first bNAb to be isolated was b12 in 1994 (Burton et al., 1994).

Particular properties are described for bNAb, such as an extended CDRH3 (complementary determining region of the heavy chain) (Burton et al., 2012). The antibody binds to the antigen with its complementary determining region formed by variable region of the antibody´s light and heavy chain. In order to increase affinity to the antigen, the antibody might undergo somatic hypermutation, which is induced by enhancement of Ig gene transcription and erroneously repaired double-strand breaks in the DNA (Di Noia and Neuberger, 2007; Gray et al., 2011). Since epitopes of bNAb are predominantly hidden on the HIV surface, somatic hypermutation allows adaptation of these antibodies to engage certain structural modifications to reach occluded sites on Env.

1.7.1 Broadly neutralizing antibodies binding to gp120

Despite the high variability and strong glycosylation of gp120, a number of neutralizing antibodies against several HIV-1 clades were identified since 1994. Those antibodies are mostly directed against two conserved sites of gp120 which accessible during the infection process: the CD4 binding site and the coreceptor binding site. Since HIV-1 clade B is prevalent in US and Western Europe, most monoclonal antibodies were isolated from clade B infected patients (Montefiori and Mascola, 2009). Thus, the largest part of literature describes the protein structure of predominantly clade B-specific bNAb, sometimes in interaction with the corresponding epitope and limits the knowledge of bNAb epitope repertoire.

The monoclonal antibody b12 (Burton et al., 1994) is a highly potent neutralizing antibody directed against gp120, recognizing and thus blocking the CD4 binding site of the virus. The broadly neutralizing capacity of b12 was described to be associated with its capability to bind monomeric and oligomeric Env (Sattentau and Moore, 1995), whereas non-neutralizing antibodies did not bind oligomeric Env structures on the viral membrane, most likely due to steric hindrance by the heterodimer itself or other Env molecules (Kwong et al., 2000; Wyatt et al., 1998). The crystal structure of the antibody (Saphire et al., 2001) shows specific properties, which are closely related to its broadly neutralizing capacity. Of note, the antibody contains an unusual long “long finger like” (Pantophlet and Burton, 2003) CDRH3 as it was described for other bNAb. These specific structures included a tryptophane at the apex and specific residues at the base of this loop which enables its binding to the hydrophobic pocket of the CD4 binding site in gp120 (Zwick et al., 2003). Neutralization assays with pseudoviruses containing Env clones from primary HIV-1 isolates (Binley et al., 2004; Moulard et al., 2002) showed cross-clade neutralization capacity and inhibit infection of HIV-1 clades A, B, C, D, E, F and G.

2G12 was the first isolated bNAb that bound a glycosylated and discontinuous epitope in V4/C4 of gp120 (Sanders et al., 2002). Binding of the monoclonal antibody to its epitope depends in detail on α-1-2-linked mannose residue on asparagines at the positions 295, 332 and 392 (Scanlan et al., 2002), whereupon the glycans are recognized via the variable region of the antibody´s heavy chain (Calarese et al., 2003). Since 2F12 recognizes only glycans as target and not the protein itself, antigens to induce this kind of antibody might be difficult to resemble. The antibody neutralizes clades A, B, D, F (Binley et al., 2004) but not clade C viruses which cause the highest percentage of infections worldwide.

During the last 4 years, a large number of new potent bNAb have been isolated compared to previous years of bNAb identification. Two antibodies, PG9 and PG16, were identified from a clade A infected donor exhibiting broad and potent neutralizing serum activity (Walker et al., 2011; Walker et al., 2009), exceeding the potency of the control antibodies b12, 2G12, 2F5 and 4E10. Neutralization assays using a multiclade pseudovirus panel of HIV-1 showed neutralization of 119 and 127 out of 162 tested viruses for PG3 and PG16, respectively. This demonstrates higher neutralization potency compared to previously isolated bNAb. PG9 and PG16 bind to a unique epitope, recognizing conserved regions in V1/V2 and V3 loops in combination with glycans on the gp120 trimer. Analysis of the antibodies’ variable genes showed a long CDRH3 loop, which was previously associated with polyreactivity (Ichiyoshi and Casali, 1994), but neither PG9 nor PG16 bound to autoantigens.

During the same time period three bNAb (VRC01, VRC02 and VRC03) were isolated by screening of B-cells for the strongest antibodies binding to the CD4 binding site (Wu et al., 2010). These antibodies neutralized 90% of circulating HIV-1 variants with an IC_{50}<1 µg/ml. Of note, a newly discovered panel of 17 neutralizing monoclonal antibodies exhibited cross-clade neutralization and some of those were 10 to 100-fold more potent than previously identified bNAbs (Walker et al., 2011). Furthermore, new neutralization targets were identified on gp120, similar to those of PG9 and PG16.

Antibody	Reference	Epitope	Neutralization breadth	Characteristics
2G12	Trkola et al., 1996 Scalan et al., 2002	V4/C4 region CD4 binding site Glycosylation N295, N332, N392	Clades A, B, D, F	Complement activation ADCC activity Polyreactive
b12	Burton et al., 1994 Zwick et al., 2003 Saphire et al., 2001	V3 crown Overlapping CD4 binding site Discontinuous epitope	Clades A, B, C, D (Binley et al., 2004) Some clade E, F, G (Moulard et al., 2002)	Interaction of gp120 with H3 required for antibody binding (Zwick et al., 2003) Polyreactive
HJ16	Corti et al., 2010	Adjacent to CD4 binding site Discontinuous epitope	Clades A, B, C, D (Corti et al., 2010)	n/a
HGN194	Corti et al., 2010	V3 crown (RRSVRIGPGQTF)	(A, B, C, AG) (Corti et al., 2010)	n/a
PG9 PG16	Walker et al., 2009 McLellan et al., 2011	V1/V2/V3 Conformational epitope glycan dependent	Clades A, B, C, few clade D, AG, AE, F (Walkeret al., 2009 and Huang et al., 2012)	Binds glycans, long CDRH3 loop mediates neutralization (Pejchal et al., 2010), no polyreactivity
PGT 127 PGT 128	Walker et al., 2011 Pejchal et al., 2011	V3 loop / glycan shield Conformational epitope	~70% of circulation HIV strains	Neutralization mediated by crosslinking Env trimers (Pejchal et al., 2011)
VRC01/02	Wu et al., 2010	CD4 binding site Conformational epitope	Clades A, B, C, D, AE, AG, G, BC Wu et al., 2010	Zhou et al., 2010 Increased neutralization potency of improved antibody NIH45-46 (Scheid et al., 2011)
3BNC117	Scheid et al., 2011	CD4 binding site	Clades A, B, C, D, AE, AG, BC Huang et al., 2012	n/a
VRC-PG04	Falkowska et al., 2012	CD4 binding site Conformational epitope	Clades A, AE, AG, B, C, D, F, G	n/a
CH01-04 VRC-CH30-34	Bonsignori et al., 2012	CD4 binding site / V2/V3 Conformational epitope	Clades A, B, C, D, AE (Bonsignori et al., 2012)	Long CDRH3 loop in CH01-04 mAb
447-52D	Gorny et al., 1994	V3 region, GPGR Conformational epitope,	Clade B (Gorny et al., 1994)	n/a
697-D	Gorny et al., 1994	V2 loop, Conformational epitope	Clade B (Gorny et al., 1994)	n/a

Table 1.2: Overview of isolated bNAb specific for gp120.

1.7.2 Neutralizing antibodies binding to gp41

More than ten bNAb targeting gp41 have been isolated from infected individuals in the last decade (Table 1.3). The potent bNAbs 2F5, 4E10 and Z13 were initially identified and extensively investigated, all three binding to epitopes located in the tryptophane-rich membrane proximal external region (MPER) of gp41. Binding of the MPER by these bNAbs inhibits the membrane fusion process and therefore blocks viral entry (Binley et al., 2003). MPER amino acids are

relatively conserved (Liu et al., 2009; Salzwedel et al., 1999) and thus represent a vulnerable target for these kinds of antibodies. The precise function of the MPER is still unknown but changes in amino acid composition in the MPER hamper and inhibit the fusion process (Munoz-Barroso et al., 1999; Salzwedel et al., 1999). The bNAb 2F5 recognizes strongly conserved amino acids (ELDKWA; GenBank: AF324493) located within the MPER of gp41 (Muster et al., 1993; Zwick et al., 2005), the core epitope is defined as (L)DKW (McGaughey et al., 2003). Neutralization breadth of 2F5 is restricted to viruses exactly containing the epitope sequence ELDKWA, whereas viruses of clade C with a modified MPER sequence are not neutralized or only neutralized to some extent by high concentrations of 2F5 (Binley et al., 2004; Parren et al., 1998; Trkola et al., 1998) due to altered amino acids within the core epitope. The potent neutralizing capacity of 2F5 is restricted to the strong avidity to its epitope. Previous reports indicated that 2F5-like antibodies were found in 0.3% of infected individuals, developing 15 to 20 months after infection (Shen et al., 2009).

The second potent bNAb also targeting the MPER (4E10) was identified in 2001 (Zwick et al., 2001) together with another bNAb (Z13). 4E10-like antibodies are detected in 3% of HIV-1 infected individuals and these antibodies appear a few years after infection (Sather et al., 2008; Sather and Stamatatos, 2010). The epitope of 4E10 (WNWF(N/D)IT) is located in the membrane embedded part of the MPER in gp41, C-terminal to the 2F5 epitope and close proximity to the transmembrane domain.

In the last two years, about four neutralizing antibodies targeting gp41 were discovered (Table 1.3), although with limited neutralization breadth compared to 2F5 or 4E10. D5, DN9 or HK20 predominantly neutralize clade B viruses and recognize epitopes often covered during the fusion process (Gustchina et al., 2010; Luftig et al., 2006).

Antibody	Reference	Epitope	Neutralization breadth	Characteristics
10E8	Huang et al., 2012	WNWFNITNWLWYIR WNWFN blocked neutaliz.	Clades A, AC, AD, AE, AG, B, C, D	No polyreactivity detected
2F5	Trkola et al., 1995 Bryson et al., 2009	MPER (aaELDKWA) Parker 2001	Clades A,B,D,E, F (Binley et al., 2004)	Polyreactive
4E10	Zwick et al., 2001 Cardoso et al., 2005/2007	MPER (aaWNWF(D/N)IT)	Clades A, B, C, D, E, F, G (Binley et al., 2004)	Polyreactive
Z13 (Fab) Z13e1 IgG1	Zwick et al., 2001 Nelson et al., 2007	MPER (aaWASLWNWF(D/N)ITN)	Clades B, E, C (Binley et al., 2004)	n/a
M66/M66.6	Zhu et al., 2011	MPER (aaDKW, L(660,663)	Clades A, AE, AG, B	Long CDR-H3 loop M66.6 reactivity to self-antigens
D5	Miller et al., 2005 Luftig et al., 2006	Hydrophobic pocket in NHR (aaLLQLTVWGIKQLQARIL)	Clade B	No atypically long CDR
HK20	Corti et al., 2010 Sabin et al., 2010	NHR (aaQQHLLQLTVWGIKQL)	Clades A, B, C, D	Enhanced neutralization by Fab compared to IgG
DN9	Nelson et al., 2008	Trimeric NHR, overlap with D5 epitope	Clades B, C	n/a
8K8	Nelson et al., 2008	Trimeric NHR, overlap with D5 epitope	Clades B, C	n/a

Table 1.3: Overview of isolated bNAb binding to gp41. (CDR) complement determining region.

1.8 Passive immunization using neutralizing antibodies

The next step after identification and isolation of bNAbs implicated *in vivo* experiments to investigate whether isolated bNAbs are able to prevent or at least inhibit infection with HIV-1. In

order to test the inhibitory effect of bNAbs, passive immunization was performed in macaques challenged with hybrid viruses containing simian immunodeficiency virus sequences combined with HIV Env (SHIV).

The first passive transfusion studies with passively administered bNAbs were performed in 1999, using the mAb 2G12 or 2F5, given alone or in combination (Mascola et al., 1999; Shibata et al., 1999). The study of Mascola and his colleagues showed a correlation of protection and passive administration of a combination of bNAb before SHIV challenge, although macaques who received 2F5 or 2G12 alone prior challenge became infected and developed high-level plasma viraemia. 2G12 was likewise later shown to protect *in vivo* from SHIV infection when administered in combination with other NAb (Baba et al., 2000; Ferrantelli et al., 2004; Hofmann-Lehmann et al., 2001; Mascola et al., 1999; Mascola et al., 2000b). The antibody was also used for infusion experiments of HIV infected patients under retroviral therapy (Trkola et al., 2005), showing that administration of 2G12 was essential for the delay of viral rebound after stop of the therapy.

Several combinations of the bNAb were tested in a number of intravenous (Baba et al., 2000; Mascola et al., 1999), intravaginal (Parren et al., 2001), oral (Baba et al., 2000; Hofmann-Lehmann et al., 2001), or intrarectal (Hessell et al., 2010) challenge regimens, demonstrating protection in macaques.

The bNAb b12 was also tested in passive immunization of macaques, demonstrating that the antibody protects *in vivo* against challenge virus (Parren et al., 2001; Veazey et al., 2003), which has been likewise demonstrated for the MPER directed antibodies 2F5 and 4E10 (Hessell et al., 2010).

A number of studies demonstrated thereafter protection from infection after intravenous SHIV challenge, although high titers of antibodies were required for infusions. Further development of these studies allowed different challenge regimens in order to simulate the “more natural” route of infection via mucosal contact to virus. These studies required lower titers of NAb present in plasma to prevent infection (Hessell et al., 2009a; Hessell et al., 2009b).

1.9 Additional characteristics of vaccine induced antibodies

Additional aspects with regards to antibody function and control of virus infection by other mechanisms than neutralization have been brought to focus in the recent years (Kwong et al., 2011). One important vaccination study described the induction of binding antibodies responsible for protection in the macaque challenge model, although this protection did not correlate with neutralizing capacity (Demberg et al., 2007). Of note, closer evaluation of Thailand RV144 study outcome also indicated a correlation of non-neutralizing antibodies with modest protection (Rerks-Ngarm et al., 2009).

This protection was identified to be related to other effector functions of binding or neutralizing antibodies besides Fab-mediated neutralization of virus. Such antibodies mediate biological activity through binding with their C-terminal Fc region to the Fcγ receptor located on a number of different effector cells. Natural killer cells (NK), dendritic cells, monocytes and neutrophils are those effector cells which mediate ADCC (antibody dependent cellular cytotoxicity) or ADCVI (antibody dependent cell mediated virus inhibition) after binding of infected cells. In both ADCC and ADCVI,

the antibody binds with its Fab region to the HIV-1 infected cell, presenting the antibodies' epitope. The Fc region of the antibody then binds the FcγIII receptor on the effector cells which further induces lysis of the infected cell. In an experimental setup, infected cells are labled and ADCC activity can be measured by reduction of intact, labeled cells. ADCVI assays measure inhibition of virus replication by NK or other cells (Forthal et al., 2005). In addition to these effector functions, antibody-mediated activation of these effector cells can also induce production of antiviral chemokines and cytokines (Forthal et al., 2001) to inhibit HIV-1 infection.

1.10 Vaccine development against HIV

1.10.1 Live-attenuated and inactivated vaccines

The discovery that immunizations with a *nef*-deleted SIV (SIVΔ*nef*) variant induced protection after challenge with a pathogenic SIV strain in macaques served as important benchmark for life attenuated vaccine development (Daniel et al., 1992). However, long-term studies of this immunization regimen showed a low-grade persistent infection of the virus vaccine, which protected macaques from challenge with pathogenic virus but did not protect against superinfection (Girard et al., 2011). Follow-ups of these studies also indicated that the attenuated virus vaccine regains its virulence and causes disease, especially in neonate macaques (Baba et al., 1995; Baba et al., 1999; Hofmann-Lehmann et al., 2003). This regimen was never tested in humans due to lack of safety.

Attempts to induce protective antibody responses with inactivated virus particles (Huang et al., 2012) or VLP (virus-like particle) (Kim et al., 2007) have not yet been very successful, although immunogenicity of VLPs was enhanced when priming with DNA (Wardemann and Nussenzweig, 2007).

1.10.2 Subunit immunogen design

Protein based subunit vaccines are known as strong antibody inducers and are predominantly used in HIV-1 immunogen design. First immunization regimens used monomeric and soluble derivatives of the HIV-1 envelope protein in combination with an aluminum salt used as adjuvant for immunization regiments: gp160, gp120 only or gp140 (Belshe et al., 1993; Dolin et al., 1991). These experiments induced neutralizing antibodies directed against V3 of gp120, although effective neutralization and protection after challenge was only observed with autologous virus (Berman et al., 1990; Fultz et al., 1992). Gp120 was further used in a number of clinical trials in combination with alum, QS-21, MF59 or MPL® (McElrath et al., 1996) but these immunization regimens did not induce a potent bNAb response.

Trimerization of immunogens creates structures resembling native Env conformation and enables the generation of antibodies more likely binding to the trimeric structure on native viruses. This was tested with derivatives of the envelope protein (Kovacs et al., 2012; Yang et al., 2001). Trimerization can also be stabilized by co-expression of other "carrier" proteins to favor trimeric structures (Yang et al., 2002). Trimeric molecules elicited low titers of neutralizing antibodies, only neutralizing autologous virus (Barnett et al., 2001; Li et al., 2006; Xu et al., 2006).

Glycosylation of antigens and distinct formation of quaternary structures represent other attempts for immunogen design since the broadly neutralizing antibodies 2G12 recognize a carbohydrate-dependent epitope on the outer domain of gp120 (Trkola et al., 1996). PG9 and PG16 as recently discovered bNAb recognize similar epitopes, indicating a potent target site for antigen design as it was reported for the bNAb CH01-CH04 (Bonsignori et al., 2011).
One of the main targets of neutralizing antibodies within Env of HIV-1 represents the CD4 binding site since four new bNAbs were recently identified to bind to this region (Bonsignori et al., 2012; Falkowska et al., 2012; Scheid et al., 2011). Presentation of the outer domain of gp120 using SU as recombinant protein (Feng et al., 2012; Kovacs et al., 2012), expressed by viral particles, as a DNA vaccine or on *in silico* designed scaffolds for relevant epitopes induced neutralizing antibodies in a number of studies although with weak cross-clade neutralizing capacity (Nkolola et al., 2010; Sundling et al., 2010).

Antigen design to target the largely conserved TM protein of HIV-1 is a challenging field due to extensive coverage by gp120. Furthermore gp41 undergoes several structural modifications during the fusion process, presenting a number of conformational epitope variants at different time points and time periods (Figure 1.6), which impedes antigen design due to non-static presentation of the potential antigen.
Specifically the MPER located in gp41, juxtaposed to the viral membrane, is a highly conserved region and target of five bNAbs: 2F5, 4E10, Z13, M66.6 and the recently identified 10E8 (Huang et al., 2012). This region represents one of the main targets important for vaccine design, although its proximity to lipids complicates immunogen design since the lipid environment could modify conformation of the MPER compared to the protein in aqueous solution (Dennison et al., 2011b; Sun et al., 2008). This target region was used in different vaccination approaches such as synthetic peptides in combination with virosomes and recombinant proteins (Bomsel et al., 2011; Wang et al., 2011), DNA immunization strategies and virus-like particles (Arnold et al., 2009; Ye et al., 2011) comprising the MPER sequence achieved moderate success to induce MPER specific antibodies capable of neutralising HIV-1, but not with broad neutralising capacity.

1.10.3 Lipopeptides and liposomes

Liposomes are phospholipid bilayer vesicles and were primarily used as immunological adjuvants in 1974 (Allison and Gregoriadis, 1974; Gregoriadis and Allison, 1974). At that time, about eight liposome-adjuvanted vaccines were tested in clinical trials, focussing on immunization against influenza, hepatitis but also on HIV vaccines (Leroux-Roels et al., 2010; Moris et al., 2011). Since that, lipid vesicle carrier and liposomes were established as important adjuvant system for vaccines to induce both humoral and cellular responses. Liposomes were shown to be well tolerated and safe as demonstrated by extensive studies with liposomal adjuvanted anti-cancer drugs (Airoldi et al., 2011; North and Butts, 2005; Stewart et al., 1998). In addition to that, liposomes showed low anti-lipid antigenicity when liposomes were applied as adjuvants for various vaccines (Even-Or et al., 2011; Regules et al., 2011). Thus, around eight different liposome carrier formulations are currently approved for human use (Watson et al., 2012).

The lipid particles are composed of phosphatidylcholine or cholesterol, occurring naturally as cellular membranes in the organism and thus not recognized as foreign. According to different lipid combinations, size and inclusion of additional proteins or immunostimulatory agents, liposomes can be differentiated into several categories:

- Cationic liposomes include a neutral helper lipid (cholesterine or phosphatidylcholine) together with one cationic lipid (1,2-dioleoyl-3-trimethylammonium propane = DOTAP, 3β-[N-(N´,N´-dimethylaminoethane)-carbamoyl]cholesterol = DC-Chol or DDA = dimethyldioctadecyl-ammonium).
- Ordinary liposomes are composed of neutral or anionic lipids combined with an immunostimulatory adjuvants such as stimulators of Toll-like receptors.
- Virosomes are reconstituted membranes derived from influenza virus including phospholipids, influenza antigens and additional phosphatidylcholine.
- Lipoplex/Lipopolyplex are types of vesicles with cationic lipid, mostly in complex with plasmid DNA and helper lipids such as cholesterol; lipopolyplex particles includes an additional polycation.
- Niosomes are composed of cholesterol and single-alkyl chain non-ionic surfactant.

Agonists for diverse pattern recognition receptors (Toll-like receptors (TLR) or NOD-like (nucleotide-binding oligomerization domain) receptors are often added to liposome formulations for subunit vaccines (Forthal and Moog, 2009) in order to increase immunogenicity of liposomes. Potent stimulators are predominantly TLR-agonists like MPLA (monophospholipid A), activating stimulatory pathways via TLR4 (Alving and Rao, 2008) or unmethylated cytosine-phosphate-guanine motives (CpG) binding to TLR9 (Bunnik et al., 2007; Cecilia et al., 1998). MPL® is a low-toxicity derivative of LPS (MPLA), licensed in Europe in combination with Glaxo-Smith-Kline´s subunit vaccine against Hepatitis B (Forthal et al., 2001).

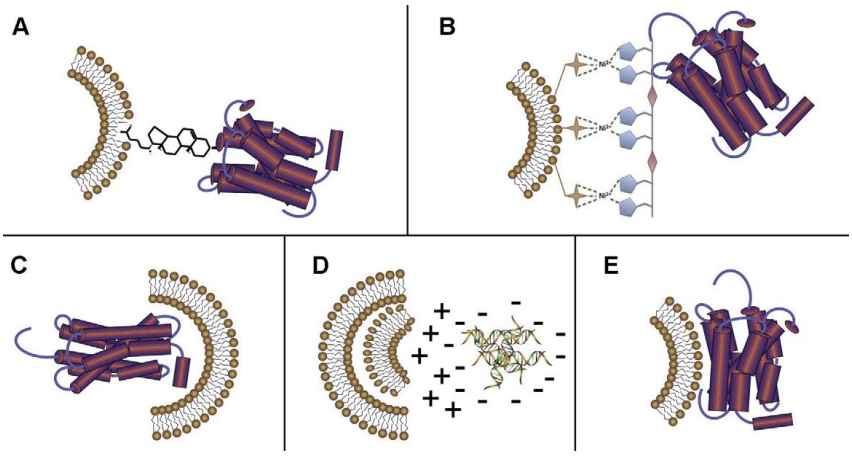


Figure 1.8: Mode of antigen attachment to liposomes (Watson et al., 2012). (A) Covalent conjugation to the liposomal surface by cholesterol. (B) Non-covalent attachment. (C) Encapsulation of the antigen within the lumen of the vesicle. (D) Antigens in complex with lipids with opposite charge. (E) Inserted into the liposomal membrane with lipophilic residues or adsorbed on the surface (Watson et al., 2012).

One of the most important steps in liposome preparation is the physical or chemical association of the antigen with the vesicles (Figure 1.8). Peptides or proteins can be associated before or after

formation of the vesicles by covalent lipid conjugation, non-covalent attachment (by affinity between Ni-NTA and 6x histidine tags), encapsulation, adsorption on the surface of liposomes or insertion by lipophilic residues into the lipid membrane. With regards to this, covalently associated antigen to the surface of liposomes induced superior antibody responses as reported previously (Therien et al., 1990; Vannier and Snyder, 1988).

1.10.4 Candidate HIV vaccines and clinical trials

Although extensive efforts were made to design a vaccine capable to prevent HIV-1 infection, only three vaccines have been tested in phase IIB or phase III clinical trials with weak or no effect.

One of the earlier phase III clinical trials included recombinant Env immunizations with gp120 (AIDSVAX), administered in combination with alum adjuvant, which failed to inhibit HIV-1 infection, reduce viral loads or prolong progression towards AIDS (Flynn et al., 2005). The STEP study performed by Merck was the second attempt of a phase IIb clinical study, performed in North America, the Caribbean, South America and Australia. The participants received three injections of the MRKAd5 HIV-1 vaccine, composed of a mixture of three different replication-defective Ad5 DNA vectors, one expressing Gag, one expressing Pol and one expressing Nef – each gene derived from a different HIV-1 strain (Buchbinder et al., 2008). Despite the positive preclinical and phase I clinical trial outcomes, the vaccine failed to prevent HIV-1 infection or to inhibit virus replication. Of note, the vaccinated group showed a higher risk for HIV-1 acquisition compared with the placebo group for a reason which still has to be investigated.

The most promising and recent phase III clinical study in Thailand (RV1444) was conducted as a heterologous prime-boost regimen using a canary poxvirus vector (ALVAC, vCP1452) expressing the products of several HIV-1 genes combined with recombinant gp120 from the AIDSVAX study (Rerks-Ngarm et al., 2009). The effective outcome of the study is still controversially discussed, but despite the failure to control virus load or inhibit decrease of $CD4^+$ cells, the vaccine had a slight effect on acquisition of HIV-1. This effect was not attributed to elicitation of neutralizing antibodies or to anti-HIV-1 CD8+ mediated T-cell responses. It is being suggested that the inhibitory effect may correlate with effector functions of non-neutralizing antibodies (Haynes et al., 2012a; Montefiori et al., 2012; Tomaras et al., 2013).

1.11 The porcine endogenous retrovirus

1.11.1 Phylogeny and genome organization

The genus of gammaretroviruses, such as the porcine endogenous retrovirus (PERV), koala retrovirus (KoRV) and feline leukaemia virus (FeLV) belong to the family of *Retroviridae* (Figure 1.9). Gammaretroviruses are transmitted vertically via the germ line and integrate as provirus into the genome of the corresponding host species. The 9 kb provirus of PERV is ubiquitous distributed in the genome of all pig species and can be further differentiated into three replication competent PERV subtypes based on the class of the used receptor for cell entry: PERV-A, PERV-B and PERV-C (Ericsson et al., 2001; Le Tissier et al., 1997; Mang et al., 2001; Patience et al., 2001).

PERV proviruses are organized like typical retroviruses with identical LTRs on the 5´ and 3´ end for reverse transcription. The PERV genome comprises two different reading frames, the first encoding Gag and retrovirus specific viral enzymes (Pol), the second one the envelope proteins. The genome of PERV does not include regulatory or accessory proteins as it is described for lentiviruses.

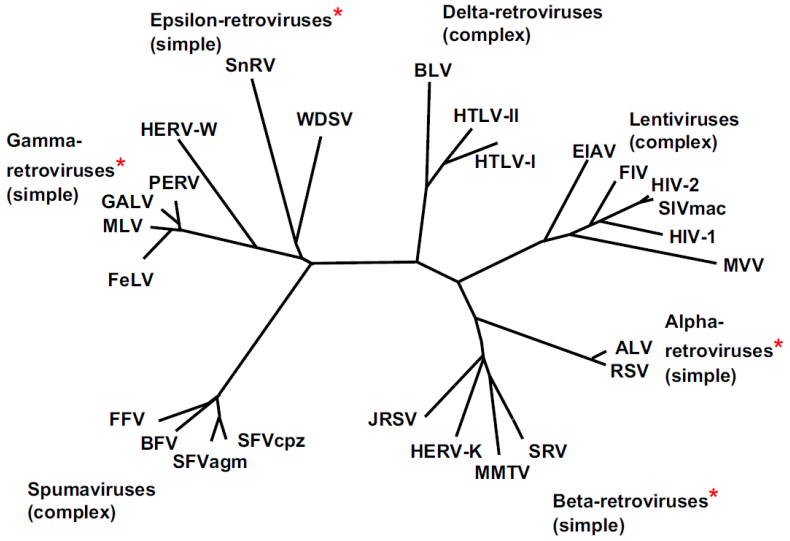


Figure 1.9: Phylogeny of retroviruses (Weiss, 2006). Retroviruses are classified according to complexity of their genomes: simple retroviruses (Alpha-, Beta-, Gamma-, Epsilonretroviruses) and complex retroviruses (Lenti-, Delta-, and Spumaviruses). Simple retroviruses (as indicated with asterisks) became endogenous in the corresponding host.

1.11.2 Morphology, replication and pathogenic potential

The morphology of PERV virions is those of typical retroviruses, comprising two identical RNA copies together with IN, RT and PR assembled within the capsid. The capsid is further enclosured with the matrix proteins. The gammaretroviral structure and composition of the envelope proteins is strongly similar to those of HIV-1, also determining tropism of the virion.

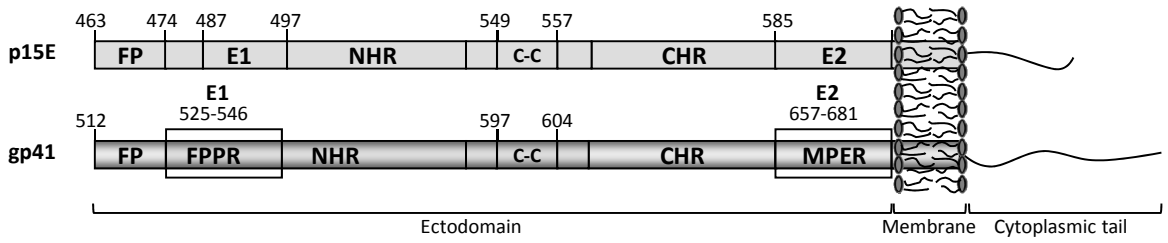


Figure 1.10: Schematic presentation of the TM proteins gp41 (HIV-1) and p15E (PERV), anchored in the lipid membrane. Main structure components represent the fusion peptide (FP), fusion peptide proximal region (FPPR), N-heptad repeat (NHR), cysteine loop (C-C); C-heptad repeat (CHR) and the membrane proximal external region (MPER). Definition of epitope region one and two (E1/E2) are equivalent with corresponding FPPR and MPER domains (Fiebig et al., 2009).

The PERV envelope protein is composed of SU (gp70) and TM (p15E), assembled as heterodimers in a trimeric configuration on the viral membrane as described for HIV-1. SU of PERV-A and PERV-B comprise seven and five N-glycosylation sites, respectively, which are

essential for receptor binding (Ericsson et al., 2003). There is also some evidence that changes in the glycosylation pattern hamper infectivity of PERV (Hazama et al., 2003).

Tropism is determined by gp70, which comprises sequence differences between three PERV subtypes (PERV-A, PERV-B, PERV-C), located at a distinct “receptor binding domain”. TM is homologous within the three subtypes of PERV and comprises the same subdomains with a similar function as it was described for HIV-1 (Figure 1.10). Binding of SU to the corresponding cellular host receptor of PERV induces conformational changes within SU and TM, resulting in fusion of viral and cellular membrane (Barnett et al., 2003; Barnett et al., 2001) with a similar mechanism as proposed for HIV (1.6.1, Figure 1.6).

PERV-A and PERV–B infect human and non-human cell lines *in vitro*, whereupon PERV-C infection is restricted to porcine cells. Pathogenic potential of PERV was not observed in the natural host, although random integration of proviruses might bear tumor-inducing potential (Dieckhoff et al., 2007).

1.12 The rationale of hybrid protein design based on gamma-retroviruses

Viral envelope proteins of HIV-1 and PERV share structural homologies despite their evolutionary distance (Figure 1.9) due to the same function with regards to fusion of viral and cellular membrane of the host cell. These proteins are thus relatively conserved since small mutation can already result in strong structural modifications, resulting in impairment of viral infection.

Immunization studies in a number of animal species (rats, guinea pigs, goat etc.) using the ectodomain of the PERV TM protein showed that neutralizing antibodies against PERV were easily induced (Fiebig et al., 2003; Kaulitz et al., 2011). This attempt was primarily evaluated in terms of virus safety in organ transplants (as reviewed in (Denner, 2011)). Epitope mapping of these neutralizing sera identified a cluster of epitopes located in the FPPR of p15E, designated as epitope region 1 (E1) and in the MPER (E2). In addition to these findings, combination of E1 and E2 derived peptides used in an ELISA showed enhanced avidity of p15E antibodies to its epitope. (Fiebig et al., 2003).

Further immunization studies using the recombinant TM ectodomain of other gammaretroviruses also demonstrated the induction of neutralizing antibodies in a number of animal species. This was shown for koala retrovirus (KoRV) (Fiebig et al., 2006), and feline leukemia virus (FeLV) (Langhammer et al., 2005, 2010; Langhammer et al., 2006). The neutralizing potential of these antibodies has further been demonstrated *in vivo*. When cats immunized with p15E of FeLV were challenged, 50% of the animals were protected from antigenemia (Langhammer et al., 2011).

Alignments of different gammaretroviruses´ TM proteins with gp41 of HIV-1 showed a sequence homologiy within the MPER. The E2 epitope (FEG**WFN**) in p15E is conserved within all gammaretroviruses and includes three identical amino acids, partial homologous with the epitope of the bNAb 4E10 (N**WFN**IT, identical amino acids in bold) (Fiebig et al., 2009; Langhammer et al., 2005; Langhammer et al., 2006). Epitope mappings of the bNAb 4E10 and 2F5 showed only epitopes located in the MPER (Fiebig et al., 2009), whereas other studies demonstrated additional binding of 4E10 to the FPPR of gp41 (Hager-Braun et al., 2006). Structural interaction of the MPER and the FPPR had been suggested as a reason for an increased binding of 2F5 to peptides

carrying its epitope in the presence of peptides corresponding to the FPPR (Fiebig et al., 2009; Lorizate et al., 2006), which was also demonstrated for p15E antibodies. In addition, a direct molecular interaction of FPPR and MPER sequences (Bellamy-McIntyre et al., 2007) and a stabilization of the 6-helix-bundle formation by peptides corresponding to the MPER and FPPR have been demonstrated (Noah et al., 2008). Some viruses comprising the "DKW" core epitope within the MPER are resistant to 2F5 neutralization (Montero et al., 2008; Morris et al., 2011), which suggests that additional regions in the protein are involved in formation of the epitope. These results allow speculations that an interaction of the FPPR with the MPER, possibly in a hairpin-like conformation, may be required to induce neutralizing 2F5-like and 4E10-like antibodies. Thus, hybrid proteins were designed in order to graft gp41 MPER and FPPR into a scaffold of p15E derived from PERV.

1.13 Aim of the thesis

Infection with HIV is still a death sentence for people living in poor countries with underdeveloped health-care systems. Despite improvement of efficacy in antiretroviral therapy, a vaccine to prevent initial infection would be an important step to safe thousands of lives. Efforts to achieve vaccine-induced protection have met with limited success when using gp120 as antigen, due to high variability and promotion of escape mutants. Thus, vaccine development targeting gp41 represent a good alternative since the transmembrane envelope protein provides relatively conserved domains for some potent bNAbs, like 2F5 and 10E8. The exact mechanism required to induce these bNAbs is still to be investigated and a large number of approaches failed to develop antigens able to induce such antibodies. On the other hand, antibodies capable to neutralize the porcine endogenous retrovirus (PERV) were induced by immunization with the ectodomain of the transmembrane envelope protein p15E. These neutralizing immune sera bound to two epitopes in p15E, corresponding to the FPPR and MPER.

On the basis of these observations, hybrid antigens should be designed by substitution of these epitopes recognized by p15E-specific neutralizing antibodies with the corresponding FPPR and MPER derived from gp41 in order to target immune responses towards the TM protein of HIV-1 but provide the scaffold of p15E maybe essential for the induction of neutralizing antibodies. These hybrid proteins should be generated by aligning p15E and gp41 sequences to identify homologies and to optimize substitution of FPPR and MPER within the p15E scaffold. Expression constructs for these hybrid antigens should be cloned, expressed in *E. coli* and purified for immunization studies in different animal species. Immune sera derived from these immunization studies should be collected and evaluated in detail for anti-gp41 antibody responses and antibody-antigen binding properties. Furthermore, neutralization capacity of these immune sera is to be evaluated by different neutralization assays. In case of HIV-1 neutralization, HIV-1 specific antibodies should be isolated in order to verify virus specific inhibition of infection.

Materials and Methods

1.14 Materials

1.14.1 Primers

Primers and probes for real-time PCR were obtained from Sigma-Aldrich, Steinheim, Germany.

Primer	Primer description	5´-sequence-3´
002 FWD	pQE30XaN1 cloning	GCA GTT GGT ATT GGT GCA C
005 FWD	pQE30Xa sequencing	AAAATTTATTTGCTTTGTGAGCG
006 REV	pQE30Xa sequencing	CCAGATGGAGTTCTGAGGTCA
007 FWD	pQE30Xa-N3 cloning	GCA GCC GGT AGC ACC ATG
008 REV	pQE30Xa-N3 cloning	ATA CCA CAG CCA ATT GGT AA
009 FWD	pQE30XaN2 cloning	GAACCGATTAGCCTGACCCTGGCA
010 REV	pQE30Xa-N2 cloning	TCGACTACTTACTTAAACGGTCAGTAACAG
011 REV	pQE30Xa-N4 cloning	CTG CTG CTG AAC AAT ACC AGA
012 FWD	pQE30Xa-N4 cloning	AGC CTG AGC GAA GTT GTT CTG
015 FWD	DREP sequencing	TAG ATT GGT GCG TTA ATA CAC AG
016 REV	DREP sequencing	TCG TCG AAT TAA GCT CCT AGG TT
021 FWD	pDisplay-N1 with ApaI cloning	TATATAGGG CCCGAGCTGTCGGAATCG
022 REV	pDisplay-N1 with SalI cloning	GAGTAGTCGACGGCCGCACTAGTTTA
024 FWD	DREP-N5 cloning	GCAACCGCCAGGGCCGCAGCCGGGTCAACAATGGGTGCTGC
025 REV	DREP-N5 cloning	GCAGCACCCATTGTTGACCCGGCTGCGGCCCTGGCGGTTGC
026 FWD	pDisplay-N5 cloning	CCAGATTATGCTGGGGCCCGAGCAGCCGGGTCAACAATGG
027 REV	pDisplay-N5 cloning	CCATTGTTGACCCGGCTGCTC GGGCCCCAGCATAATCTGG
T7 FWD	pDisplay sequencing	TAATACGACTCACTATAGGG
T7 REV	pQE30XaN1 cloning	GAGTAGTCGACGGCCGCACTAGTTTA
BGH REV	REV pDisplay sequencing	TAGAAGGCACAGTCGAGG
68spez	HIV-1NL4-3 Real-time PCR	GGAGCAGCAGGAAGCACTATGG
69spez	HIV-1NL4-3 Real-time PCR	CCCCAGACTGTGAGTTGCAACA-
HIV-1 Probe	Real-time PCR	FAM–TGACGCTGACGGTACAGGCCAGAC-BHQI
44 FWD	GAPDH Real-time PCR	GGCGATGCTGGCGCTGAGTAC
45 REV	GAPDH Real-time PCR	TGGTCCACACCCATGACGA
GAPDH Probe	Real-time	HEX-TTCACCACCATGGAGAAGGCTGGG-BHQI

Table 2.1: Overview of primers used in cloning procedures and for real-time PCR-based neutralization assays.

1.14.2 Plasmids

Recombinant proteins were expressed in *E. coli*, using the plasmid pQE30Xa (Qiagen, Hilden, Germany). Two eukaryotic expression vectors were used for DNA immunization via Gene-Gun to present membrane associated recombinant proteins. Therefore, the pDisplay vector (Appendix) was used, which included the export signal and provided an internal transmembrane domain for anchoring the proteins in the cell membrane. The DREP vector (Nordstrom et al., 2005), kindly provided by Prof. Peter Liljeström), was modified according to a previously reported internalization of export signal due to the fact that the provided vector was designed to express the reporter gene EGFP in the cytosol (2.2.7).

1.14.3 Enzymes

Enzyme	Company
Fast-Digest ® Restriction Rnzymes	Fermentas, St. Leon-Rot, Germany
T4 Ligase	Fermentas, St. Leon-Rot, Germany
Shrimp Alkaline Phosphatase	Invitrogen, Karlsruhe, Germany
Pfu DNA Polymerase	Invitrogen, Karlsruhe, Germany
Pfu Turbo DNA Polymerase	Stratagene, Heidelberg, Germany
AmpliTaq Gold DNA Polymerase	Applied Biosystems, Darmstadt, Germany
Proteinase K	Invitrogen, Karlsruhe, Germany
Benzonase	Invitrogen, Karlsruhe, Germany

Table 2.2: Overview of enzymes used for cloning procedures, purification of proteins and real-time PCR-based neutralization assays.

1.14.4 Bacterial strains

Bacterial strain	Genotype	Application	Reference
One Shot® Top10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Plasmid expansion/ cloning procedures	Stratagene, Heidelberg, Germany
SCS1/pSE111	*E. coli recA1 endA1 gyrA96 thi-1 hsdR17* (*r*K– *m*K+) *supE44 relA1* (for SCS1)	Recombinant protein expression	(Bussow et al., 1998)
C3030H	*MiniF lys*Y (Cam^R) */ fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal λatt::pNEB3-r1-cDsbC* (Spec^R, *lacI^q*) *ΔtrxB sulA11 R(mcr-73::miniTn10--*Tet^S*)2 [dcm] R(zgb-210::Tn10 --*Tet^S*) endA1 Δgor Δ(mcrC-mrr)114::IS10*	Recombinant protein expression	New England Biolabs, Frankfurt, Germany
BL21(DE3)pLysS	*E. coli* B F$^-$*dcm ompT hsdS*(rB$^-$ mB$^-$) *gal* λ(DE3)	Recombinant protein expression	Promega, Mannheim, Germay

Table 2.3: Overview of bacterial strains used for cloning procedures and expression of recombinant proteins.

1.14.5 Bacterial media

Media	Composition
LB	1% tryptone, 0.5% yeast extract, 1% NaCl,
TB	1.2% tryptone, 2.4% yeast extract, 72 mM K_2HPO_4, 17 mM KH_2PO_4, 0.4% glycerol
2YT	1.6 % tryptone, 1% yeast extract, 0.5% NaCl
SOC	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10mM $MgSO_4$, 20 mM glucose

Table 2.4: Overview of bacterial strains used for cloning procedures and expression of recombinant proteins.

1.14.6 Peptides, Pepspot-Membranes and Peptide-Microarrays

Peptides used for ELISA were all purchased from Genaxxon (Ulm, Germany).

Peptides	Amino acid sequence
FPPR / gp41	AASMTLTVQARQLLS
MPER / gp41	ELLELDKWASLWNWFNITNWL

Table 2.5: Overview of peptides used for ELISA to determine concentrations of peptide specific serum antibodies.

Peptide amino acid sequences for membranes and microarrays correspond to the ectodomains of gp41 (clade B, HIV-1_{NL4-3}, Genbank: AF324493; clade B, HIV-1_{HXB2}, GenBank: K03455) or p15E (PERV C, Genbank: AJ133817). Nitrocellulose PepSpot™ membranes or microarray glass slide epitope mappings were purchased from JPT, Berlin, Germany.

1.14.7 Antibodies

Antibodies	Dilution	Company
2F5 (human monoclonal antibody	varied	obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. H. Katinger (Muster et al. 2003)
4E10 (human monoclonal antibody	varied	obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. H. Katinger (Stiegler et al. 2001)
Polyclonal goat anti-mouse-HRP	1:2000 1:4000	DakoCytomation, Glostrup, Denmark
Polyclonal goat anti-rat-HRP	1:4000	DakoCytomation, Glostrup, Denmark
Polyclonal goat anti-human-HRP	1:5000	DakoCytomation, Glostrup, Denmark
Polyclonal rabbit anti-goat-HRP	1:2000	DakoCytomation, Glostrup, Denmark
Polyclonal goat anti-rabbit-HRP	1:2000	DakoCytomation, Glostrup, Denmark
Polyclonal goat anti-guinea pig-HRP	1:1000	DakoCytomation, Glostrup, Denmark
RGS-His antibody	1:4000	Qiagen, Hilden, Germany

Table 2.6: Overview of antibodies used for ELISA, Western blot analysis, neutralization assays and immunufluorescent staining.

1.14.8 Cell lines

Cell line	Cell type/origin	Reference
293T	Human embryonic kidney cells	Pear et al., 1993
TZM-bl (JC53-bl, Clone 13)	HeLa cells (cervical cancer)	NIH AIDS reagent program
C8166	Human leukaemia T-cell s	Sodroski et al., 1984

Table 2.7: Overview of cell-lines used for transfection, neutralization assays and assays for *in vitro* protein expression.

1.14.9 Viruses

The molecular clone of HIV-1_{NL4-3} and one HIV-1 primary isolates (clade F, HIV-$1_{BR\ 93/020}$) was obtained from the NIH Research and Reference Reagent Program.

1.15 Methods

1.15.1 Molecular biology

1.15.1.1 Plasmid amplification/isolation

Amplification of plasmids was performed by transformation of the required circular plasmids into electro- or chemocompetent Top10 *E. coli*. 1.5 ml bacterial cultures for Mini Plasmid Preparations (Invitek, Berlin, Germany) were incubated overnight and plasmids isolated according to the manufacturer´s protocol. Endo-Free Maxi Preparations (Qiagen, Hilden, Germany) of plasmids were required for transfection and DNA immunization of eukaryotic expression constructs due to inhibition of transfection efficacy by endotoxins present in the DNA preparation and were performed according to recommendations of the manufacturer.

The concentration of nucleic acids was measured using ND-1000 Nano-Drop® (Peqlab, Erlangen, Germany).

1.15.1.2 Purification of DNA fragments

DNA fragments were purified in order to eliminate template DNA after PCR or enzymes by agarose electrophoresis. Desired fragments were cut out of the gel, extracted with the Invisorb® Spin DNA Extraction Kit (Invitek, Berlin, Germany) and eluted in 30 µl nuclease free water (Promega, Mannheim, Germany). Desalting and concentration of DNA fragments was performed with the MSB® Spin PCRapace Kit (Invitek, Berlin, Germany).

1.15.1.3 Transformation

Plasmid DNA was transformed into electrocompetent Top10 *E. coli* after cloning procedures using the Gene-Pulser 2 (Bio-Rad, Munich, Germany). Competent *E. coli* Top10 bacteria were thawn on ice, 1 µl of ligation mix added to bacteria and transferred to pre-chilled cuvettes. Transformation was performed by pulsing the bacteria with 25 µF/200 Ω. 250 µl of SOCS media were added after transformation, incubated under shaking conditions at 37ºC for 60 min and subsequently plated on pre-warmed agar plates containing the required antibiotics.

Retransformation of plasmids and transformation into protein expression strains was performed using chemical transformation. The DNA was incubated with thawed bacteria for 20 min, heat-shocked for 60 sec at 42ºC, recovered 5 min on ice and incubated with SOCS media under shaking conditions for at least 30 min. Bacterial cultures were then plated on agar plates containing the required antibiotics.

1.15.1.4 Colony PCR and sequencing of positive clones

For the detection of bacterial colonies carrying the plasmid with the insert of interest, at least 10 colonies were picked from the agar-plates, plated out on a new agar-plate, 1.5 ml of required *E. coli* growing medium were inoculated for subsequent Mini-Prep plasmid isolation of positive clones and the rest of the colony was scratched into PCR tubes for colony PCR. In order to perform the colony

PCR, 20 µl of PCR master-mix containing 1x PCR Buffer, 15 mM $MgCl_2$, 0.8 mM dNTP mix (both Fermentas, St. Leon-Roth, Germany), 4 pmol of forward and reverse primer, 1 unit Hot-start Taq polymerase were resuspended in PCR tubes containing the colony. Cycling conditions were as follows: initial denaturation at 95ºC for 15 min, followed by 30 cycles of denaturation (95ºC for 30 sec), annealing (55ºC for 30 sec) and elongation (72ºC for 30 sec). The PCR reactions were analysed on 1% agarose gels. Positive bacterial clones were inoculated in 1.5 ml LB-media, shaken overnight (O/N) at 37ºC and plasmid DNA of positive clones was isolated the next day.
DNA of positive clones was used for sequencing PCR with BigDye Premix 3.1 (Applied Biosystems, Darmstadt, Germany) according to the manufacturer´s protocol. Briefly, 150-300 ng of plasmid DNA were used in each 10 µl sequencing reaction (10 pmol vector-specific, forward/reverse sequencing primer; 1 µl ABI sequencing buffer (Applied Biosystems, Darmstadt, Germany)). Cycle conditions were as follows: initial denaturation at 96ºC for 2 min, followed by 25 cycles of denaturation (96ºC for 10 sec), annealing (55ºC for 5 sec) and elongation (60ºC for 4 min). Samples for sequencing were stored at -20ºC until purification by gel filtration and read-out of the sequencing reaction was carried out by the sequencing core facility at the Robert Koch-Institute. Sequences were analysed using the BioEdit and Geneious software.

1.15.1.5 Cloning strategies for recombinant hybrid proteins (prokaryotic expression system)

DNA constructs of hybrid proteins N1 and N2 were purchased from Geneart (Regensburg, Germany) with a codonoptimized sequence corresponding to the expression organism. N1 was cloned into the pQE30Xa expression vector using the restriction sites *Stu*I/*Bam*HI. N2 was generated by PCR amplification using primers 009/010 (Table 2.1) and inserted blunt end into the pQE30Xa expression vector via the *Stu*I restriction site. Primers 007/008 amplified the construct N3 with pQE30Xa-N1 used as template and also inserted blunt end via *Stu*I into the multiple cloning site. The linear PCR product was subsequently purified by 1% agarose electrophoresis, extracted from the gel with the Invisorb® Spin DNA Extraction Kit (Invitek, Berlin, Germany) and religated blunt end. The construct pQE30Xa-N4 was cloned from pQE30Xa-N3 by truncation of 26 amino acids via PCR using the primers 011/012. Purification and ligation of the PCR product was conducted as described for N3. The detailed amino acid sequences of the hybrid proteins are described in the appendix.

1.15.1.6 Cloning strategies for recombinant hybrid proteins (eukaryotic expression system)

Two different mammalian expression systems for DNA immunization of hybrid proteins were applied to compare the quality of immune responses in prokaryotic and eukaryotic expression systems. The pDisplay™ (Invitrogen, Karlsruhe, Germany) was selected to present hybrid proteins on the cell surface and simulate viral spike-like presentation of HIV-1 envelope sequences to the immune system. The vector included all required components such as (i) a human cytomegalovirus promoter to provide high expression of the recombinant protein, (ii) a murine Ig κ-chain leader sequence to target the expressed proteins to the secretory pathway and (iii) a bowine growth

hormone derived polyadenylation signal. The HIV-1 transmembrane domain was included in all expressed construct and used as membrane anchor to provide insertion of the hybrid proteins into the cellular membrane and facilitate optimal folding. Thus, hybrid proteins were cloned in-frame with promoter and signal peptide sequence into the multiple-cloning site of the pDisplay™ vector. A STOP-codon at the C-terminal end of the hybrid protein sequence prohibited transcription of the platelet-derived growth factor receptor transmembrane domain (PDGFR-TM) downstream of the sequence encoding the hybrid protein and a *myc*-tag which was already included in the vector. Hybrid proteins N1 and N5 (N3 + transmembrane domain) were used for DNA immunization using the Gene-Gun vaccine application system (3.6). The construct N1 was purchased from GeneScript (Piscataway, USA) and codonoptimized for the mouse organism since mice were used for immunization experiments. Additional restriction sites *Apa*I/*Sal*I were included by PCR using the primer 021/022 (Table 2.1) and inserted into pDisplay™. N5 was amplified by QuickChange™ *in vitro* mutagenesis (Stratagene, Heidelberg, Germany) using the vector pDisplay-N1 as template with the primers 028/29, which were designed accordingly to the manufacturer´s instructions. This method is primarily used for *in vitro* site directed mutagenesis in the target sequence but was here applied for cloning procedures to delete 13 amino acids from the pDisplay-N1 construct. Primers with a length of 25 to 45 bp were designed, overlapping about 20 bp on both sides of the sequence to be deleted. After amplification of the construct, circular DNA of pDisplay-N1 was digested with methylation sensitive *Dpn*I to deplete non-mutated vector background, which was then used for transformation into electrocompetent Top10 *E. coli*. Clones were picked, sequenced and Endofree Maxi preparations of plasmid DNA were performed to isolate DNA for preparation of the gene gun ammunition. Design and cloning procedures of Semliki Forest Virus vectors expressing N1 or N5 are described in section 1.7 and the appendix.

1.15.2 Working with proteins and peptides

1.15.2.1 Expression of recombinant hybrid proteins (prokaryotic expression)

All constructs cloned in to the pQE30Xa expression plasmid contained N-terminally located hexahistidine tags for purification procedures. Plasmids were transformed into *E. coli* expression strains (BL21(DE3)pLysS, C3030H, SCS1/SE111) and 3 clones of each bacterial strain were tested for optimal expression conditions (Figure 3.3) by SDS-PAGE and Western blot analysis using mAbs 2F5 and RGS-His. The SCS1/pSE111 strain (Bussow et al., 1998) remained the only tested strain expressing the recombinant hybrid proteins of interest. Transformants were grown in 2YT medium at 37°C containing 100 µg/ml ampicillin and protein expression was induced at OD_{600} of 0.7 with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 h at 220 rpm. The cells were pelleted at 10,000 rpm/4ºC for 20 min. Supernatant and pellet were tested for expressed protein and aggregated protein was only detected in the pelleted bacteria. Thus the pellets were used for further processing.

1.15.2.2 Purification procedures of recombinant hybrid proteins

Recombinant proteins N1 and N2 were aggregated in inclusion bodies and thus difficult to solubilize in physiological and denaturing buffers. Thus, cell pellets from expression cultures were

resuspended in 6 M guanidine hydrochloride buffer (Buffer C, Qiagen, Hilden, Germany) and subsequently sonicated (three pulses of 60 sec, break 60 sec using a Branson Sonifier II 250, Danbury, CT, USA), followed by centrifugation (0.5 h/10,000 rpm at room temperature). This procedure was repeated five times and supernatants were collected. The 4th and 5th supernatant fractions containing purified proteins N1 or N2 were dialysed against double distilled water (ddH_2O) for further concentration.

N3 and N4 were purified by affinity chromatography using the ÄKTAexplorer 100 (GE Healthcare, Uppsala, Sweden) with denaturing purification protocols (8 M Urea buffer) for initial immunizations in rats, guinea pigs and one rabbit. Pelleted bacteria expressing N3 or N4 were resuspended in 8 M urea buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM TrisCl, lysozyme, pH=8) and lysed by sonication as described above. Crude lysates were centrifuged (1 h, 20,000 rpm at room temperature), supernatants were added to Ni-NTA agarose (Qiagen, Hilden, Germany) and incubated for 1 h/RT (room temperature) on a rotating shaker. The column was then washed with 30 column volumes (cv) of urea buffer (pH=6.3) and eluates were collected in 1.5 ml fractions. Eluates containing purified protein were dialysed against ddH_2O and used for immunizations.

In addition, the purification protocol was further modified to increase solubility of the purified proteins and promote optimal refolding by milder purification conditions. Thus, pelleted bacteria expressing recombinant proteins N3 or N4 of 1L expression culture were resuspended in 10 ml PBS containing lysozyme, sonicated as described above and centrifuged for 60 min/4ºC at 16,000 g. Supernatants were discarded, the remaining pellets resuspended in 10 ml PBS, containing 1% N-Laroyl-Sarcosine (Sigma-Aldrich, Steinheim, Germany) with 20 mM imidazole and incubated at 4ºC O/N on a rotating shaker. The suspensions were then centrifuged at 16,000 g/30 min and the supernatants diluted with 90 ml PBS/20 mM imidazole to obtain a final concentration of 0.1% N-Laroyl-Sarcosine. Purification of recombinant proteins was performed using the ÄKTAexplorer 100 (GE Healthcare, Uppsala, Sweden) with a 5 ml HIS FF crude column (GE Healthcare, Uppsala, Sweden). Recombinant proteins were eluted with a gradient protocol by increasing the imidazole concentration from 20 mM to 500 mM over 30 column volumes. 1.5 ml elution fraction were then dialysed against PBS/0.1% N-Laroyl-Sarcosine and used for immunization of rats and 1 goat.

1.15.2.3 Purification procedures of analytical recombinant proteins

The recombinant protein comprising the ectodomain of p15E (expression plasmid kindly provided by Danny Kaulitz) used for analysis of rat preimmune sera was expressed and purified as previously described (Fiebig et al., 2003; Kaulitz et al., 2011).

The histidine-tagged recombinant ectodomain of gp41 (aa533-681, GenBank: AB221005), provided by Rayk Behrendt) used for the analysis of induced anti-rgp41 responses in immune sera was expressed analogous to N3 and N4. Pelleted bacteria obtained from 2L of expression culture were resuspended in 120 ml of 6 M guanidine hydrochloride buffer (6 M guanidine hydrochloride, 10 mM Tris base, 100 mM NaH_2PO_4), centrifuged for 10 min/16,000 g and subsequently centrifuged for 30 min/20,000 g. The recombinant protein was purified using the ÄKTAexplorer 100 (GE Healthcare, Uppsala, Sweden) with a 5 ml HIS FF crude column (GE Healthcare, Uppsala, Sweden). Clarified lysates were applied to the purification column and eluted with a stepwise

gradient purification protocol. The column was washed with an 8 M urea buffer (8 M urea, 20 mM imidazole, 150 mM NaCl, pH=8) and proteins eluted into 1.5 ml fractions by increasing application of elution buffer (8 M urea, 500 mM imidazole, 150 mM NaCl, pH=8).

The expression construct gp41-CHR (HIV-1; Genbank: K03455, aa605-681, the expression construct was kindly provided by Daniel Effert) was fused with GST for native purification. Pelleted bacteria of 1 L BL21 (DE3) expression culture expressing the recombinant CHR of gp41 were resuspended in 50 ml PBS/1% glycerine/10 mM dithiothreitol, sonicated as described above and centrifuged for 20 min at 25,000 g/4ºC. Then, the supernatant was applied to the Glutathion Sepharose 4B (GE Healthcare, Uppsala, Sweden) and incubated on a rotating shaker at 4ºC for 2 h. After five subsequent washing steps with PBS/1% glycerine, the recombinant protein was eluted in 2 ml elution buffer fractions (50 mM Tris HCl, 10 mM reduced glutathione, pH=8). Purification of 1 L expression culture resulted in 5 mg of purified protein, which were dialysed against PBS.

1.15.2.4 Insertion of recombinant proteins N1, N3 and N4 into Liposomes

Recombinant hybrid proteins N1, N3 and N4 were sent to Polymun (Vienna, Austria) for insertion of the proteins into the lipid bilayer of liposomes. Immunization experiments using N1 în liposomes included two different vaccine batches with different liposome preparations.

Recombinant hybrid proteins were mixed with a lipid/MPLA/ethanol solution via the patent-registered process technology from Polymun to prepare the both batches. Afterwards, the liposome suspension was diluted with PBS for the first batch of recombinant protein N1 in liposomes to reduce residual ethanol or detergent and subsequently ultra- and dia-filtrated in order to remove detergent, ethanol or non-incorporated proteins. Lipid composition in the protein/liposome suspension was defined by Polymun as 5 - 7 mg/ml dimyristoylphosphatidylcholine (DMPC), 0.5 - 0.8 mg/ml dimyristoylphosphatidylglycerol (DMPG), 2 - 3 mg/ml cholesterol, and 0.15 – 0.2 mg/ml MPLA.

Preparation of the second batch of N1 in liposomes was performed by filtration of recombinant hybrid protein solution through a 0.2 µm filter prior to lipid incorporation and filtration of the recombinant hybrid proteins via the Polymun liposome processing technique.

Recombinant hybrid proteins N3 and N4 were processed as described for the first batch of N1 liposome insertion, although the last step of ultrafiltration to remove residual unbound recombinant hybrid protein was omitted, since both proteins did not include a transmembrane domain and were thus not expected to be inserted into the lipid bilayer of the liposomes.

1.15.3 Immunological methods

1.15.3.1 PAGE and Western blot analysis

The purified recombinant proteins were characterized by 10% tricin sodium dodecylsulfat polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using the mAbs 2F5 and 4E10. Briefly, 0.5 to 5 µg of recombinant protein were mixed 1:2 with Laemmli sample buffer (62.5 mM Tris-HCl pH=6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% Bromphenol Blue) and boiled for 5 min at 95°C in non-reducing sample buffer (62.5 mM Tris-HCl pH=6.8, 40%

glycerol, 0.01% Bromphenol Blue). Protein samples were loaded on SDS-PAGE and subsequently blotted as described below.

Sera of immunized animals were assayed for antibody properties binding to purified recombinant gp41 or the p15E ectodomain. Electrophoresis was performed with a complete preparative 10% SDS-PAGE using 5 µg of purified rgp41 protein or rp15E, which was equivalent to 0.5 µg per lane. Proteins were transferred to a 0.45 µm nitrocellulose membrane via semi-dry blotting (Trans-Blot® SD semi-dry transfer cell, Biorad, Munich, Germany). Membranes were washed twice with phosphate buffered saline (PBS, pH 7.2), and blocked by incubation with blocking buffer (5% nonfat milk powder in PBS/0.05% Tween 20) overnight at 4ºC. After washing procedures for three times with washing buffer (PBS containing 0.05% Tween 20), the membranes were clamped into a Mini-Protean Multiscreen apparatus (Biorad, Munich, Germany) and subsequently incubated with diluted sera (1:400 in blocking buffer) for 2 h at room temperature. Following serum incubation, blots were washed for 0.5 h with washing buffer and incubated with horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) for 1 h in blocking buffer at room temperature at concentrations described in section 2.1.7. After secondary antibody incubation, membrane was washed five times with washing buffer for five minutes each and detection was performed using Pierce ECL substrate (Thermo Scientific, Bonn, Germany).

1.15.3.2 Isolation of immunoglobulins

Total IgG fractions were isolated from serum using Protein G HP Spin Trap (GE Healthcare, Munich, Germany) according to the manufacturer´s protocol. Briefly, decomplemented serum was diluted 1:4 with resin binding buffer (20 mM sodium phosphate, pH 7.0) and incubated with Protein G Sepharose for 15 min. Four washing steps with binding buffer were followed by two elution steps using 200 µl elution buffer (0.1 M glycine-HCl, pH=2.7). Elution fractions were immediately neutralized with 1 M Tris-HCl, pH 9.0, pooled and dialysed against PBS for 48 h at 4ºC to allow refolding of the purified antibodies. IgG concentration was normalized to the IgG concentration in serum by an ELISA, using rgp41 as antigen.

1.15.3.3 Isolation of gp41-CHR specific antibodies from goat serum

The gp41-CHR recombinant protein was coupled to CNBr Sepharose in order to isolate and concentrate CHR-specific antibodies from goat serum. 0.5 g of freeze-dried CNBr-activated sepharose 4B (~1.5 g gel matrix) were washed with 100 ml of 1 mM HCl and subsequently equilibrated with freshly prepared matrix coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl). Gp41-CHR protein was dialysed against coupling buffer and concentrated to 0.5 µg/ml. 20 ml of protein solution was incubated with the sepharose O/N at 4ºC on a rotation shaker to couple the protein to the sepharose. Unbound ligand was washed away with 20 ml of coupling buffer and unspecific binding sites were blocked with blocking buffer (0.1 M Tris-HCl buffer, pH=8) for 2 h. Then, the protein-coupled sepharose was washed three times with altering pH (0.1 M NaAc, 0.5 M NaCl, pH=4 and pH=8), applied to the column and subsequently washed with PBS. Goat serum obtained three and eight weeks after the third immunization was diluted 1:3 and applied to the column, circulating O/N. The next day, the column was washed with PBS and CHR binding antibodies were eluted in 1.5 ml fractions with elution buffer (0.1 M glycine.HCl, pH=2.7) and immediately

neutralized with 1 M Tris-HCl, pH=10. Eluted fractions were analysed by SDS-PAGE and fractions containing high antibody concentration were pooled and dialysed against PBS. Antibody concentration was determined by NanoDrop 1000 Spektrophotometer at 280 nm (Thermo Scientific, Bonn, Germany).

1.15.3.4 Peptide and anti-lipid ELISA

96-well plates (Nunc, Roskilde, Denmark) were coated with 100 ng of rgp41 or peptides corresponding to the FPPR or MPER of gp41 (aa532-546 or aa657-681; Genbank: K03455, AB221005) per well. Evaluation of antibody reactivity with lipids was performed by coating 1 µg cardiolipin or sphingomyelin (Sigma), respectively (Haynes, 2005; Matyas et al., 2009a). Plates were blocked with 0.05% Tween 20 and 5% BSA in PBS for 2 h at 37ºC and subsequently washed with PBS containing 0.05% Tween 20 (washing buffer). Sera were diluted in PBS containing 2.5% BSA and 0.05% Tween 20 and incubated for 1.5 h at 37ºC. Plates were washed three times, followed by incubation with peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) diluted in PBS containing 2.5% BSA and 0.05% Tween 20 for 1 hour at 37ºC. Prior addition of freshly prepared *o*-phenylenediamine dihydrochloride/H_2O_2 solution (Merck, Darmstadt, Germany), plates were washed seven times with washing buffer. The colour reaction was stopped after a maximum time of 12 min with 5N H_2SO_4 and optical density was measured at 492/620 nm. Endpoint titers of binding antibodies were defined as reciprocal of the highest serum dilution that gives a value at $OD_{492/620}$ nm above the cut-off. The cut-off value was equivalent to the mean value of the negative control sera plus three times the standard deviation value.

1.15.3.5 Epitope mapping

Two different methods of epitope mapping were performed, using 15-mer peptides, overlapping 12 amino acids spotted on microarray glass slides or linked to nitrocellulose membrane (both JPT, Berlin, Germany). Each slide or membrane comprised the complete ectodomain of gp41 and was incubated with sera diluted 1:400. Incubation procedures were performed using the suppliers´ standard protocol. Binding of antibodies to peptides was detected by peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) and Pierce ECL substrate (Thermo Scientific, Bonn, Germany). The readout of the microarray glass slides, incubated with Dylight649 (Thermo Scientific, Bonn, Germany), was performed using a Genepix 4000B Microarray scanner (Molecular Devices, Ismaning, Germany).

1.15.3.6 Surface plasmon resonance analysis (SPR)

Apparent K_D values of 50% binding equilibrium of the mAbs 2F5 and 4E10 to the N3 and N4 antigen were estimated using a BIAcore X100 (GE Healthcare, Waukesha, Wisconsin, USA). A sensor chip CM5 containing a carboxymethylated dextran matrix was coated with 4,000 RU of N3 on flow cell 2 (Fc2) and flow cell 1 (Fc1) was left uncoated as control. Affinity determination by steady-state analyis was carried out in 1x HBS-EP+ running buffer containing 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20, pH 7.4 at a constant temperature of 25°C. To determine apparent K_D values, mAbs 2F5 and 4E10 were diluted in running buffer and tested by injecting 50 µl (600 sec, flow rate 5 µl/min) of diluted protein solution at concentrations ranging from

113 ng/ml to 1.13 ng/ml for 2F5 and 119.4 µg/ml to 0.1194 µg/ml for 4E10. Apparent affinity of 2F5 to the hybrid protein N4 was measured at concentrations ranging from 22.6 µg/ml to 0.226 µg/ml. 4E10 was tested at equivalent concentrations as used for affinity testing to N3. The chips were regenerated for 60 sec with 50 mM glycin/HCl pH 1.5 and results evaluated with the Biacore X100 software 1.0.

1.15.4 Animal experiments

1.15.4.1 Animals used for immunization experiments with recombinant antigens

Species	Number of Animal	Antigen/Adjuvant	Amount antigen	Number of immunizations
Guinea pigs	N1g#1 N1g#2 N1g#3 N1g#4	Recombinant N1 / CFA or IFA	250 µg	4
Rats	N1r#1 N1r#2 N1r#3 N1r#4	Recombinant N1 / CFA or IFA	250 µg	4
Rats	N2r#1 N2r#2 N2r#3 N2r#4	Recombinant N2 / CFA or IFA	250 µg	4
Guinea pigs	N3g#1 N3g#2 N3g#3 N3g#4	Recombinant N3 / CFA or IFA	250 µg	4
Rats	N3r#1 N3r#2 N3r#3 N3r#4	Recombinant N3 / CFA or IFA	250 µg	4
Rabbit	N3h#1	Recombinant N3 / Montanide	500 µg	2
Rats	N3r#5 N3r#6 N3r#7 N3r#8	Recombinant N3 / CFA or IFA	250 µg	4
Rats	N4r#1 N4r#2 N4r#3 N4r#4	Recombinant N4 / CFA or IFA	250 µg	4
Rats	N4r#5 N4r#6 N4r#7 N4r#8	Recombinant N4 / CFA or IFA	500 µg	4
Rats	N4r#9 N4r#10 N4r#11 N4r#12 N4r#13 N4r#14 N4r#15 N4r#16	Recombinant N4 / CFA or IFA	250 µg	3
Goat	N4z#1	Recombinant N4 / CFA or IFA	500 µg	3

1.15.4.2 Animals used for immunization studies with recombinant hybrid antigens on liposomes

Species	Number of Animal	Antigen/Adjuvant	Amount antigen	Number of immunizations
Guinea pigs	$N1_{Lip}g$#5 $N1_{Lip}g$#6 $N1_{Lip}g$#7 Lipg#1	Recombinant N1 on Liposomes / MPLA Empty Liposomes / MPLA	250 µg	3
Goat	$N1_{Lip}z$#1	Recombinant N1 on Liposomes / MPLA	250 µg	3
Rats	$N3_{Lipr}$#9 $N3_{Lipr}$#10 $N3_{Lipr}$#11 $N3_{Lipr}$#12	Recombinant N3 on Liposomes/ MPLA	250 µg	3
Rats	$N4_{Lipr}$#17 $N4_{Lipr}$#18 $N4_{Lipr}$#19 $N4_{Lipr}$#20 Lipr#1	Recombinant N3 on Liposomes / MPLA Empty Liposomes / MPLA	250 µg	3

1.15.5 Application of antigens

1.15.5.1 Application of recombinant hybrid protein antigens

Preimmune sera of rats, guinea pigs, rabbits or goats were collected before the first immunization. The two groups of guinea pigs immunized with N1 and N3, respectively, were chosen as initial test of responses due to previously observed antibodies against rp15E in naïve rats (Behrendt et al., 2012b). When preimmune sera of rats were tested negatively for p15E antibodies, rats were included in this immunization study. Immune sera were taken 19 days after each immunization (Figure 2.1). Animals were immunized subcutaneously (s.c.) and/or intramuscularly with recombinant proteins diluted in PBS and mixed 1:1 with complete Freund´s adjuvant (Thermo Scientific, Bonn, Germany) for primary immunizations and with incomplete Freund´s adjuvant (Thermo Scientific, Bonn, Germany) for booster immunizations unless otherwise noted. Four applications of 250 µg or 500 µg of recombinant antigen were chosen for primary immunization studies in a 3 week immunization schedule unless otherwise noted. Immunizations with recombinant proteins in liposomes and DNA vaccination studies were performed with 3 applications of 250 µg antigen in a three week immunization schedule.

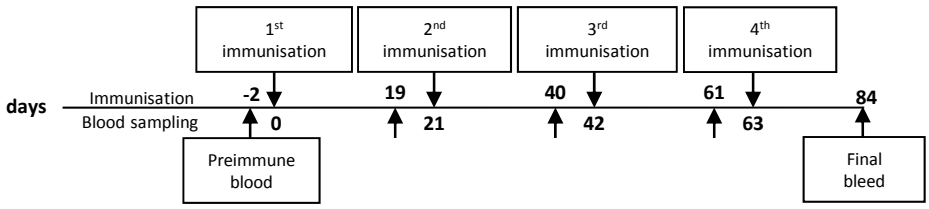


Figure 2.1: Immunization schedule of guinea pigs and rats immunized with 250 µg of recombinant hybrid proteins N1, N2, N3 or N4 mixed with complete Freund´s adjuvant for initial immunization or incomplete Freund´s adjuvant for booster immunization.

1.15.6 Cell culture

1.15.6.1 Transfection of 293T cells

Expression of DNA vectors used for DNA immunization experiments in mice was tested by transfecting 293T cells and surface expression of hybrid proteins was verified via immunofluorescent staining. 293T cells were maintained with complete D-MEM (10% FKS, 100 µg/ml penicillin/streptomycin, 4 mM glutamine). The day prior transfection, $4x10^5$ cells were seeded per 6-well in order to obtain 80-90% confluency the day of transfection. Four µg of vector was diluted in D-MEM and mixed with 6 µl of Transfectin (BioRad, Munich, Germany) transfection reagent, incubated for 20 min and subsequently added to the cells. Media was changed after 4 hours and cells were harvested after 48 h to assess expression of transfected constructs.

1.15.6.2 Immunofluorescent staining of transfected 293T cells

6 wells containing transfected 293T cells were trypsinized and resuspended with 1 ml of D-MEM. Cells were subsequently washed three times with warm PBS, fixed in 2% PFA for 1 h at 37°C, again washed with PBS and incubated in PBS overnight. The cell pellet obtained from one 6-well was resuspended in 100 µl PBS and one drop (20 µl) of the suspension was used per immunofluorescence sample to stain. Cells were dropped on Poly-L-Lysine coated slides, dried at 37°C and rehydrated in PBS. Samples were blocked with blocking buffer (PBS with 0.1% BSA) for 60 min and incubated with 5 µ/ml 2F5 and 4E10 primary antibodies diluted in blocking buffer for 45 min. Slides were subsequently washed with PBS and incubated with the secondary antibody coupled to AlexaFluor®488 for 30 min. Nuclei of cells were then stained with 0.25 µg/ml bis-enzimide diluted in PBS. After washing with PBS, slides were monted with Mowiol and stored at 4°C until analysis by Laser Scanning Microscopy (LSM).

1.15.6.3 Virus propagation

HIV-1_{NL4-3} was produced by transfecting 293T cells with the moleclular clone. Media was changed 4 hours after transfection, supernatants collected after 48 hours and stored at -80°C.

The primary isolate of clade F (HIV-$1_{BR\ 93/020}$) was propagated on PBMC isolated from buffy coats by Ficoll density gradient centrifugation.

Titration of virus stocks was performed on TZM-bl cells in a 96-well format. Briefly, $2x10^4$ cells were seeded per well and grown for 24 hours. Virus was serially diluted 1:10 in D-MEM, added to the cells and incubated for 48 h. Then, cells were washed with PBS and fixed with 2% paraformaldehyde. Staining of HIV-1 infected cells was performed by adding 80 µl of β-galactosidase solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, X-gal, diluted in PBS) to previously washed cells and incubated for 16 h. Virus titers were determined by counting blue cells per well, ranging from $5*10^5$ to 10^7 infectious particles/ml

1.15.6.4 *In vitro* neutraliziation assays

1.15.6.4.1 TZM-bl neutralization assay

The HIV-1 neutralizing activity of the sera or the purified IgG antibodies was measured in a 96-well format using reporter TZM-bl cells (Montefiori, 2009) and HIV-1_{NL4-3} (clade B, Genbank: AF324493).

Briefly, $2x10^4$ cells were seeded per well and grown for 24 h. Sera or purified IgG antibodies used at concentrations comparable to those in serum were diluted 1:20 in Dulbecco´s Modified Eagle´s Media followed by four steps of two-fold serial dilution. 25 µl of virus (infectious titer $5x10^5$/ml) and 25 µl serum/IgG dilution were pre-incubated for 30 min at 37°C and subsequently transferred to the cells. After incubation for 48 h at 37°C, neutralization activity was estimated using the Bright-Glo™ Luciferase Assay System (Promega, Mannheim, Germany). In this assay, luciferase activitiy induced by HIV-1 Tat protein was measured by a GloMax®-96 luminometer (Promega, Mannheim, Germany). The cut-off for neutralization was set at 50% RLU (relative luminescence units) value measured with an untreated virus infectivity control.

1.15.6.4.2 Real-time PCR-based neutralization assay

Sera which recognized the 2F5 epitope as shown by the epitope mappings in the first immunization study or sera inhibiting HIV-1 infection detected by TZM-bl neutralization assay were also tested in a neutralization assay based on the measurement of the reduction of provirus integration taken as a marker for neutralization capacity (Behrendt et al., 2009; Fiebig et al., 2009). Briefly, 10 µl serum dilution was mixed with 90 µl virus dilution (MOI=1) and incubated for 30 min/ 37°C. C8166 cells or PHA (5 µg/ml) stimulated PBMC ($5x10^4$ cells in 100 µl) were added and incubated for 65 hours, followed by aspiration of the supernatants. Cell pellets were lysed as previously described and provirus integration was assessed by duplex real-time PCR using HIV-1_{NL4-3} specific probe (2.1.1 Table 2.1) and primers (68spez, 69spez). Equal load of cells was simultaneously monitored by amplification of human GAPDH as a housekeeping gene (GAPDH probe, primers 44FWD, 45REV). Duplex real-time PCR was performed using MX3000P (Stratagene, La Jolla, USA) with the following cycle conditions: 95ºC/10 min, 95ºC/1 min, 52ºC/1 min, 72ºC/30 sec. Delta-CT values of preimmune serum plus 3 times the standard deviation were taken as cut-off value and a inhibition of infection more than 50% was defined as positive neutralization.

1.15.7 DNA vaccination with the Semliki Forest Virus vector system

1.15.7.1 Expression system overview

Semliki Forest virus (SFV) belongs to the family of *Togaviridae*, genus of *Alphaviruses*. The SFV genome comprises a single stranded RNA (11.4 kb) of positive polarity, packaged into a capsid that is further enveloped by a lipid bilayer. The genomic RNA constitutes two open reading frames (ORF), the 5´ ORF encoding the viral replicase or non-structural proteins nsp1-4. Capsid protein (C) and spike proteins (E1, E2, E3 and 6K) are encode within the second reading frame of the SFV genome. A translational enhancer (b1) is additionally encoded within the capsid gene in order to provide high expression levels of structural proteins (Sjoberg et al., 1994).

After release of the viral RNA into the host cell, the early RNA polymerase (nsp1-3 as a polyprotein, nsp4 as cleaved protein) transcribes a complementary, negative strand RNA (Koff et al., 2006). The polyprotein of nsp1-3 is thereafter cleaved and assembles to form the replicase complex together with nsp4. This complex therafter transcribes a positive-sense genomic RNA and a subgenomic RNA. Structural proteins are translated as polyprotein and subsequently cleaved into capsid protein and the spike proteins (E1, E2, E3 and 6k).

Peter Liljeström and Henrik Garoff developed the Semliki Forest virus vector system (Liljestrom and Garoff, 1991), substituting the genes of the SFV structural proteins by the relevant gene of interest. The vector system was primarily developed to produce recombinant SFV particles in order to generate high amounts of heterologous protein, which was further modified to an alphaviral replicon based (DREP) DNA vector system (Berglund et al., 1998). A number of studies demonstrated enhanced antibody responses and antigen specific CD8+ T-cell responses after immunization with DREP compared to the immunogenicity of a conventional DNA vector (Leitner et al., 2006; Naslund et al., 2011; Naslund et al., 2007; Nordstrom et al., 2005).

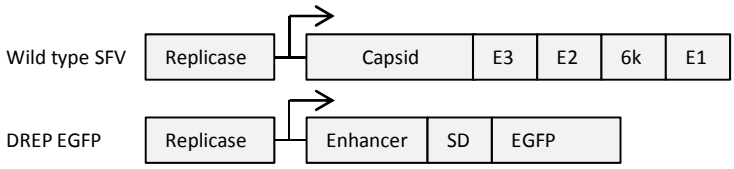


Figure 2.2: Schematic presentation of the DREP expression system in comparison with the wild type SFV genome. Arrows represent internal subgenomic promotors, start of translation for the foreign gene by the vector encoded replicase. Expression constructs included the translational enhancer (enhancer), the ribosomal slippery sequence (SD) and the antigen of interest.

Animals and antigens used for the DNA immunization study are described in the following table:

Species	Number	Antigen/Adjuvant	Amount antigen	Number of immunizations
Mice	N1m#1 N1m#2 N1m#3 N1m#4 N1m#5	pDisplay-N1 with GM-CSF	2 µg pDisplay-N1 2 µg GM-CSF	3
Mice	N1m#6 N1m#7 N1m#8 N1m#9 N1m#10	DREP-N1	2 µg pDisplay-N1	3
Mice	N1m#11 N1m#12 N1m#13 N1m#14 N1m#15	rN1	100 µg Incomplete Freud´s adjuvant	3
Mice	N5m#1 N5m#2 N5m#3 N5m#4 N5m#5	pDisplay-N5 with GM-CSF	2 µg pDisplay-N5 2 µg GM-CSF	3
Mice	N5m#6 N5m#7 N5m#8 N5m#9 N5m#10	DREP-N5	2 µg pDisplay-N5	3
Mice	N5m#11 N5m#12 N5m#13 N5m#14 N5m#15	rN3	100 µg Incomplete Freud´s adjuvant	3

The DREP vector was used in this project for the induction of potent antibody responses against HIV-1, expressing two different hybrid proteins. Recombinant hybrid proteins N1 and N5 were cloned into the DREP vector as described in section 3.7 and the appendix.

1.15.7.2 Preparation of gene-gun ammunition and application of DNA expression plasmids

Ballistic immunization procedures were utilized with antigen/adjuvant-coated Tefzel tubes in combination with the Helios GeneGun® (Biorad) according to the manufacturer´s protocol. Briefly, Tefzel tubes were washed with 100% ethanol and dried with nitrogen for 1 h prior antigen coating. 25 mg of gold particles were resuspended in 100 µl of 0.05 M sperimidin and subsequently vortexed and sonicated. 100 µg plasmid DNA of the expression vectors encoding hybrid antigens and 100 µg of the GM-CSF-encoding plasmid were added to the gold particle mixture, followed by dropwise addition of 1 M $CaCl_2$ solution for DNA precipitation. After 10 min of incubation, golden particles coated with precipitated DNA were washed with 100% ethanol and then resuspended in 3 ml of 0.025 mg/ml Polyvinylpyrrodidon (in 100%) ethanol. The plasmid-gold particles were coated onto the inner surface of the Tefzel tubes using the Biorad Tubing Prep Station® by rotation and subsequent drying via nitrogen. The tube was then cut into 1-2 cm segments, resulting in 1 µg of vaccine and adjuvant expression plasmid each per shot.

Application of antigens in the DNA immunization study was performed using Helios® GeneGun system. Plasmid DNA was delivered intracutaneously to shaved abdominal skin of mice via two shots, 1 µg of DNA each at a helium pressure of 400 p.s.i.

2. Results

2.1 Design of hybrid proteins

Four recombinant hybrid proteins (N1, N2, N3, N4) possessing p15E and gp41 sequences were designed on basis of protein sequence alignment of the PERV and HIV-1 TM proteins (Figure 3.1) in order to optimize substitutions of sequences in the p15E backbone.

Figure 3.1: (A) Alignment of HIV-1 and PERV TM protein sequences for the design of hybrid proteins. (B) Composition of protein sequences in hybrid proteins. Boxed sequences in blue indicate HIV-1 sequences, sequences boxed in green squares correspond to PERV sequences. Multiple sequence alignments were performed with Seqman software (DNAStar, Madison, USA).

Hybrid proteins N1 and N2 included the fusion peptide, the transmembrane region and a truncated intraviral tail in contrast to hybrid proteins which were previously designed by Rayk Behrendt (Behrendt, 2009). Incorporation of these gp41 subunits were proposed to be necessary for generation of native-like prehairpin intermediate stuctures, may be required to induce HIV-1 neutralizing antibodies (Denner, 2011; Gao et al., 2013). Homologies between the bNAb 4E10 epitope in the MPER of gp41 and a sequence located in the MPER of p15E, recognized by PERV neutralizing sera, were also observed in these alignments as previously reported (Fiebig et al., 2003). Sequences of the HIV-1 TM protein were “grafted” into the TM protein p15E of PERV based on prior alignments with regard to sequence homologies after removal of the epitopes in p15E which were recognized by these PERV neutralizing sera (Figure 3.1B).

2.2 N1 and N2 hybrid proteins

2.2.1 Design, expression and characterization of recombinant hybrid proteins N1 and N2

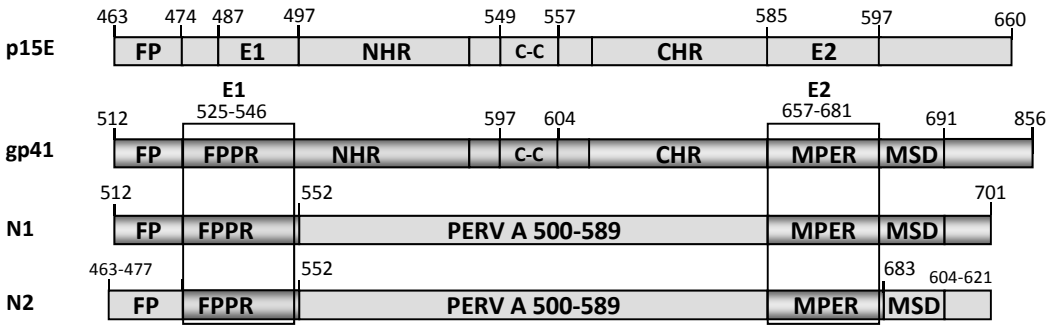


Figure 3.2: Schematic presentation and characterization of gp41, p15E and antigens N1 and N2 containing sequences of gp41 of HIV-1_{NL4-3} (GenBank: AF324493) and p15E of PERV-A (GenBank: AY953542). Dark grey squares indicate sequences of gp41 of HIV-1; light grey squares represent sequences of p15E of PERV-A. The FPPR and MPER domains are derived from gp41, substituting analogue domains in p15E. N1 and N2 contain the fusion peptide (FP), the membrane spanning domain (MSD) and a truncated intraviral/intracytoplasmic tail from gp41 (N1) or p15E (N2).

Initially, hybrid proteins N1 and N2 contained the fusion peptide (FP), the membrane spanning domain (MSD) and a truncated intraviral/intracytoplasmic tail as flanking regions for the HIV-1 FPPR and MPER. These constructs were designed in order to provide the approximate full length TM protein to allow native-like folding of the MPER in presence of flanking regions (Hager-Braun et al., 2006; Montero et al., 2012). The recombinant hybrid protein N1 contained FP, MSD, a truncated intraviral/intracytoplasmic tail and the FPPR and MPER domains derived from gp41 (Figure 3.2) grafted into the backbone of p15E. For the design of construct N2, only sequences in p15E recognized by PERV neutralizing sera obtained from immunization studies using the PERV TM protein as antigen were substituted by the corresponding FPPR and MPER from gp41. N2 was designed to allow a similar folding as in p15E in order to present potential epitopes for HIV-neutralizing antibodies in a p15E scenario.

Both constructs remained difficult to express with ordinary expression strains. Optimization of recombinant hybrid antigen expression was performed with *E. coli* bacterial strains SCS1SE111, C3030H and BL21(DE3)pLysS at 25°C or 37°C (Figure 3.3). Sufficient overexpression of hybrid proteins was only detected when the expression strain SCS1/SE111 was used.

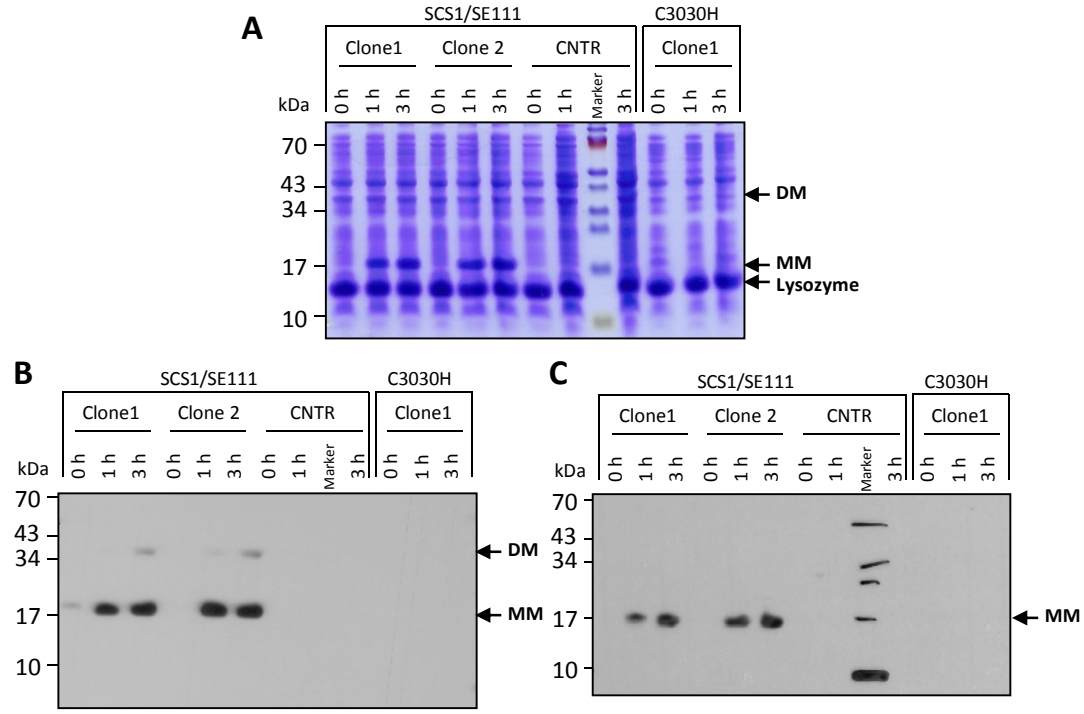


Figure 3.3: Representative expression optimization of hybrid protein N1 with two different expression clones in *E. coli* expression strains SCS1/SE111 and C3030H at 37°C. Samples of lysed expression cultures were taken before induction (0 h), 1 h and 3 h after induction with 1 mM IPTG. (A) SDS-PAGE of *E. coli* lysates after expression were stained with Coomassie brilliant blue. (B) Expression of recombinant protein N1 was detected on Western blot with monoclonal antibodies 2F5 and RGS-His (C). Arrows indicate monomeric (MM) or dimeric (DM) recombinant proteins.

Purification using the N-terminal histidine tag (RGS-His) was insufficient due to aggregation of the recombinant proteins N1 and N2 in inclusion bodies and insolubility in native buffers without detergent. Thus, the bacterial lysate was therefore washed with PBS and the remaining pellet subsequently solubilized with guanidine hydrochloride in order to solubilize hydrophobic proteins, resulting in a hybrid protein solution with a purity of 70%. The purified recombinant proteins N1 and N2 were recognized by the two bNAb 2F5 and 4E10 in Western blot analysis (Figure 3.4). N1 was used for immunization of 4 rats and 4 guinea pigs, the recombinant hybrid protein N2 was used for immunization of 4 rats. Animals were immunized four times in a three week immunization schedule (2.2.5.1, Figure 2.1).

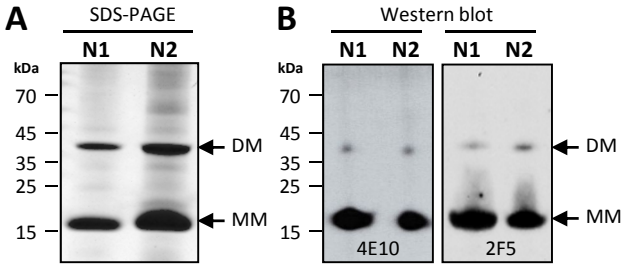


Figure 3.4: Characterization of the purified proteins N1 and N2 on reducing SDS-PAGE (A) stained with Coomassie brilliant blue. (B) Western blot analysis of purified proteins with monoclonal antibodies 2F5 and 4E10. Arrows indicate monomeric (MM) or dimeric (DM) recombinant proteins.

2.2.2 Immune responses induced by immunizations with N1 and N2

Sera specimens derived from guinea pigs (N1g#1-4) and rats (N1r#1-4; N2 r#1-4) after the third booster immunization were screened for reactivity against the recombinant ectodomain of gp41 by Western blot analysis and ELISA in order to evaluate HIV-1 specific humoral immune responses. All immune sera obtained from rats and guinea pigs immunized with N1 or N2 recognized recombinant gp41 in Western blot analysis (3.5C).

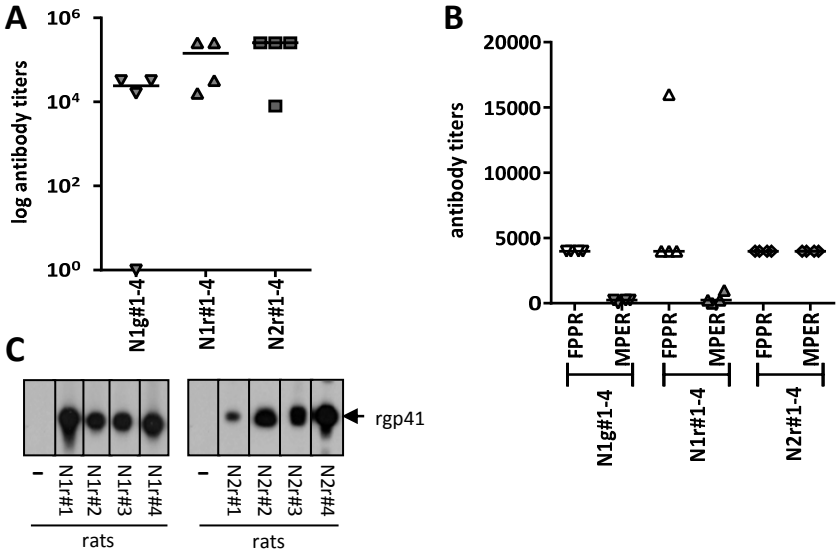


Figure 3.5: Serum antibody titers against rgp41 (A) or peptides corresponding to either FPPR or MPER of gp41 (B). (C) Western blot analysis of rat immune sera derived after the third immunization boost, using rgp41 as antigen on SDS-PAGE.

Sera collected after the fourth immunization were further tested in ELISA using rgp41 (Figure 3.5A) and peptides corresponding to the gp41 FPPR or MPER (Figure 3.5B) to evaluate gp41-specific antibodies. Moderate anti-rgp41 titers between 10^4 and 10^6 were detected in every immunized animal but one guinea pig immunized with N1. Anti-MPER responses were weak (1:250) or not detected in guinea pigs or rats, which received N1 recombinant protein. All sera from rats immunized with N2 reacted with FPPR and MPER peptides.

Epitope mappings of immune sera derived from rats immunized with N1 induced antibodies binding to the FPPR, which confirmed previous ELISA results (Figure 3.6). Sera from rats immunized with N2 also recognized the sequence TLTVQARQL in N-terminal region of gp41. Peptides corresponding to the MPER were not recognized by sera from animals immunized with N2, although these sera reacted in an ELISA with a longer peptide comprising the complete MPER sequence (Figure 3.5B), suggesting an influence of the peptide-length and -conformation on antibody binding.

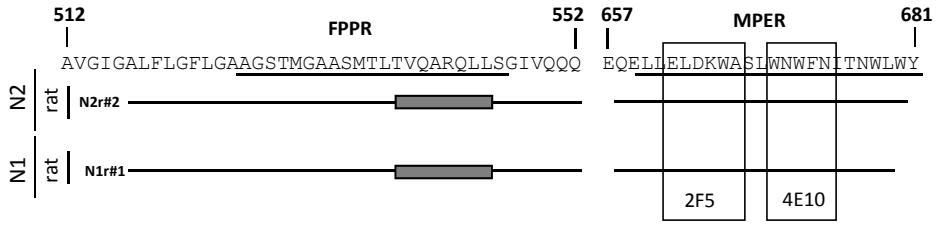


Figure 3.6: Representative epitope mapping of one rat immunized with N1 or N2, respectively. Sequences corresponding to the FPPR and the MPER of gp41 in the hybrid constructs are underlined. Boxed sequences indicate epitopes of 2F5 (ELDKWA) and 4E10 (NWFNIT).

Sera derived from each immunization study were tested for neutralizing capacity, using HIV-1_{NL4-3} as standard virus, which was previously described as neutralization sensitive to bNAb 2F5 and 4E10 (Binley et al., 2004). Antisera derived after the third booster immunization with N1 or N2 were all tested in the TZM-bl neutralization assay but none of the sera or isolated IgG inhibited HIV-1_{NL4-3} infection (Figure 3.7A-C).

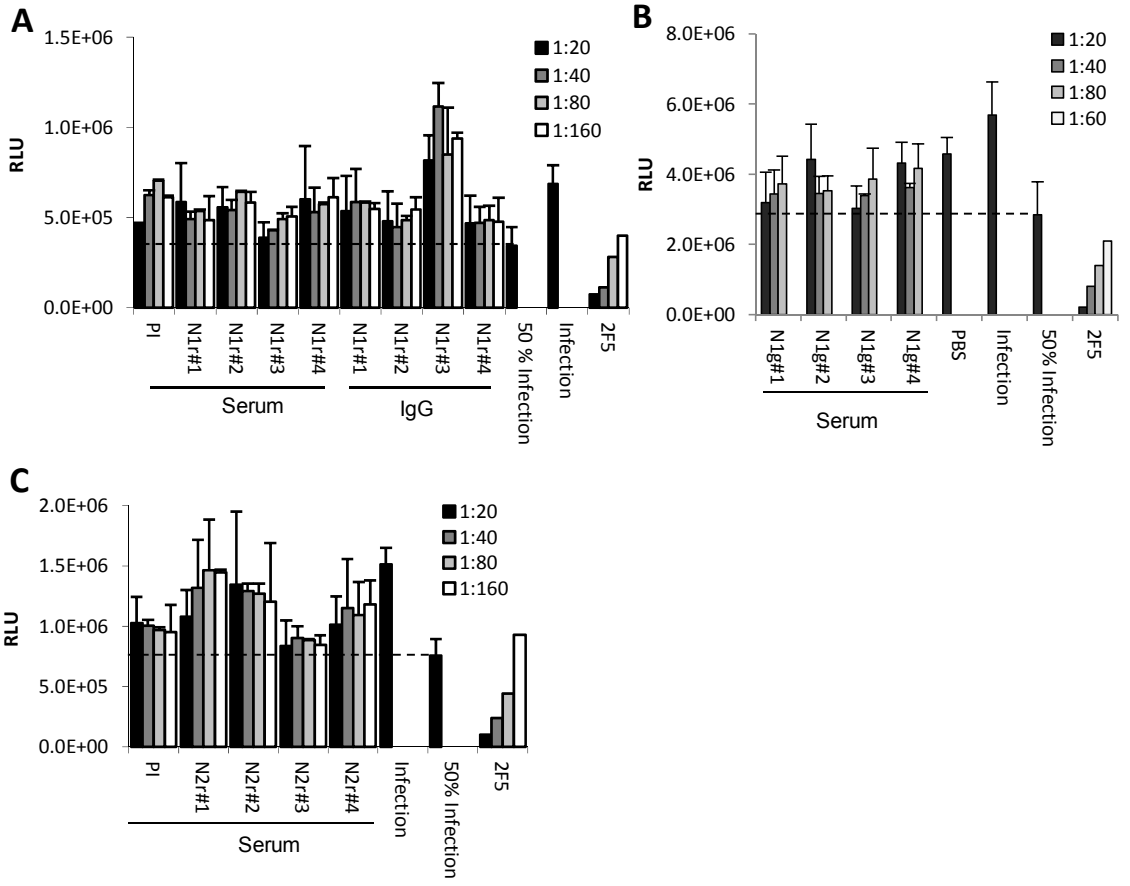


Figure 3.7: Neutralization assay with immune sera or isolated IgG from rats (A) and guinea pigs (B) immunized with N1 derived after the third immunization boost. (C) Neutralization assay off immune sera after four immunizations with recombinant hybrid protein N2. PI describes preimmune sera used as negative control, PBS represents PBS control wells, PBS was added instead of serum. Purified IgG isolated from immune sera were used in dilutions (corresponding to the dilution in the original serum) from 1:20 followed by 1:2 dilution steps. 2F5 was used as control in concentrations of 50/12.5/3.125/0.78 µg/ml. Luciferase reporter activity is presented as RLU (relative luminescence units) and the dotted line indicates the cut-off defined as 50% of untreated virus infectivity control. Error bars represent the standard deviation (n=3).

To summarize the results above, immune sera from rats and guinea pigs obtained after immunization with N1 induced HIV-1 FPPR-specific antibodies, whereas FPPR and MPER-specific antibodies were observed after immunization with N2, although no specific epitope was identified for MPER-specific antibodies. Moreover, immune sera of immunized rats or guinea pigs inhibited infection of HIV-1.

2.3 N3 and N4 hybrid proteins

2.3.1 Design, cloning, expression and characterization of recombinant hybrid protein N3

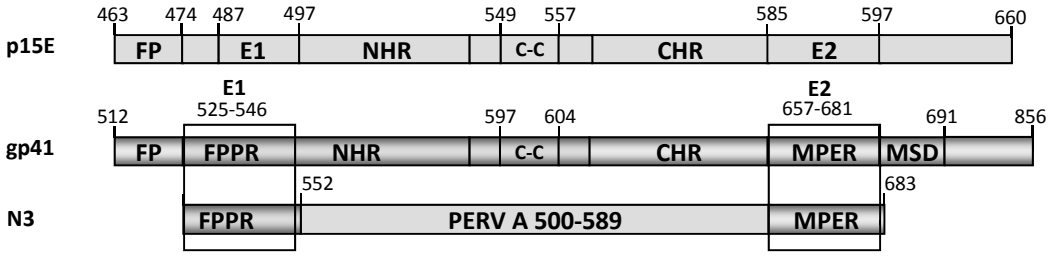


Figure 3.8: Schematic presentation of gp41, p15E and antigen N3 containing sequences of gp41 of HIV-1_{NL4-3} (GenBank: AF324493) and p15E of PERV-A (GenBank: AY953542). Dark grey squares indicate sequences of gp41 of HIV-1; light grey squares represent sequences of p15E of PERV-A. The FPPR and MPER domains are derived from gp41, substituting analogue domains in p15E. Construct N3 was designed to remove FPPR and MPER flanking regions to prevent potential masking of bNAb binding sites.

The hybrid N3 was designed to eliminate potential masking of FPPR or MPER by flanking regions removing hydrophobic FP and MSD sequences and to increase solubility of the recombinant protein (Figure 3.8). N3 was cloned, expressed and initially purified by affinity chromatography under denaturing conditions using 8M Urea, achieving a purity of 95% (Figure 3.9). The recombinant protein was purified under denaturing conditions and used for immunization experiments in four rats, four guinea pigs and one rabbit. Further analysis of the proteins showed dimerization on reducing SDS-PAGE (red.) and oligomers on non-reducing SDS-PAGE (non-red.). Presence and accessibility of the MPER was tested by mAbs 2F5 and 4E10 in Western blot analysis (Figure 3.9B).

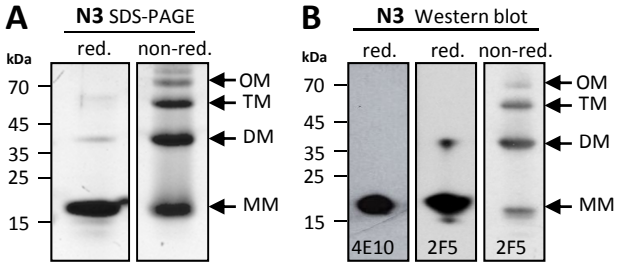


Figure 3.9: (A) Characterization of purified recombinant protein N3 on 10% SDS-PAGE stained with Coomassie brilliant blue G 250. Protein samples were treated with sample buffer containing β-mercaptoethanol (red.) or native sample buffer without reducing agent (non-red.). Arrows show recombinant proteins as monomers (MM), dimers (DM), trimers (TM) and oligomers (OM). (B) Western blot analyses of purified recombinant N3 using the monoclonal antibodies 4E10 and 2F5.

Recombinant hybrid protein N3 was further characterized by SPR analysis and binding affinities were assessed for the bNAb 2F5 and 4E10 to the recombinant antigens. 2F5 bound N3 with an apparent K_D value of 0.3448 nM, showing a 100-fold higher affinity value to N3 compared to that measured for 4E10 (32.0 nM). N4 was bound by 2F5 with a 20-fold lower apparent K_D value of 18.34 nM compared to the affinity for 4E10 (344.8 nM) to the recombinant hybrid protein.

2.3.2 Immunization of 4 guinea pigs and 4 rats and 1 rabbit with N3

To evaluate the immunogenicity of N3, four guinea pigs, four rats and one rabbit were immunized with the recombinant hybrid protein. Guinea pigs and rats received 4 injections of 250 µg antigen, mixed with CFA/IFA in a 3-week immunization schedule (2.2.5.1, Figure 2.1).

Sera specimens derived from immunized animals were screened for reactivity against the recombinant ectodomain of gp41 by Western blot and ELISA. All immunized animals reacted specifically with rgp41 in Western blot analysis (Figure 3.10C) which was also demonstrated in ELISA, using rgp41 and peptides corresponding to the FPPR or MPER (Figure 3.10A and B). Anti-rgp41 titers between 10^4 and 10^6 were observed in immune sera of the rats and guinea pigs, immunized with N3. Strong reactivity against the FPPR-derived peptide was observed in every anti-serum from four rats and three guinea pigs immunized with N3. This guinea pig showed a strong anti-MPER response and an anti-FPPR antibody titer. To be noticed, a significant increase of anti-MPER responses was detected in antisera of rats immunized with N3 compared to N1 immunized rats. Differences in the arrangement of HIV-1 derived sequences in the p15E backbone in N1, N2 and N3 might have modified accessibility of the HIV-1 MPER and thus increased the antibody response towards the MPER.

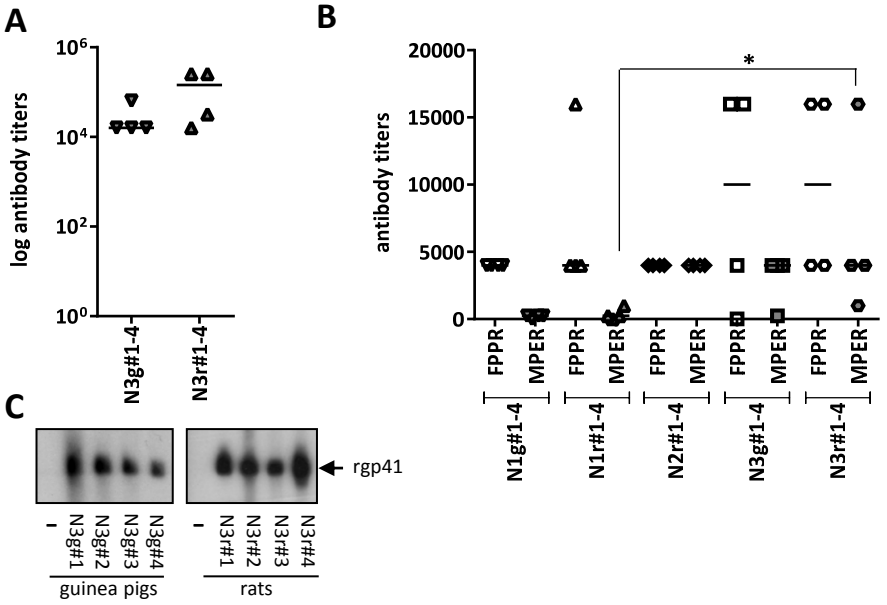


Figure 3.10: (A) Titers of antibodies specific for rgp41 in the sera of animals immunized four times with the hybrid protein N3. (B) Comparison of anti-FPPR and anti-MPER antibody titers (using FPPR and MPER derived peptide as antigens). Asterisks indicate significant differences in antibody titers between two groups of animals evaluated by Mann-Whitney test ($p<0.05$). C) Western blot of immune sera from animals immunized with N3, using rgp41 as antigen.

Sera from rats immunized with N3 also recognized epitopes in the N-terminal region of gp41, mainly the sequence TLTVQARQL (Figure 3.11). Antisera from three guinea pigs and three rats immunized with N3 reacted with MPER derived peptides, specifically binding the core epitope of 2F5 (ELDKWA) (Figure 3.11). Representative epitope mapping on a nitrocellulose membrane of the serum from guinea pig N3g#3 shows one epitope in the FPPR and another in the MPER including the 2F5 epitope. Reactivity of the serum with two additional peptides derived from gp41 was observed although this part of the protein was substituted by corresponding regions in p15E. Other serum proteins might have bound non-specifically to the membrane presumably due to insufficient regeneration since the membrane was used repeatedly for epitope mapping. Furthermore, microarray chips used for epitope mapping of this serum did not show binding to these peptides.

Importantly, the serum from guinea pig N3g#4 immunized with N3 recognized only the ELDKWA epitope in the MPER, but did not recognize sequences in the FPPR in epitope mappings. The recognition pattern of the immune serum corresponds exactly to the binding charasteristics of bNAb 2F5 in the peptide epitope mapping.

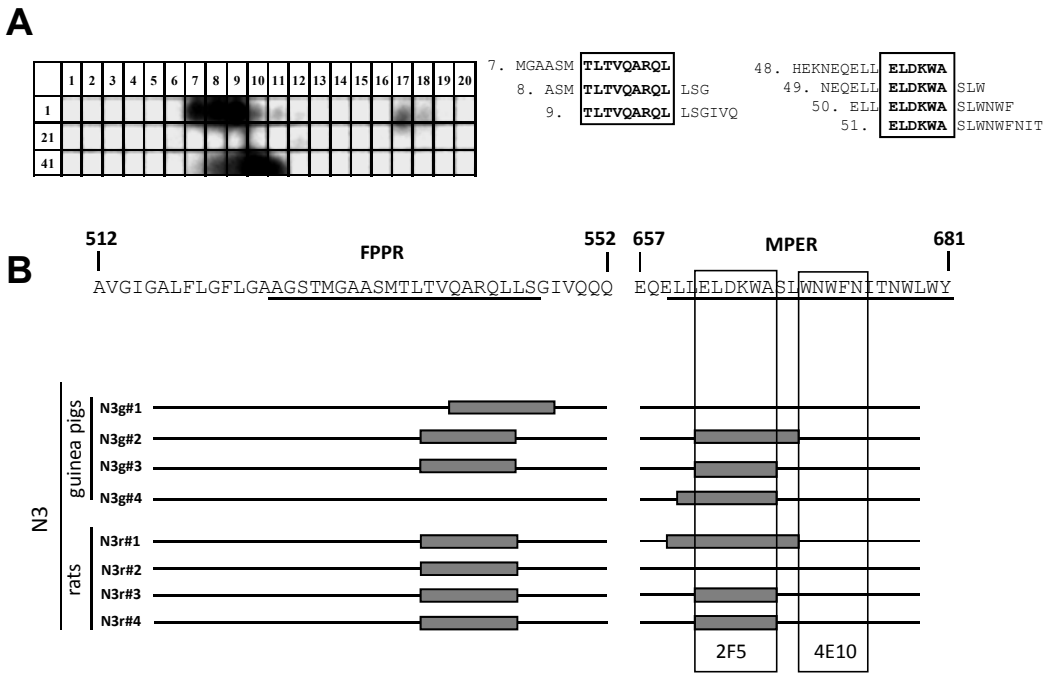


Figure 3.11: (A) Epitope mappings of the antisera derived from animals immunized with the recombinant hybrid protein N3. (A) Representative epitope mapping on a nitrocellulose membrane of the serum from guinea pig N3g#3, showing one epitope in the FPPR and another in the MPER including the 2F5 epitope. (B) Summary of epitope mappings of all sera from guinea pigs and rats immunized with N3. Sequences corresponding to the FPPR and the MPER of gp41 in the hybrid constructs are underlined. Boxed sequences indicate epitopes of mAb 2F5 (ELDKWA) and mAb 4E10 (NWFNIT).

Sera from animals immunized with N3 which recognized rgp41 were further tested for neutralization activity by a neutralization assay based on TZM-bl cells with HIV-$1_{NL4-3,}$ but no neutralizing activity of immune sera or IgG isolated from these sera was observed, despite that some of these sera bound to the same epitope as the broadly neutralizing antibody 2F5 (Figure 3.12A and B). Sera and IgG from rats and guinea pigs immunized with N3 which reacted

with the 2F5 epitope were also analysed in a neutralization test based on C8166 cells or PHA stimulated PBMCs. None of the analysed sera in different dilutions (1:20 or 1:50) or isolated IgG were able to inhibit provirus integration in C8166 cells to a greater extent than the corresponding preimmune serum (Appendix; A1A and B). Surprisingly, the serum of guinea pig N3g#4 weakly inhibited provirus integration in the PBMC-based real-time PCR neutralization assay despite the negative results in the two other neutralization assays.

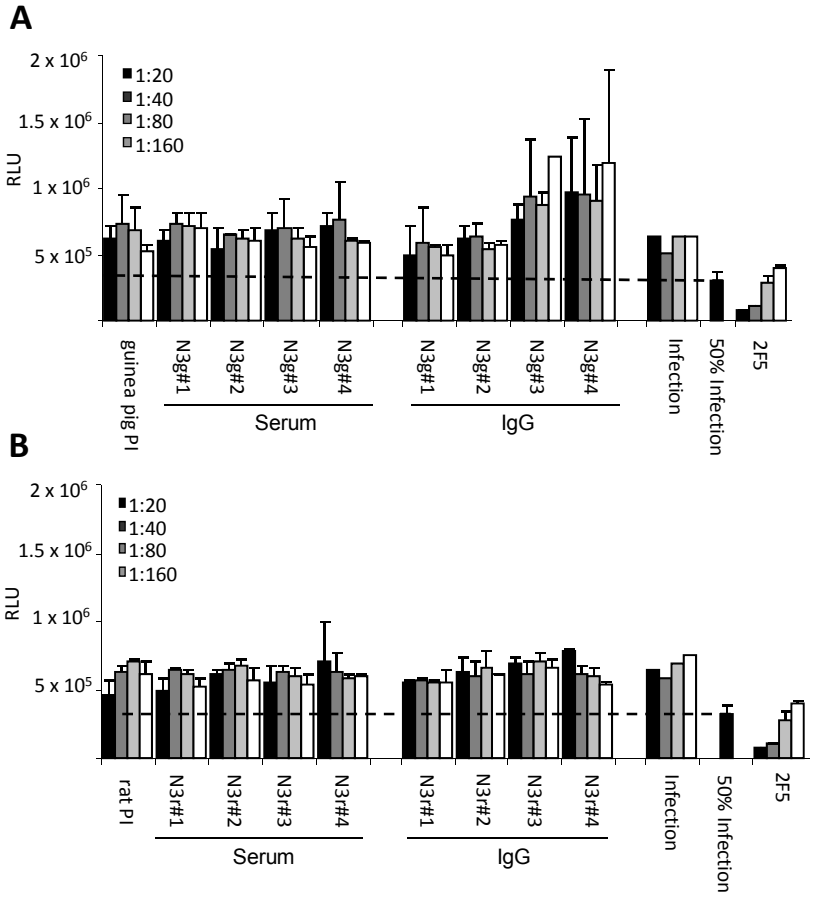


Figure 3.12: (A) Comparison of mean values of rgp41 binding antibodies in sera and isolated IgG fractions. (B) Neutralization assay using sera and isolated IgG from guinea pigs (A) and rats (B) immunized with N3. PI indicates IgG isolated from preimmune serum. Purified IgG were used in dilutions (corresponding to the dilution in the original serum) from 1:20 followed by 1:2 dilution steps. 2F5 was used as control in concentrations of 50/12.5/3.125/0.78 µg/ml. Luciferase reporter activity is presented as RLU (relative luminescence units) and the dotted line indicates the cut-off defined as 50% of untreated virus infectivity control. Error bars represent the standard deviation (n=2).

The immunization study with the rabbit was performed in Hungary in cooperation with our Euroneut-41 cooperation partners at the Central Agricultural Office in Budapest in order to evaluate the immunogenicity of N3 in an additional animal species. Two batches of 250 µg antigen for each immunization were mixed each 1:1 with Montanide at a total volume of 500 µl and sent to the testing site. The rabbit was immunized for primary and booster immunization with 100 µl i.m. in each hind leg and received 300 µl subcutaneously. Serum was taken after each immunization (S1, S2) and sent back to the Robert Koch-Institute for evaluation of antibody responses induced by immunization.

Since the rabbit was only immunized twice, immune sera from this immunization study were first tested for reactivity against the N3 and rgp41, showing antibodies binding to the proteins already three weeks after the first immunization (Figure 3.13A). Titers of anti-gp41 antibodies in immune sera obtained after the fourth immunization were comparable to those observed in rats and guinea pigs after the same number of booster immunizations (Figure 3.13B). Antibody responses against the FPPR were moderate, whereas anti-MPER responses remained weak and were thus not detected in epitope mapping (Figure 3.13C).

When testing the rabbit immune sera in the neutralization assay, no inhibition of HIV-1_{NL4-3} infection was observed (Figure 3.13D).

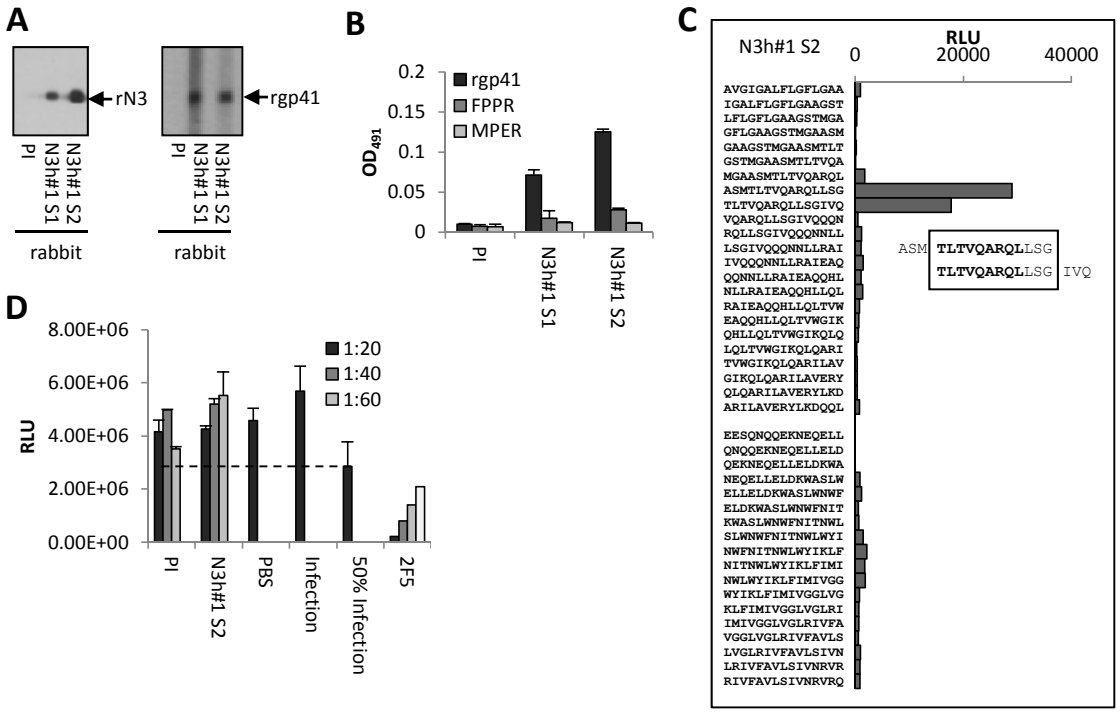


Figure 3.13: (A) Immune sera of the rabbit immunized with N3 derived after 1st and 2nd immunization (S1, S2), recognizing N3 and gp41, diluted 1:400. (B) Comparison of antibodiy responses after 1st and 2nd immunization against rgp41, FPPR and MPER derived peptides; serum diluted 1:2000. Error bars represent the standard deviation (n=3). (C) Microarray-based epitope mapping of rabbit immune serum. (D) Neutralization assay with preimmune (PI) and immune serum derived after the 2nd immunization (S2). Error bars represent the standard deviation (n=2).

It was demonstrated that immunizations with N3 of guinea pigs and rats induced increased titers of anti-MPER antibodies compared to previous immunizations with hybrid proteins N2 or N2, although the amount of gp41 and FPPR binding antibodies in immune sera remained comparable to those observed after N1 or N2 immunizations. N3 vaccination of one rabbit induced moderate titers of anti-FPPR antibodies but no MPER specific antibodies. Despite successful induction of antibodies binding exactly to the epitope of 2F5 after immunization with N3 in rats and guinea pigs, none of the sera or isolated IgG neutralized HIV-1_{NL4-3} in the TZM-bl or C8166 cell-based neutralization assay. Only the serum of one guinea pig showed a weak inhibition of HIV-1_{NL4-3} infection in the PBMC-based real-time PCR assay.

2.3.3 Design, expression and characterization of recombinant hybrid protein N4

Previous immunization studies with N3 in rats and guinea pigs resulted in antibodies responses binding precisely to the ELDKWA epitope, showing a moderate focus on the MPER but no neutralization of HIV-1. Since a number of previous studies have shown MPER binding antibodies but no effective neutralization (Dennison et al., 2011b; Hinz et al., 2009; Jain et al., 2010; Kamdem Toukam et al., 2012; Law et al., 2007; Shen et al., 2010), the construct N4 was designed with focus on optimal amino-acid composition and location of different domains within the hybrid protein. Thus, 26 amino acids were deleted in the p15E backbone of N3 (Figure 3.14), expecting a closer proximity of the FPPR and MPER region within the folded protein. In addition, purification procedures were optimized for N3 using imidazole (2.2.2.2) to reduce denaturation of the protein during purification.

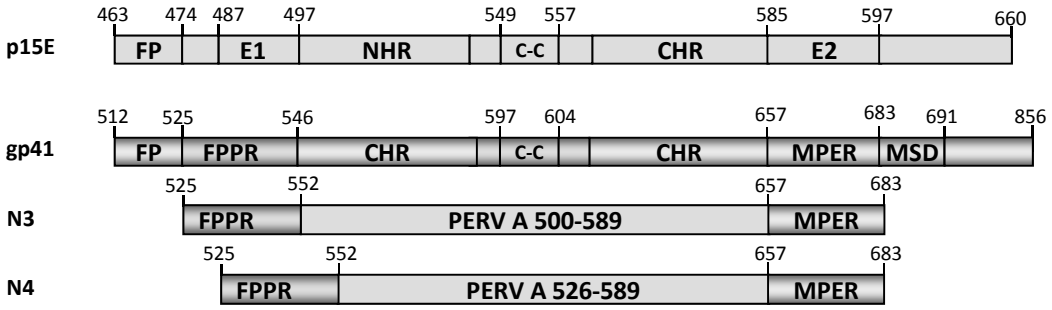


Figure 14: Schematic presentation of gp41, p15E and antigen N3 and N4 containing sequences of gp41 of HIV-1_{NL4-3} (GenBank: AF324493) and p15E of PERV-A (GenBank: AY953542). Dark grey squares indicate sequences of gp41 of HIV-1; light grey squares represent sequences of p15E of PERV-A. The FPPR and MPER domains are derived from gp41, substituting analogue domains in p15E. Construct N3 was designed to remove FPPR and MPER flanking regions to prevent potential masking of bNAb binding sites.

The recombinant protein was expressed and purified with an identical purification protocol using imidazole as elution component, achieving a purity of 95% (Figure 3.15A). Analysis of N4 on denaturing and reducing SDS-PAGE showed monomers and dimerization of the recombinant protein with Coomassie brilliant blue staining (Figure 3.15A), also detected with 2F5 and with 4E10 (Figure 3.15B). N4 treated with native sample buffer without reducing agent (non-red.) showed formation of mono-, di-, tri-, and oligomers on SDS-PAGE, which were bound by 2F5 and 4E10.

Recombinant hybrid protein N4 was also characterized by SPR analysis and binding affinities were assessed for the bNAb 2F5 and 4E10 to the recombinant antigens. N4 was bound by 2F5 with a 20-fold lower apparent K_D value of 18.34 nM compared to the affinity for 4E10 (344.8 nM) to the recombinant hybrid protein.

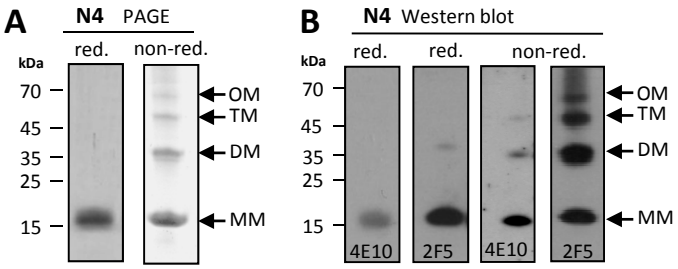


Figure 3.15: (A) Characterization of the purified recombinant protein N4 on 10% SDS-PAGE stained with Coomassie brilliant blue G 250. Protein samples were treated with sample buffer containing β-mercaptoethanol (red.) or native sample buffer without reducing agent (non-red.). Arrows show recombinant proteins as monomers (MM), dimers (DM), trimers (TM) and oligomers (OM). (B) Western blot analyses of purified recombinant proteins using the monoclonal antibodies 4E10 and 2F5.

2.3.4 Immunization of rats with N3 and N4

Purified recombinant proteins N3 and N4 were used for further immunizations of rats in groups of four animals each. One group received 250 µg N3 (N3r#5-8), four animals were immunized with 250 µg (N4#r1-4) or 500 µg N4 (N4r#5-8) in a three week immunization schedule, respectively (2.2.5.1, Figure 2.1). Hybrid protein N3 was used in this immunization regiment in order to compare immune responses after immunization of rats directly side by side and evaluate the impact on immunogenicity of gp41 FPPR and MPER due to backbone modification.

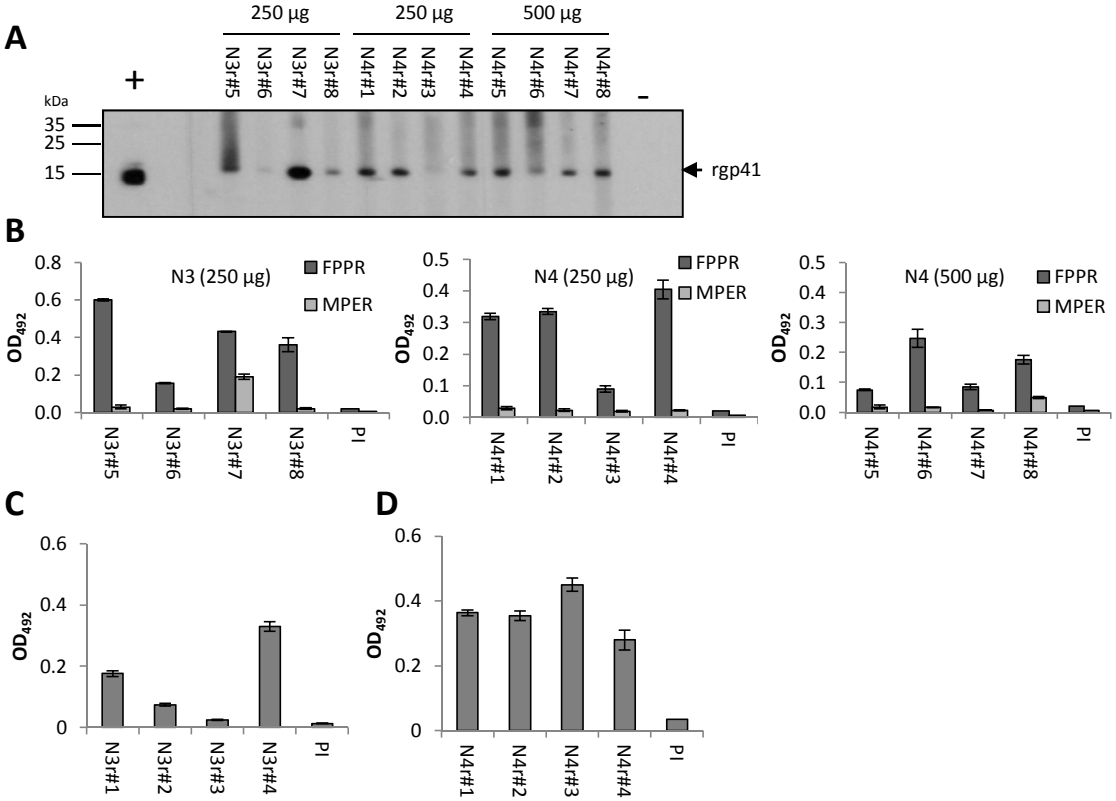


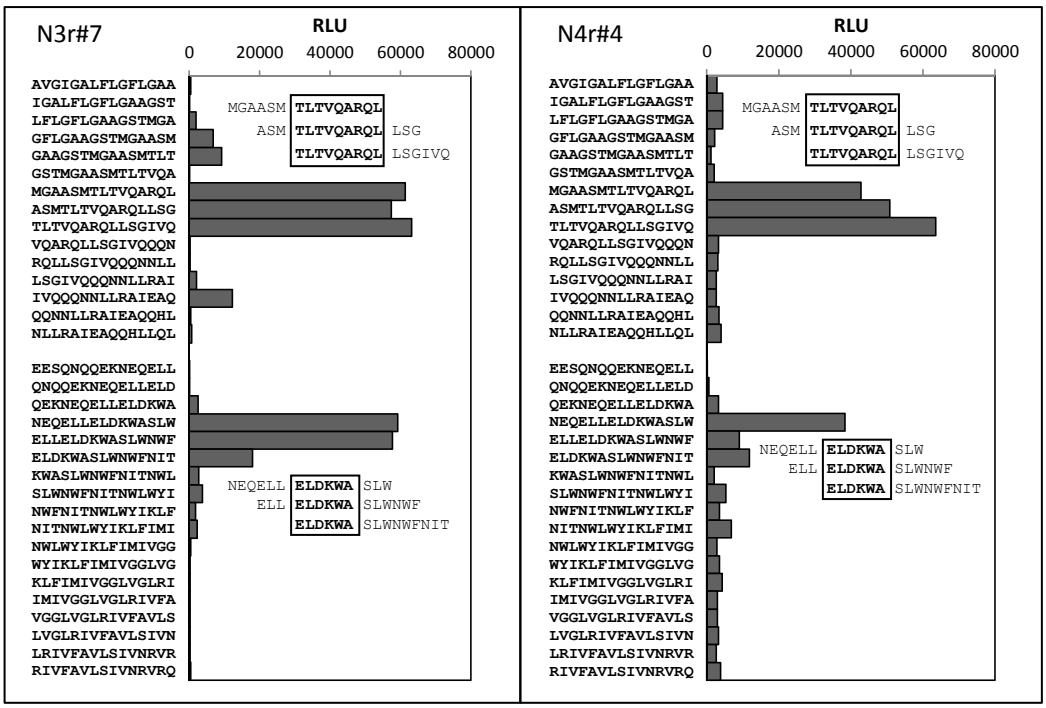
Figure 3.16: (A) Western blot analysis of immune sera (after 4th immunization), diluted 1:400 from animals immunized with N3 (N3r#5-8) or N4 (N4r#1-8), using rgp41 as antigen. (+) 2F5 diluted 1:100 000 and (PI) or (-) represents preimmune serum. (B) OD_{492} values of serum antibodies diluted 1:2000 specific for FPPR and MPER derived peptides of animals immunized four times with the hybrid protein N3 or N4. (C) OD_{492} values of MPER-specific antibodies in immune sera after immunization with N3 or N4 (D), diluted 1:250. Error bars represent the standard deviation (n=3).

Anti-rgp41 Western blot analysis showed moderate antibody titers against the ectodomain of the HIV-1 TM protein (Figure 3.16B) in N3 and N4 immunized animals. Anti-rgp41 titers in sera from N3 immunized animals (3.16B) were comparable to those observed in the previous immunization experiment (Figure 3.10A) but remained higher compared to those observed in immune sera obtained after immunization with 250 µg N4. Detailed analysis of binding antibodies by ELISA using FPPR and MPER derived peptides as antigens (Figure 3.16B) showed predominant FPPR directed responses in immune sera derived from N3 or N4 immunized animals.

Comparing immune sera of rat groups immunized with 250 µg or 500 µg, respectively, administration of 500 µg N4 recombinant protein had an inhibitory effect on induction of rgp41-specific antibodies compared to immune sera derived from the rats which received 250 µg (Figure 3.16B). Moreover, immunization with 500 µg of N4 recombinant protein induced lower antibody titers of anti-FPPR antibodies compared to rats immunized with half the amount of antigen. Anti-MPER responses of N3 and N4 immunized animals tested in ELISA remained overall lower in this immunization study (Figure 3.16B) except rat N3r#7. These results were surprising since potent MPER-specific antibody responses were observed in the previous immunization study using N3 as antigen (Figure 3.16C and D). Even though immunization with N3 induced higher titers of rgp41-specific antibodies, stronger MPER-specific antibody responses were observed in immune sera of rats immunized with N4 (Figure 3.16C and D).

Epitope maps of serum derived from one animal immunized with N3 (N3r#7) are consistent with previous peptide ELISA results, demonstrating a MPER responses with binding antibodies specific for the 2F5 epitope (Figure 3.17). Immunization with N4 induced higher titers of MPER-specific antibodies compared to N3 immunization studies (Figure 3.16 D), the ELDKWA epitope was also recognized by the immune sera (Figure 3.17A and B).

A



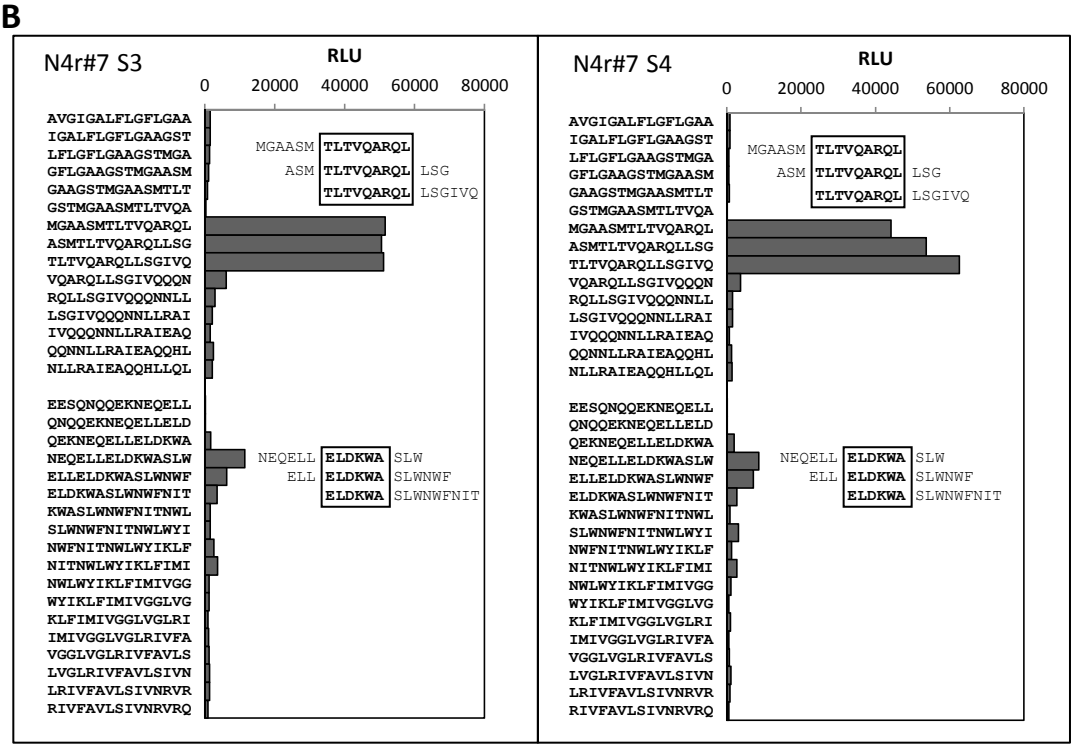


Figure 3.17: Representative epitope mapping of antisera derived from animals immunized with the recombinant hybrid protein N3 or N4. Mappings were performed on microarray chip at a serum dilution of 1:400, showing epitopes in the FPPR and MPER region in the MPER including the 2F5 epitope.

To summarize the results above, gp41-specific antibody titers of induced by immunizations with N3 were comparable to those observed in the previous study. Vaccination with N4 induced lower titers of anti-gp41 antibodies, compared to N3 immunizations, although a stronger anti-MPER antibody response was induced after immunization with N4.

Since all sera reacted with rgp41, neutralization assays were performed with HIV-1_{NL4-3}. Surprisingly, three rat immune sera obtained after the third immunization showed an inhibitory effect on HIV-1 infection (Figure 3.18) in the TZM-bl neutralization assay compared to the PBS immunized control animal, whereas this effect was not observed in immune sera derived after the fourth immunization. As a consequence, IgG were isolated from these sera derived after the third immunization and the neutralization assay was repeated, using the same concentration of rgp41 specific antibodies as in the immune sera. IgG isolated from rat sera N4r#2 and N4r#4 showed weak inhibition of virus infection at a dilution of 1:20. In addition to that, antisera from those three rats derived after first and second immunizations were tested to further investigate the influence of primary and booster immunizations on neutralization capacity of the sera. Sera from animals N4r#2 and N4r#7 derived after the third immunization with N4 exhibited the strongest virus inhibition capacity compared to sera obtained after the other immunizations. Of note, the serum S2 from animal N4r#4 but not S3 showed the strongest inhibition of virus infection in the assay, although S3 was still capable to inhibit HIV-1 infection to a minor extent.

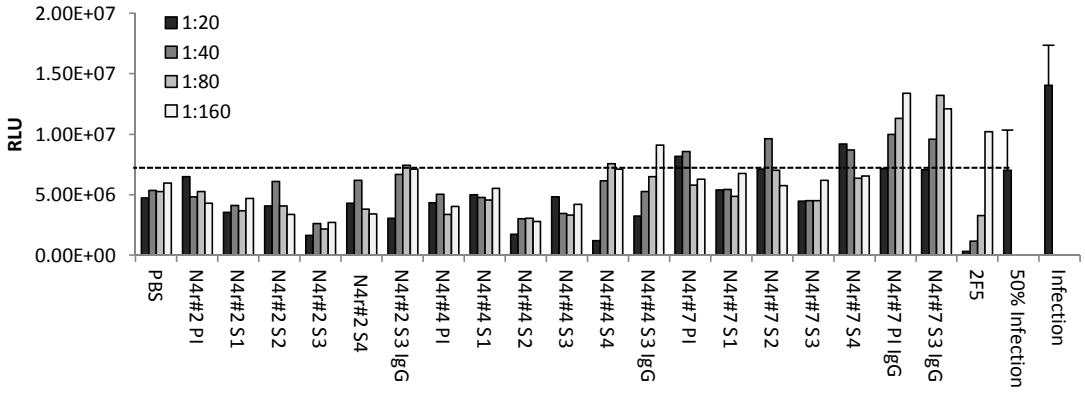


Figure 3.18: TZM-bl neutralization assay of sera and isolated IgG from animals N4r#2, N4r#4, N4r#7 derived after each immunization (S1 = serum derived after 1st immunization, S2 = 2nd immunization, S3 = 3rd immunization, S4 = 4th immunization). Sera and IgG were used in two-fold dilutions from 1:20 to 1:160. 2F5 was used as control in concentrations of 50/12.5/3.125/0.78 µg/ml. The cut-off line represents 50% of RLU measured for virus infection without serum or antibodies.

In addition to the TZM-bl neutralization assay, sera from animals N4r#2, N4r#4 and N4r#7 derived after the third and fourth immunization were then furthermore tested in the real-time PCR-based neutralization assay (Figure 3.19).

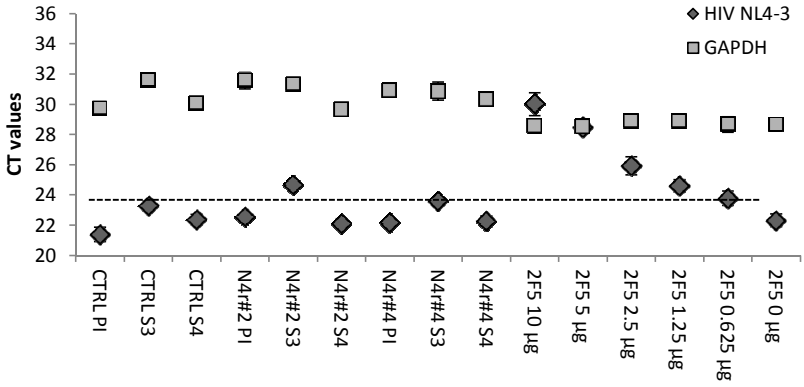


Figure 3.19: Real-time PCR-based neutralization assay of sera from animals N4r#2, N4r#4, N4r#7 derived after third and fourth immunization (S3 = 3rd immunization, S4 = 4th immunization). Sera were used in a 1:20 dilution. Serum from a control animal which received only PBS was used as negative control. Error bars represent the standard deviation (n=3).

Serum from animals N4r#4 and N4r#7 derived after the third immunization did not inhibit HIV-1_{NL4-3} infection compared to serum from the PBS control animal (Figure 3.19). Of note, S3 from rat N4r#2 significantly reduced virus infections comparable to 1.25 µg of 2F5, although this effect is not observed when testing sera derived after the fourth immunization.This was also observed in the TZM-bl neutralization assay, but isolated IgG from this serum did not inhibit virus infection (Figure 3.18). Due to limited serum volumes derived from rats after the third immunization (1 ml), additional neutralization tests could not be performed and therefore the immunization study was repeated with eight rats and one goat.

2.3.5 Immunization study of four rats and one goat with N4

Since immune sera after the third immunization derived from the previous immunization regimen with antigen N4 exhibited weak neutralizing activity and serum volumes were insufficient for additional neutralization tests, the immunization of rats was repeated with three instead of four immunizations to obtain more blood from the final bleed.

Immunization of 8 rats with 250 µg recombinant hybrid protein N4 induced anti-gp41 titers of 10^4-10^6 after the second boost, which was comparable to those observed in the previous immunization experiment with N3 as antigen (Figure 3.10A and B). Further detailed evaluation of immune responses directed against the FPPR or MPER domains showed again a dominant FPPR response and relatively weak anti-MPER responses but animal N4r#11, exhibiting a balanced anti-FPPR and anti-MPER response (Figure 3.20C). In this experimental set-up, animal N4r#10 died during the bleed after the first, animal N4r#12 after the second and animal N4r#16 after the third immunization since the animal facility used another anesthetic for taking the blood sample instead of the previously used isoflurane. Thus, no serum of animals #10 and #12 was available after the third immunization.

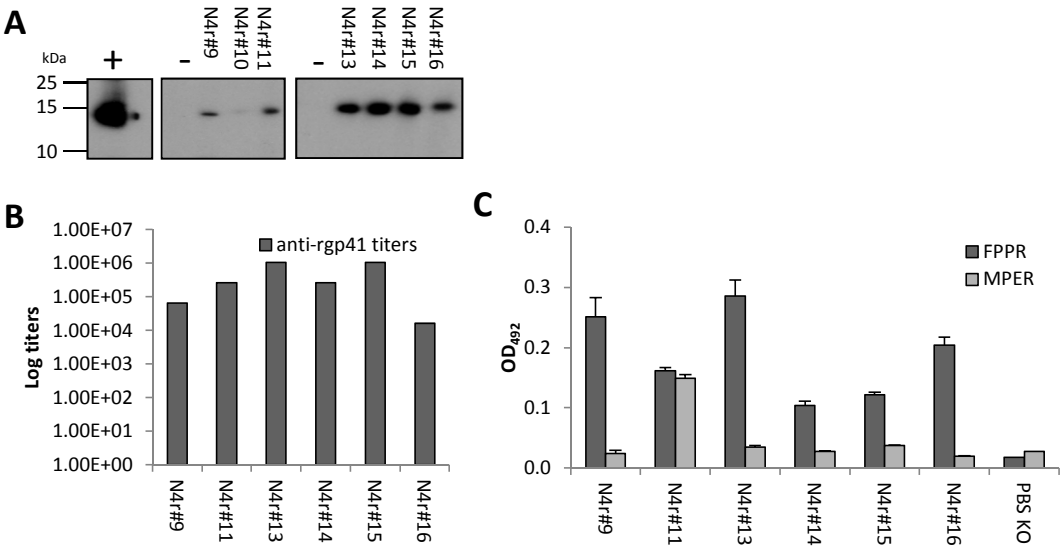


Figure 3.20: (A) Western blot analysis of rat immune sera (obtained after the 3rd immunization: N4r#9; N4#r11; N4#13-16; serum of animal N4r#10 obtained after the 1st immunization), diluted 1:400, using rgp41 as antigen. (+) 2F5 diluted 1:100 000 and (-) represents preimmune serum. (B) Anti-gp41 antibody titers of rat immune sera obtained after the third immunization, diluted 1:1000. (C) FPPR and MPER-specific OD_{492} values of rat sera obtained after the third immunization, diluted 1:400. PBS KO represents serum from the control animal immunized three times with PBS plus corresponding adjuvant. Error bars represent the standard deviation (n=3).

Evaluation of neutralization mediated by immune sera after the third immunization showed a 50% inhibition of HIV-1 infection at a dilution of 1:40 by the immune serum of animal N4#r9, comparable to a concentration of 0.3 µg/ml 2F5 (Figure 3.21A). It was thus necessary to isolate IgG from these sera but the inhibitory effect was not detected in subsequent neutralization assays when using the same amount of rgp41 specific antibodies as in the serum (Figure 3.21B).

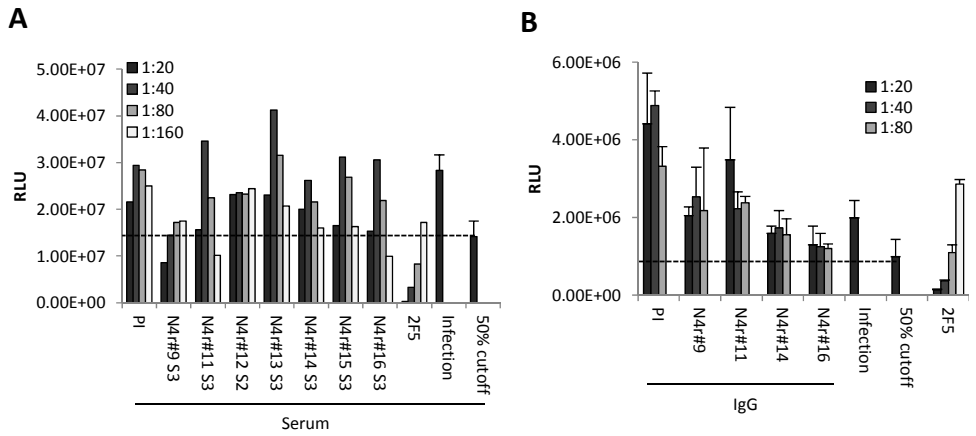


Figure 3.21: TZM-bl neutralization assay of sera (A) and isolated IgG (B) from rats derived after second (S2) or third (S3) immunization. Sera and IgG were used in two-fold dilutions from 1:20 to 1:160. 2F5 was used as control in concentrations of 50/12.5/3.125/0.78 µg/ml. The cut-off line represents 50% of RLU measured for virus infection without serum or antibodies. Error bars represent the standard deviation (n=2).

Immunization experiments with N4 induced predominantly FPPR-specific antibodies in rats. Sera after the third immunization showed weak HIV-1 neutralization in the TZM-bl neutralization assay, which was not caused by isolated IgG from these sera (Figure 3.21). Serum volumes of rats received from the animal facility varied between 300 and 800 µl after each bleed which were insufficient volumes for antigen specific antibody purification. Therefore, a goat was immunized three times with 500 µg of the recombinant hybrid protein N4 and bled after each immunization. Anti-rgp41 antibody responses were detected after the first immunization (Figure 3.22A and B) whereas a moderate rgp41 and peptide response was detected after the second immunization boost (Figure 3.22B).

The sera were then tested in the TZM-bl neutralization assay, showing an inhibitory effect on HIV-1 infection by the serum derived after the second booster immunization, diluted 1:20. This effect was stronger than 50% of the RLU value measured with virus infection and as well stronger than the preimmune serum diluted 1:20 and thus defined as neutralizing. Of note, the goat was bled again 9 weeks after the third immunization (S4), and this serum did not exhibit virus neutralizing activity.
To evaluate whether this neutralizing effect was attributed to antibodies induced by immunization with the hybrid protein, CHR/MPER-specific antibodies were isolated from the serum by affinity purification, using a recombinant protein comprising the CHR of gp41 (aa605-681, Genbank: K03455). Isolation of CHR/MPER-specific antibodies resulted in a twofold concentration compared to the concentration of CHR/MPER-specific antibodies detected in the immune serum, which bound exactly the core epitope of the bNAb 2F5 (Figure 3.22C).

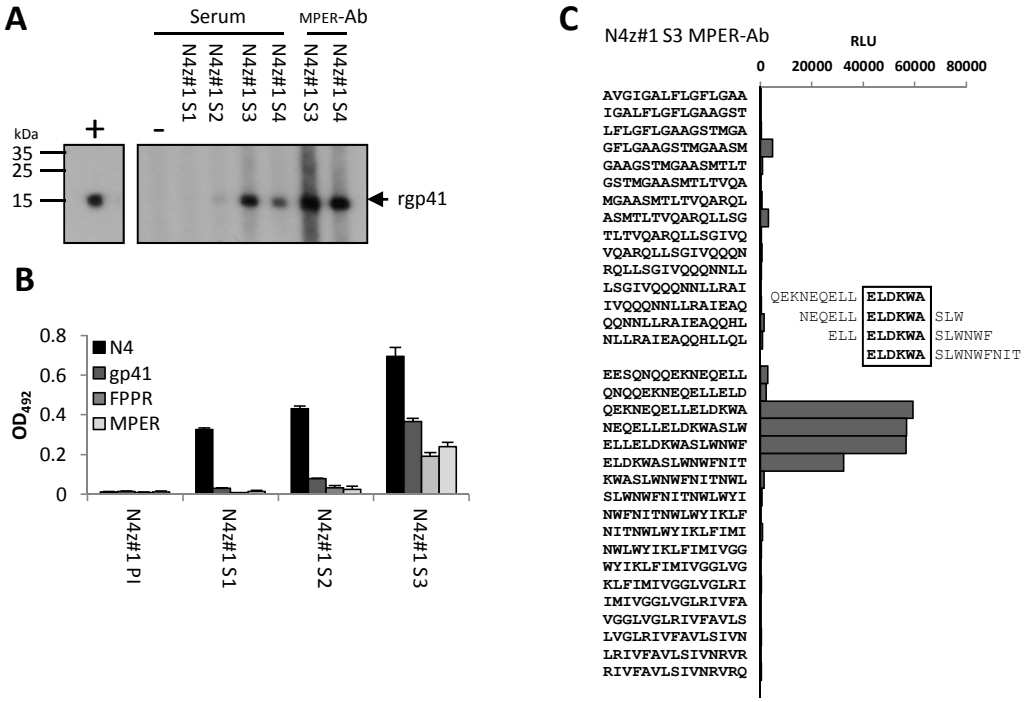


Figure 3.22: (A) Western blot analysis of goat immune sera (N4z#1) obtained after the each immunization (S1-S3), 6 weeks after the third immunization (S4) and isolated gp41-CHR-specific antibodies from sera S3 and S4 (MPER-Ab). Sera and antibodies were diluted 1:400. (+) 2F5 diluted 1:100 000 and (-) represents preimmune serum. (B) OD_{492} values of N4, rgp41, FPPR or MPER-specific antibodies in goat immune serum obtained after each immunization, serum was diluted 1:1000. Error bars represent the standard deviation (n=3). (C) Epitope mapping of $rgp41_{CHR}$-specific antibodies, binding to ELDKWA as core epitope.

Sera and isolated concentrated antibodies were tested again for neutralization of HIV-1_{NL4-3} and a primary isolate of HIV-1 clade F. Although the goat immune serum obtained after the third immunization with N4 inhibited HIV-1_{NL4-3} in the TZM-bl neutralization assay, neutralization was not mediated by MPER antibodies (Figure 3.23). Furthermore, neither serum nor CHR/MPER-specific antibodies neutralized HIV-$1_{BR\ 93/020}$.

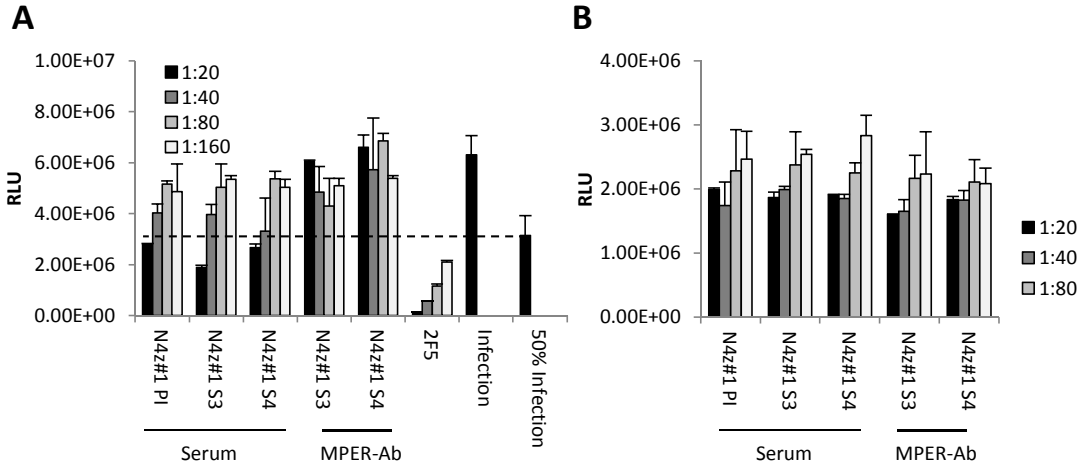


Figure 3.23: TZM-bl neutralization assay of sera and isolated IgG with viruses HIV-1_{NL4-3} clade B (A) and a clade F primary isolate HIV-$1_{BR\ 93/020}$. Sera and CHR-specific antibodies were used in two-fold dilutions from 1:20 to 1:160. 2F5 was used as control in concentrations of 50/12.5/3.125/0.78 µg/ml. The cut-off line represents 50% of RLU measured for virus infection without serum or antibodies. Error bars represent the standard deviation (n=2).

In summary, immunization of one goat with N4 induced CHR/MPER-specific antibodies, which also bound the epitope of 2F5 as observed in previous immunizations of rats. The immune serum obtained after the third immunization neutralized HIV-1_{NL4-3} but this effect was not mediated by CHR/MPER-specific antibodies.

2.4 Immunization with recombinant N1 in liposomes

Since immunization of the N1 hybrid protein predominantly induced only FPPR specific responses, presentation of potential targets for neutralizing epitopes within the MPER was modified. The recombinant protein was thus incorporated into liposomes in order to present the MPER in a lipid context, since native MPER structure depends on lipid environment (Coutant et al., 2008; Sun et al., 2008). N1 purified recombinant protein was sent to Polymun (Vienna, Austria) and inserted into the lipid bilayer of manufactured liposomes in combination with the adjuvant MPLA (2.2.2.4). Liposome technology of Polymun was already tested in a phase I clinical trial (Katinger et al., 2012) and was shown to induce potent immune responses against a gp41 derivative.

The protein/liposome suspension ($N1_{Lip}$) was analyzed on reducing SDS-PAGE. Recombinant hybrid proteins inserted into liposomes formed monomers and dimers on SDS-PAGE, also bound by 2F5 (Figure 3.24), which demonstrates accessibility of the MPER in the monomeric and dimeric form, at least in Western blot analysis

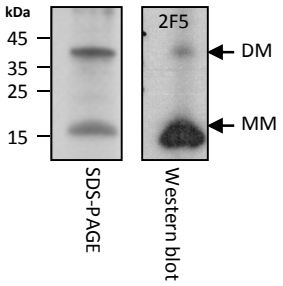


Figure 3.24: Analysis of immunogen $N1_{Lip}$ (batch 1) received from Polymun by Coomassie brilliant blue stained 10% SDS-PAGE and Western blot analysis with 2F5.

The non-filtrated batch one of $N1_{Lip}$ was used for immunization of two guinea pigs and one goat, one guinea pig received batch two (recombinant protein suspension was filtrated prior liposome preparation) and one guinea pig received empty liposomes in a three week immunization schedule with one primary immunization and two boosts.

Immune responses of four immunized guinea pigs and one goat against rgp41, FPPR and MPER after immunization were tested by Western blot analysis and ELISA. Sera from guinea pigs immunized three times with batch one of $N1_{Lip}$ developed weak antibody responses against rgp41 (1:1000), focused on the FPPR. Immunization of one guinea pig with batch two did not result in significantly stronger anti-rgp41 responses compared to the control animal, which received empty liposomes. Immunization of the goat did not induce detectable anti-gp41 antibodies even after the third immunization. Neutralization assays did not show any inhibition of $HIV\text{-}1_{NL4\text{-}3}$ infection by pre-incubation with neither goat (Figure 3.25B) nor guinea pig (Figure 3.25A) sera obtained after immunization with $N1_{Lip}$.

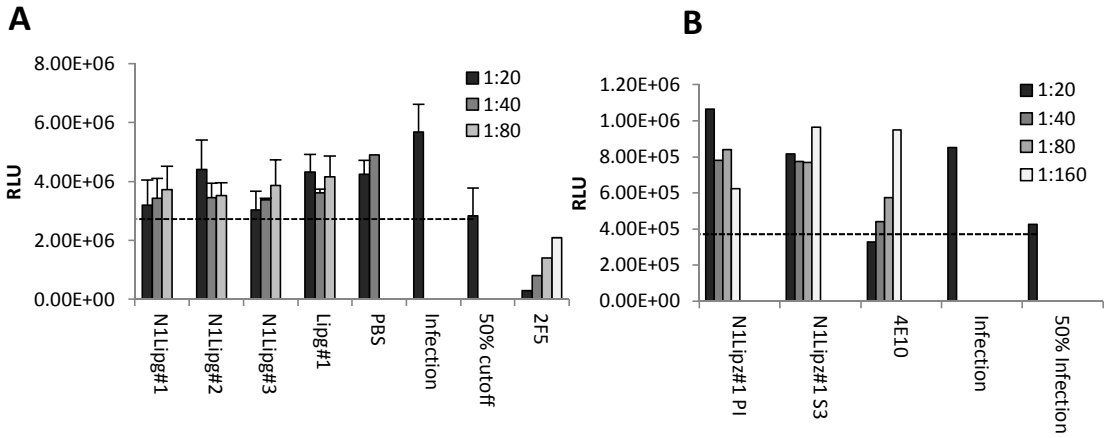


Figure 3.25: TZM-bl neutralization assay of sera from goat (B) and guinea pigs (A) immunized with $N1_{Lip}$. Sera were used in two-fold dilutions from 1:20 to 1:160. Four-fold serial dilution of 4E10 and 2F5 was used as positive control, starting at a concentration of 25 µg/ml. The cut-off line represents 50% of RLU measured for virus infection without inhibitory agents. Error bars represent the standard deviation (n=2).

2.5 Immunization with recombinant N3 and N4 in liposomes

Immunization experiments in guinea pigs, rats and one goat with recombinant hybrid proteins N3 and N4 induced promising antibody responses against the FPPR and MPER, binding precisely to the epitope of 2F5. Sera derived from these immunized animals were capable to marginally inhibit HIV-1_{NL4-3} infection of TZM-bl cells, although this inhibition was not attributed isolated IgG or CHR-specific antibodies isolated from these sera. In order to present the MPER in a lipid context, the purified recombinant hybrid proteins N3 and N4 were also sent to Polymun for manufacturing a mixture of liposomes containing MPLA as adjuvant and hybrid proteins N3 and N4, respectively. The batches received were analyzed on reducing SDS-PAGE, showing formation of monomers and dimers, both conformations bound by 4E10 and 2F5 (Figure 3.26).

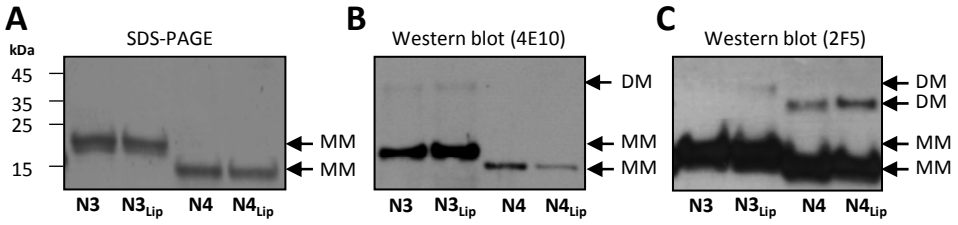


Figure 3.26: Comparative immunogen analysis of $N3_{Lip}$ and $N4_{Lip}$ received from Polymun with recombinant proteins only. (A) Coomassie brilliant blue stained reducing SDS-PAGE. (B) Western blot analysis with 2F5 or 4E10 (C). Arrows indicate recombinant proteins forming monomers (MM) and dimers (DM).

Analysis of rgp41 binding antibodies showed varying titers, whereupon the strongest anti-rgp41 responses were detected in rats immunized with $N4_{Lip}$ (Figure 3.27A). Moderate titers of anti-FPPR antibodies were measured in two rats immunized with $N3_{Lip}$ and three $N4_{Lip}$ immunized animals, whereas no anti-MPER antibodies were detected by peptide ELISA after both immunization regimens.

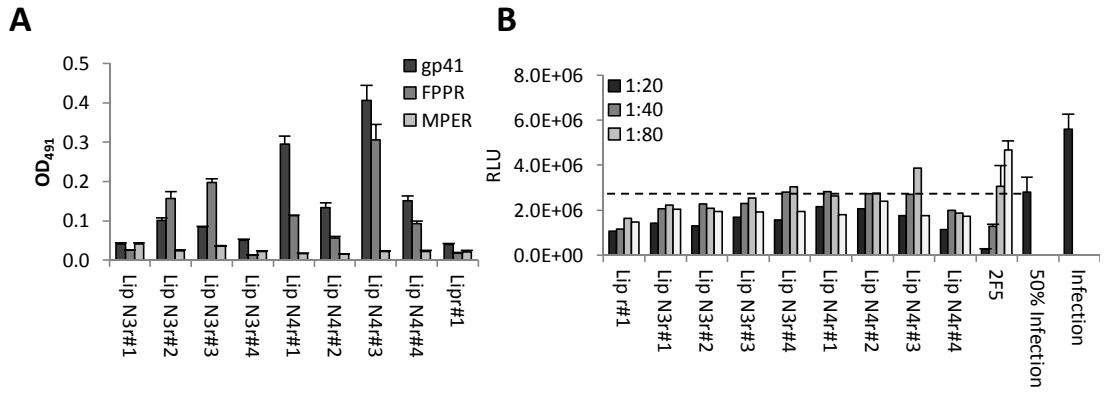


Figure 3.27: (A) OD_{492} values of rat serum diluted 1:250 after the second booster immunization, binding to rgp41, FPPR or MPER derived peptides. (B) TZM-bl neutralization assay of sera from rats immunized with $N3_{Lip,}$ $N4_{Lip}$. Sera were used in two-fold dilutions from 1:20 to 1:160. 2F5 was four-fold diluted, starting at a concentration of 50 µg/ml. The cut-off line represents 50% of RLU measured for virus infection without inhibitory agents. Lipr#1 represents control serum of one rat which received empty liposomes diluted 1:250 for the ELISA (A) or starting from 1:20 in the neutralization assay (B). Error bars represent the standard deviation (n=2).

All sera inhibit HIV-1_{NL4-3} infection of TZM-bl cells in the neutralization assay, although not to a greater extent than the immune serum of one rat which only received empty liposomes (Figure 3.27B). To evaluate the inhibitory effect on virus infection, IgG were isolated from these sera but did not inhibit HIV-1 infection. Unspecific inhibitory effects which are not antibody attributed were kindly tested by Dr. Uwe Fiebig with a real-time PCR-based reference neutralization assay, using SIVmac293 as control virus (Figure 3.28). Two sera derived after the second immunization with N3 ($N3_{Lip}$r#1) or N4 ($N4_{Lip}$r#4) showed a weak inhibitory effect on HIV-1 infection compared to the negative control sera, respectively. This was also true for SIVmac293 infection which indicates non-virus specific inhibitory effects on cell infection by the rat immune sera. A cytotoxic effect was partially excluded due to parallel measurement of the GAPDH reference gene in the same real-time PCR assay.

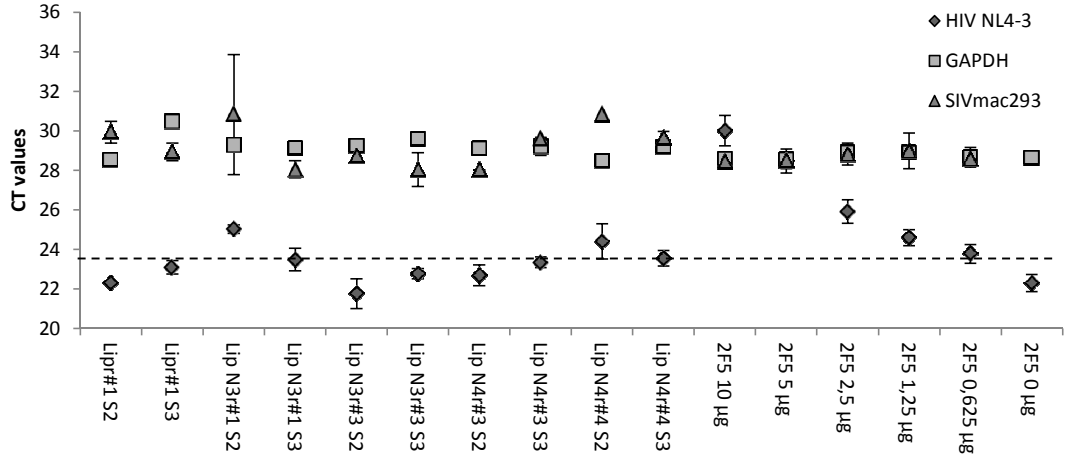


Figure 3.28: Real-time PCR-based neutralization assay of sera from rats immunized with $N3_{Lip}$, $N4_{Lip}$ or empty liposomes (Lipr#1) derived after the second (S2) or third (S3) immunization. Sera were diluted 1:20. Dotted line represents the cut-off CT-value based on negative control sera. Error bars represent the standard deviation (n=3).

In summary, immunization with N1, N3 and N4 in MPLA containing liposomes induced a weaker anti-gp41 response with focus on the FPPR than previous immunization studies with recombinant hybrid proteins in combination with complete/incomplete Freund´s adjuvant and neutralization of HIV-1_{NL4-3} infection by immune sera was observed.

2.6 DNA immunization with recombinant hybrid antigens in 2 different expression vector systems

Previous immunization experiments with the recombinant hybrid antigens expressed by prokaryotic organisms did not induce MPER-specific antibodies, which are able to significantly neutralize HIV-1. Advantages of immunizations with prokaryotically expressed proteins relied on the relatively large amount of purified antigen and immunogenicity of protein immunization. Application of a certain amount of antigen was distinct although the vigorous mixing of antigen suspension and adjuvant resulted in a viscous fluid and thus, challenging to immunize subcutaneously and intramuscularly. An additional instable factor when using recombinant proteins expressed from prokaryotic organisms was the absence post-translational modification procedures. A certain conformation of the hybrid proteins composed of PERV and HIV-1 sequences was suggested to engage a distinct conformation required to induce HIV-1 neutralizing antibodies. The approach to transfer this immunization experiment hypothesis into a DNA immunization regimen was to obtain eukaryotic *in vivo* protein expression, folding and glycosylation of the expressed protein.

Two eukaryotic expression systems were chosen to evaluate the antibody responses induced by immunization with the vector encoded hybrid proteins and HIV-1 neutralizing capacity of immune sera. One system designed for surface antigen expression was the previously tested pDisplay expression vector (Behrendt et al., 2012a), expressing hybrid proteins composed of PERV sequences combined with HIV-1 gp41 domains. These gene gun immunization experiments in rats induced antibody responses up to 1:32,000. Thus, the pDisplay expression system was applied to express modified hybrid constructs N1 and N5 (Figure 3.29). The constructs of N1 and N5 were designed and thus cloned into the pDisplay expression vector (2.2.1.6).

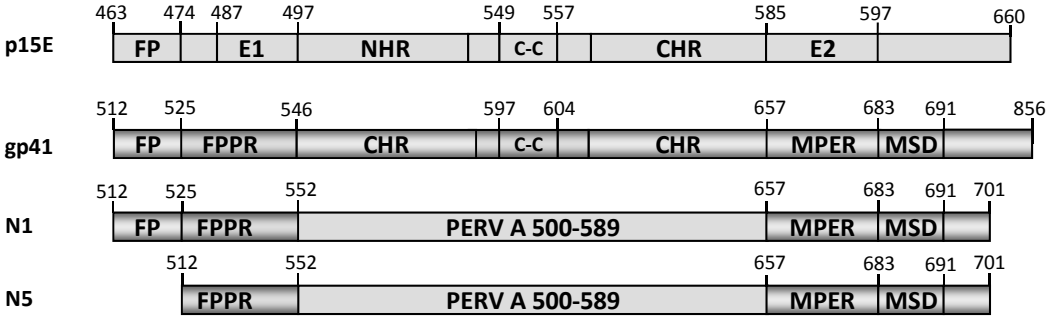


Figure 29: Schematic presentation of gp41, p15E and antigen N1 and N5 containing sequences of gp41 of HIV-1_{NL4-3} (GenBank: AF324493) and p15E of PERV-A (GenBank: AY953542). Dark grey squares indicate sequences of gp41 of HIV-1; light grey squares represent sequences of p15E of PERV-A. The FPPR and MPER domains are derived from gp41, substituting analogue domains in p15E. Construct N5 was designed on basis of N3 with the additional membrane spanning domain (MSD) and a truncated cytoplasmic tail derived from gp41 to insert the hybrid protein in the cellular membrane after *in vivo* expression.

A Semliki Forest Virus DNA replicon vector (DREP) was used as second eukaryotic expression system (kindly provided by Prof. Peter Liljeström, Karolinska Institute, Stockholm). This vector is based on the SFV expression system (Berglund et al., 1998; Nordstrom et al., 2005) and thus carries intrinsic adjuvant properties (Naslund et al., 2011). Due to presence of the Semliki Forest

Virus replicase encoded in the vector, transfection of the DNA results in production of self-replicating mRNA. The DREP vector did not include an export signal to transfer the antigen to the cell surface. Therefore the vector was modified and the export signal (ES) derived from Semliki Forest Virus was included in the vector on the basis of a previously published design (Forsell et al., 2007), showing to enhance antigen-specific antibody responses.

Sequences of N1 with the N-terminal located SFV export signal were codon optimized for the mouse organism since mice were used as test animals. The construct was synthesized by Genescript (Piscataway, USA) and cloned in-frame with the translational enhancer and the ribosomal skipping domain (SD), using *HindIII/SpeI* restriction sites. The construct N5 comprised the complete sequence of N3 plus the membrane spanning region (TM) derived from HIV-1 to anchor the translated protein into the cellular membrane (Figure 3.29, 3.30). DREP-N5 was amplified by QuickChange™ *in vitro* mutagenesis (Stratagene, Heidelberg, Germany) using the vector DREP-N1 as template with the primers 024/025, which were designed accordingly to the manufacturer´s instructions. This method was applied for cloning procedures to delete the fusion peptide (13 amino acids) from the DREP-N1 construct. The pDisplay-N1 and pDisplay-N1 expression vectors were cloned as described in section 2.2.1.6 and coated on gold particles at a 1:1 ratio with the cytokine GM-CSF (Granulocyte macrophage colony-stimulating factor) used as adjuvant, also codon optimized for the mouse organism. Since antibody responses of DNA vaccines were previously shown to be enhanced by additional immunization with plasmid-encoded GM-CSF (Brave et al., 2005; Ou-Yang et al., 2002), the adjuvant was used for the pDisplay immunization regimen encoding the hybrid proteins. DREP-N1 and DREP-N5 constructs were coated on gold particles without additional adjuvants since the DREP vector carries intrinsic adjuvant properties (Naslund et al., 2011).

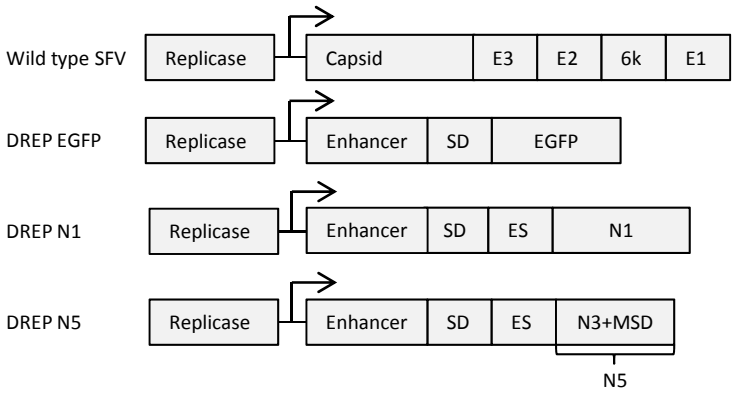


Fig 3.30: Schematic presentation of the DREP expression system with inserted hybrid antigens N1 and N5. Arrows represent internal subgenomic promotors, start of translation for the foreign gene by the vector encoded replicase. Expression constructs (DREP-N1, DREP-N5) include the viral replicase, the internal translational enhancer (enhancer), the ribosomal slippery sequence (SD) and the export signal (ES) from SFV. Foreign antigens N1 and N5 including the transmembrane domain (TM) derived from HIV-1 are cloned 5´ in frame with the second open reading frame.

In order to test expression of recombinant hybrid proteins encoded in the two different expression vectors, 293T cells were transfected with the constructs N1 and in N5 cloned into the DREP or pDisplay expression system. Transfected cells were stained extracellularly with 2F5 and 4E10 for immunofluorescence analysis, investigating localization and accessibility of the MPER on the

cellular surface. Lysates of transfected cells were used for Western blot analysis, using the antibodies mentioned above. 2F5 and 4E10 bound only to cells transfected with the expression constructs N1 and N5 in pDisplay or DREP, showing expression of the antigen in the cell lysate of 293T cells (Figure 3.31) and on the cellular membrane of transfected cells after immunofluorescent staining (Figure 3.32). Cellular lysates of 293T cells transfected with a molecular clone of HIV-1_{NL4-3} was used as a positive control and hence also recognized by 2F5. The ~13 kDa band in Western blot analysis of cells transfected with pDisplay N1 represents N-terminally degraded protein, since both proteins are recognized by 2F5 and 4E10.

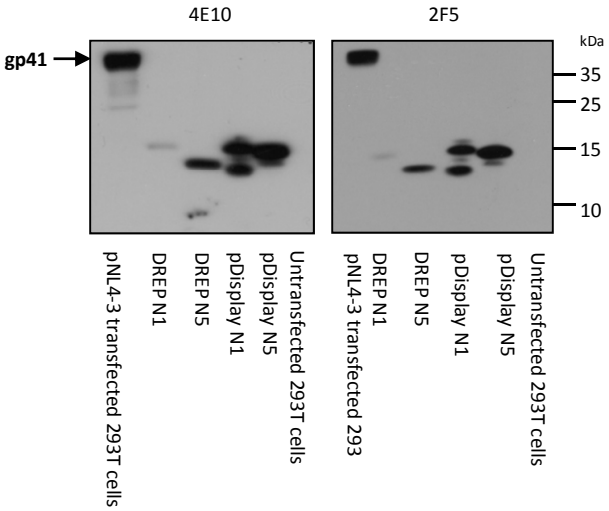


Figure 3.31: Western Blot analysis of cell lysates obtained from transfected 293T cells. Membranes were incubated with 2F5 or 4E10 1:50 000 and secondary HRP-conjugated anti-human antibody 1:5000.

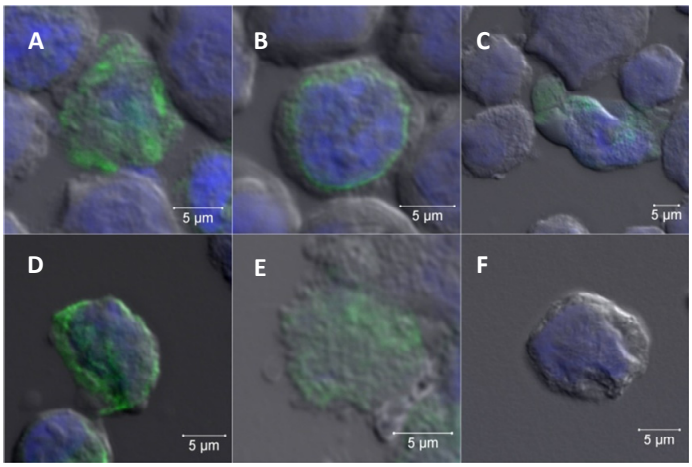


Figure 3.32: LSM analysis of 293T cells transfected with eukaryotic expression constructs N1 and N5 in pDisplay and DREP used for DNA immunization experiments stained with 2F5 at a concentration of 5 µg/ml and anti-human AlexaFluor488 diluted 1:200. (A) pDisplay-N1. (B) DREP-N1. (C) pDisplay gp41. (D) pDisplay-N5. (E) DREP-N5. (F) untransfected 293T cells stained with primary and secondary antibody.

Groups of five mice each were immunized three times with two shots containing 1 µg of plasmid encoding the codon optimized hybrid antigens for the mouse organism, the pDisplay vectors combined with 1 µg of plasmid encoding the murine GM-CSF adjuvant, in a three-week immunization schedule. Recombinant antigens N1 and N3 mixed with incomplete Freund´s

adjuvant were immunized as a positive control. Preimmune sera were taken before the first immunization, mice were sacrificed and exsanguinated three weeks after the last immunization in order to receive sufficient serum volume at the terminal bleed to test antigen specific antibody responses and neutralization of HIV-1_{NL4-3}. Immune sera derived from mice which received the hybrid proteins as DNA vaccine did not recognize rgp41 expressed by *E. coli*. Immunizations with purified recombinant hybrid antigen N1 induced anti-rgp41 antibodies in three animals out of five immunized mice, two immune sera from mice which received N5 as immunogen reacted with rgp41. Sera were in moreover tested by Western blot analysis with cellular lysate of HIV-1_{NL4-3} infected C8166 cells to evaluate reactivity of serum to eukaryotically expressed HIV-1_{NL4-3} envelope proteins but no reactivity to gp41 or gp160 was detected. This surprisingly demonstrates low immunogenicity of the plasmid-encoded antigens in the mice model in contrast to previously published immunization studies (Brave et al., 2005; Ou-Yang et al., 2002; Rodriguez et al., 2012), although GM-CSF was co-immunized with the DNA vectors encoding hybrid proteins as an antibody-stimulating adjuvant. Control groups of mice which received prokaryotically expressed recombinant antigen did not develop a robust antibody response as it was the case in previous rat and guinea pig immunization regimens.

Despite the low reactivity of immune sera against rgp41 and eukaryotically expressed gp41 derivatives, sera of mice derived after the second immunization boost were tested in the TZM-bl neutralization assay. Immune sera from mice immunized with pDisplay or DREP encoding N1 did not inhibit of HIV-1_{NL4-3} infection to a greater extent compared to the preimmune serum, neither the immune serum derived from mice immunized with recombinant N1 protein. Marginally more than 50% inhibition of HIV-1_{NL4-3} infection was mediated by sera diluted 1:20 and 1:40 derived from mice immunized with DREP expressing N5. Significant inhibition of virus infection by the immune serum sample of one animal immunized with N3 was also detected by the TZM-bl neutralization assay. Since this inhibition of HIV-1_{NL4-3} infection remained not stronger than the inhibition by sera of control animals immunized with empty (pDisplay) or EGFP expressing DREP vector and antibodies specific for rgp41 were absent in immune sera, this observed inhibitory effect was not antibody specific.

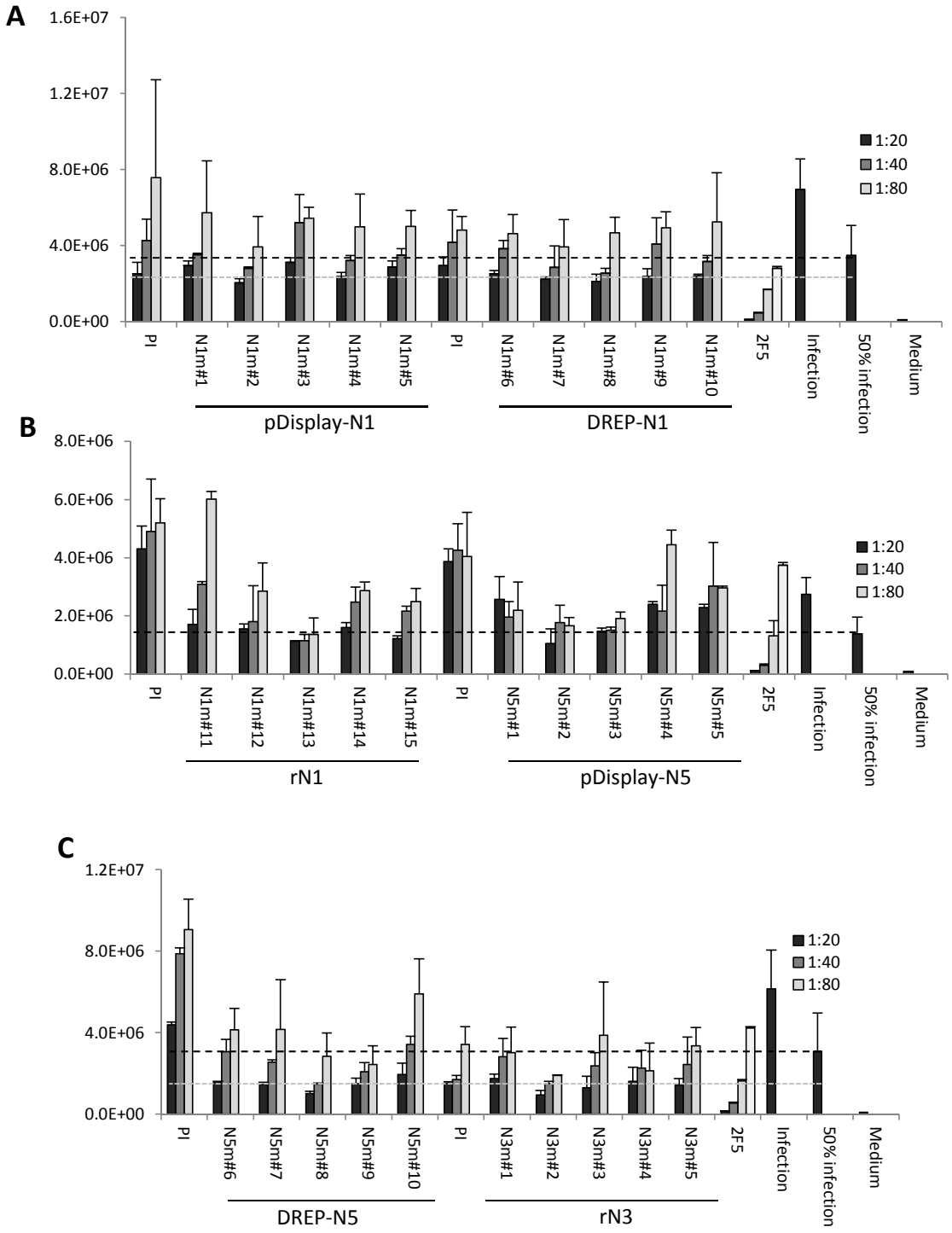


Figure 3.33: TZM-bl neutralization assay of sera from mice immunized with plasmids pDisplay or DREP expressing hybrid proteins N1 or N5. Sera were used in two-fold dilutions from 1:20 to 1:80. 2F5 was diluted 1:4, starting at a concentration of 50 µg/ml. The black and dotted cut-off line represents 50% of RLU measured for virus infection without inhibitory agents, the light grey cut-off line represent RLU value of preimmune serum at the 1:20 dilution. Medium indicates RLU negative control values of lysed TZM-bl cells without HIV-1_{NL4-3} virus particles. Error bars represent the standard deviation (n=2).

In order to summarize the above mentioned results, immunization of hybrid proteins in the DNA immunization regimen expressed in vivo by two different vector systems did not induce rgp41 specific antibodies or inhibited HIV-1_{NL-4-3} to a greater extent than the control sera.

2.7 Testing of sera for reactivity to lipids

Broadly neutralizing antibodies directed against the MPER of gp41 were controversially discussed to exhibit autoreactive properties (Haynes, 2005). Since the MPER was included in all hybrid proteins used as antigen for our immunization experiments, sera derived after these immunizations were screened for potential reactivity with two different lipid antigens (cardiolipin and sphingomyelin).

As expected, none of the anti-sera derived from immunizations with recombinant hybrid proteins N1, N2, N3 the significantly reacted with sphingomyelin or cardiolipin, which is consistent with the fact that only antibodies binding to the FPPR and the 2F5 epitope in epitope mappings were observed (Figure 3.34).

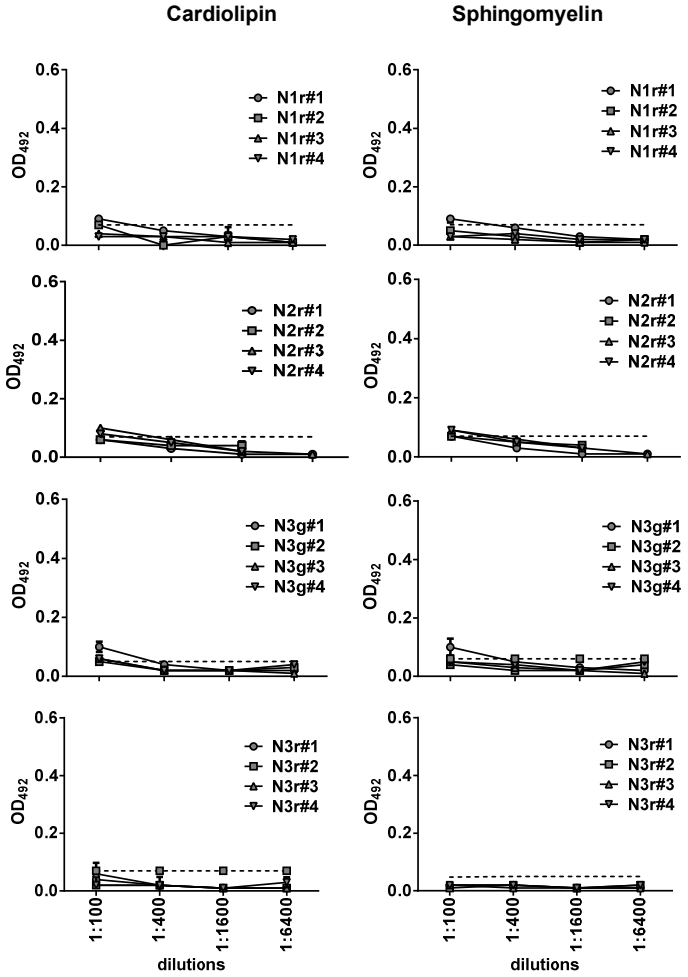


Figure 3.34: Detailed analysis of immune sera from rats or guinea pigs immunized with N1, N2 or N3 for reactivity against lipids. Dotted lines represent the cut-off value estimated by the mean value from a pool of 4 preimmune sera plus three times the standard deviation. Error bars represent the standard deviation (n=3).

Binding of 2F5 and 4E10 to sphingomyelin or cardiolipin was tested as reference for reactivity of serum to both lipids. The anti-lipid ELISA showed binding of 4E10 to cardiolipin, whereas 2F5 exhibited no affinity to both lipids (Figure 3.35) in contrast to previous reports regarding the reactivity to cardiolipin and other lipids (Haynes et al., 2005; Matyas et al., 2009b; Sanchez-Martinez et al., 2006a; Sanchez-Martinez et al., 2006b).

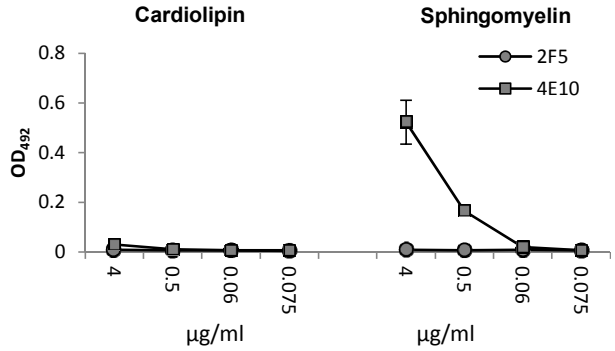


Figure 3.35: Analysis of bNAb 2F5 and 4E10 binding to cardiolipin and sphingomyelin. Error bars represent the standard deviation (n=3).

Sera of rats, guinea pigs and one goat immunized with recombinant antigens in liposomes were also tested for reactivity to lipid antigens. Surprisingly, sera from guinea pigs $N1_{Lip}$g#3 and $N1_{Lip}$g#4 derived after the third immunization bound weakly to cardiolipin but not to sphingomyelin (Figure 3.36A), although no MPER specific antibodies were observed or exhibited additional virus inhibiting properties. Immune sera of two other guinea pigs and one goat which received $N1_{Lip}$ did not bind to lipid antigens. Rat sera from animals which received $N3_{Lip}$ and $N4_{Lip}$ also did not react with cardiolipin or sphingomyelin (Figure 3.37).

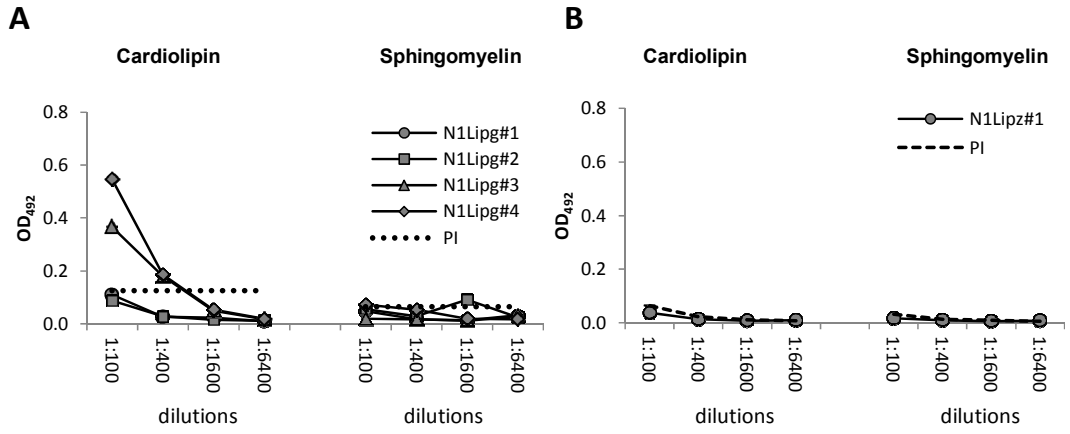


Figure 3.36: Analysis of immune sera from guinea pigs (g) or one goat (z) immunized three times with N1 in liposomes (Lip) for reactivity against lipids. Dotted lines (PI) represent the cut-off value estimated by the mean value the pool of 4 preimmune sera from guinea pigs or preimmune serum of one goat immunized with $N1_{Lip}$ plus three times the standard deviation. Error bars represent the standard deviation (n=3).

Summarizing the results above, none of the immune sera obtained after immunization with recombinant hybrid protein N3 or N3 and N4 in liposomes bound to either one of the lipids tested. Despite two guinea pig immune sera obtained after immunization with $N1_{Lip}$ reacted positive with cardiolipin at serum dilutions of 1:100 and 1:400, none of the sera obtained from the other animals immunized with $N1_{Lip}$ recognized both lipids.

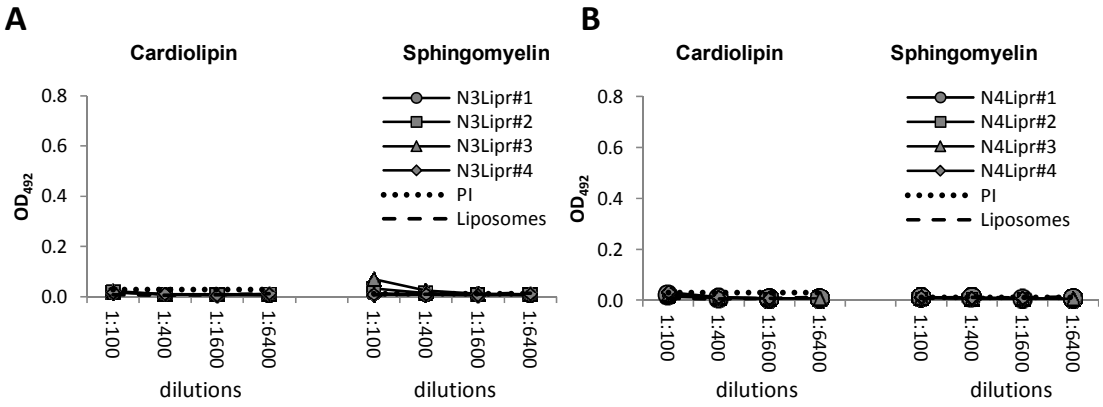


Figure 3.37: Analysis of rat (r) immune sera immunized four times with N3 (A) or N4 (B) in liposomes for reactivity against lipids. Dotted lines represent the cut-off values estimated by the mean value from a pool of 4 preimmune sera (PI) or immune serum of one rats immunized four times with empty liposomes (Liposomes) plus three times the standard deviation. Error bars represent the standard deviation (n=3).

3. Discussion

3.1 Protection against HIV infection

A vaccine, which is cheap to produce, easy to apply and to handle would be the best strategy to keep the HIV-1 epidemic in bay. This goal seems to be unachievable at the moment and thus prevention programs are substantial in countries with HIV prevalence. New infections can primarily be prevented with condoms and also protection of mucosal HIV-1 entry sites by microbicides or other pre-exposure prophylaxis has been brought to focus in the recent years. Microbicides are an essential option to prevent of sexual transmission, especially for women due to gender inequality since cooperation of the male partner is not required (Vail et al., 2004){Cutler, 2008 #1019}. In addition to that, comprehensive antiretroviral treatment for HIV-1 infected, pregnant women by treatment before, during and after birth is urgently necessary to prevent child infection {Paredes, 2013 #1020}.

With regards to HIV-1 vaccine development, the most discussed question is still which part of the immune system contributes to clearance of the virus and to prevent infection. The opinions are diverse, many researchers in the HIV vaccine field believe in essentiality of a B-cell stimulating vaccine capable to elicit bNAbs (Douek et al., 2006; Letvin, 2006), since only those antibodies will prevent entry of the virus into $CD4^+$ cells and inhibit replication and integration (Han et al., 2007). Others postulate that the induction of cytotoxic T-cells, capable to eliminate the virus rapidly after primary entry of the host would be essential for clearance of the virus (Makedonas and Betts, 2011; Plotkin, 2010). Another hypothesis implied protection depending on immunogenic tolerance and suppression of $CD4^+$ T-cell activation to inhibit effective virus replication after primary host entry. This approach recently came to focus when an oral vaccine comprising inactivated SIV with immunomodulatory *Lactobacillus plantarum* induced protection against homologous or heterologous SIV challenge (Lu et al., 2012). Despite extensive discussions in the field, antibodies are the first wall for virus entry and should thus be induced by a potential preventive vaccine.

3.2 Hybrid proteins used as immunogens

Aim of this project was to implement an innovative strategy in order to generate hybrid proteins as antigens for a HIV-1 vaccine for induction of HIV-1 preventive antibodies. These immunogens were composed of the HIV-1 FPPR and MPER, grafted into a scaffold of the transmembrane protein of PERV.

As a first step in the project, the design of hybrid proteins was accomplished by aligning the TM protein sequences of several HIV-1 clades and PERV strains (3.2, Figure 3.1). When comparing the subdomains of both TM proteins, p15E and gp41, a major difference in sequence length of different subdomains was observed. The gp41 intraviral tail comprises approximately the same number of amino acids as the corresponding ectodomain of the same protein. In contrast to that, the intraviral tail of p15E represents only one quarter of the whole TM protein, which might have implications on presentation of the MPER and distinct epitopes (Postler and Desrosiers, 2013). Besides discrepancies between gp41 and p15E in amino acid sequence composition and length of

the subdomains, distinct homologies were observed (Figure 3.1). On basis of these alignments, combination of HIV-1 and PERV TM protein sequences for hybrid proteins were optimized. Epitopes in the FPPR and MPER of p15E which have been recognized by PERV neutralizing sera derived from animals immunized with the ectodomain of recombinant p15E were substituted with corresponding FPPR and MPER regions in gp41.

Two different hybrid proteins N1 and N2 have been designed and were expressed, purified and used for immunization experiments in rats and guinea pigs to evaluate the impact of remaining lipophilic sequences such as the FP and the transmembrane region. Despite extensive optimization of purification procedures, these proteins turned out to be insoluble under native conditions. The purification procedures were performed with denaturing buffers, which hampered evaluation of qualitative antigen-NAb interaction by SPR analysis. It was assumed that both hybrid proteins aggregated in native buffers since N1 and N2 formed monomers and dimers already under denaturing and reducing conditions (3.3.1, Figure 3.4). This aggregation prohibited the antigens from migration though the native PAGE (data not shown). Nevertheless, both antigens were used for immunizations since protein aggregation potentially diminished in lipid environment of the adjuvant which was co-delivered with the antigens. Immunization of rats with N1 or N2 induced similar anti-rgp41 and anti-FPPR antibody titers, although no anti-MPER antibodies were observed in sera from animals immunized with N1. The N1 hybrid protein comprised its FP, transmembrane domain and truncated intraviral tail from HIV-1 in contrast to corresponding p15E derived regions in hybrid protein N2. These differences in N-terminal and C-terminal sequence composition had an impact on MPER presentation as previously proposed and was shielded by other protein domains (Sun et al., 2008). MPER-specific antibodies induced by immunization with N2 bound a long MPER-comprising peptide in ELISA analysis of immune sera, but none of the 15-mer peptides used for epitope mappings on both, pepspot membrane and mircroarray chip. With regards to this result, those gp41-specific antibodies might have been specific for a conformational epitope exhibited by the whole MPER region but was not presented on shorter peptides. Despite the presence of these MPER binding antibodies in immune sera after immunizations with N2, no inhibition of virus infection by immune sera or isolated IgG after immunization with N1 or N2 was observed.

In order to improve solubility of the hybrid proteins, two additional antigens were designed. Solubility of hybrid proteins N3 and N4 was enhanced by deleting the dispensable FP, the transmembrane domain and the truncated intraviral tail. Although the proteins were not soluble in PBS without addition of N-Lauroylsarkosine, concentration of the detergent was reduced and enabled evaluation on non-reducing SDS-PAGE. N3 and N4 showed trimers and oligomers in absence of reducing agents within the gel, indicating a variety of protein conformations and thus potent presentation of various epitopes.
Initial immunizations of N3 in guinea pigs and rats induced anti-rgp41 antibody responses comparable to those observed with N1 or N2. Detailed evaluation of anti-gp41 antibody responses showed the induction of FPPR binding antibodies in all immunized animals. Of note, anti-MPER responses were observed in six of eight immunized animals and epitope mappings of these

antibodies demonstrated binding to the 2F5 epitope. Deletion of FP, transmembrane domain and the truncated intraviral tail thus might have had an effect on presentation of this neutralizing epitope (Montero et al., 2012), since antibodies binding to this epitope were not observed after immunizations with N1 or N2.

In addition to that, absence of antibodies binding to other epitopes in the MPER than to those of 2F5 (ELDKWA, core epitope (L)DKW) were surprising. 2F5 exhibited higher apparent affinity in SPR analysis to both antigens compared to that measured for 4E10, indicating a better accessibility of the 2F5 epitope than other MPER domains. Nonetheless, those immune sera or isolated IgG were not capable to inhibit HIV-1 infection tested with the TZM-bl neutralization assay used.

Immunization of a rabbit with N3 co-delivered with Montanide as adjuvant induced a weaker anti-gp41 antibody response compared to antibody responses observed after the previous rat vaccination. Detailed evaluation of HIV-1 TM protein-specific antibody responses demonstrated FPPR binding antibodies but no MPER specific antibodies, although MPER antibodies were detected in rats after two immunizations with N3 in combination with complete and incomplete Freund´s adjuvant, respectively. This immunization schedule with only one booster immunization in combination with a different adjuvant did not induce a potent anti-gp41 antibody response, although Montanide and Freund´s adjuvant was previously described to induce comparable antibody responses in rabbits when immunized s.c. or i.m. (Leenaars et al., 1998).

Neutralization assay		**TZM-bl Reporter Assay**	**Real-time PCR Assay**		**p24 ELISA**
Cell type		**TZM-bl cells**	**C8166**	**PBMC**	**C8166**
Virus (HIV-1)		**NL4-3**	**NL4-3**	**NL4-3**	**MN**
Serum	N3g#1	-	-	-	-
	N3g#2	-	-	-	-
	N3g#3	-	-	-	-
	N3g#4	-	-	+	-

Table 4.1: Summary of neutralization assays performed with guinea pig sera obtained after the fourth immunization with N3. The table describes the assay readout, cells and HIV-1 used for the corresponding assay. (+) and (-) describe positive and negative results for neutralization measured with the cited neutralization assay. TZM-bl reporter assay and the real-time PCR assay were performed by the applicant. The p24 neutralization assay was performed by Dr. Steve Norley (RKI).

Immune sera of guinea pigs and rats contained antibodies binding to the 2F5 epitope, but did not neutralize in the TZM-bl neutralization assay. Thus, sera were additionally tested in other laboratories for virus inhibiting capacity. No significant neutralizing effect of sera and isolated IgG after the fourth immunization was observed in our TZM-bl cell-based, the real-time PCR-based neutralization assay with C8166 cells and an additional assay, using quantification of p24 as indication for inhibition of virus infection with two laboratory-adapted HIV-1 strains (HIV-1_{NL4-3}, HIV-1_{MN}). Only sera of guinea pig N3g#4 showed a weak virus inhibition in the PBMC based real-time PCR assay, although isolated IgG from this serum did not show any neutralizing effect (Table 4.1).

Immune sera obtained from N3 immunized guinea pigs were in addition sent to David Montefiori´s laboratory (Duke University Medical Center, Durham, USA) in order to test the sera with other neutralization assays since the outcome might differ when testing the same serum in different assays as it was previously proposed (Fenyo et al., 2009; Heyndrickx et al., 2012). Hence, the sera were tested in two different assays (Table 4.2), SIVmac293 and MuLV (murine leukaemia retrovirus) were used to evaluate non-HIV-1 specific neutralization.

Despite the fact that no significant neutralization was observed with our assays, all immune sera obtained after the fourth immunization of the four guinea pigs immunized with N3 showed a weak neutralizing effect in the engineered CEM (A3R5) cell-based neutralization (Table 4.2) assay (Montefiori et al., 2012). In this assay, immune sera were incubated with a pseudotyped HIV-1 clade C virus (Hoffenberg et al., 2013) with moderate sensitivity to antibody-mediated neutralization and subsequently added to A3R5 cells (modified CEM cells). Interestingly, the immune sera of all guinea pigs immunized with N3 showed a weak neutralizing effect. It should be noted, that sera of guinea pigs N3g#1 and N3g#4 unspecifically neutralized the control virus (SIVmac239) to a minor extent but sera were defined as neutralizing if ID50 values were three times higher compared to the negative control. Since it was demonstrated that immunization with hybrid protein N3 induced 2F5 binding antibodies, neutralization of a the clade C isolate CH58 was surprising since clade C viruses were shown to be resistant to 2F5 mediated neutralization (Binley et al., 2004) and no neutralization was observed with neutralization sensitive HIV-1 strains in previously performed neutralization assays. In addition to this observation, the HIV-1 strain TRO.11 was previously published to be a HIV-1 clade B virus and was tested sensitive to 2F5 mediated neutralization (Li et al., 2005), although a mutation in the core epitope ((L)DKW) was observed by sequence alignments of the MPER (Table 4.3) which was shown to cause resistance to 2F5 mediated neutralization.

Neutralization assay		**TZM-bl Reporter Assay**					**A3R5 Reporter Assay**	
Cell type		**TZM-bl cells**					**A3R5 (modified CEM)**	
Virus (HIV-1)		**MLV (Neg. Ctrl.)**	**MN**	**W61D-TCLA**	**RHPA4259.7**	**TRO.11**	**SIV (Neg. Ctrl.)**	**CH58**
Serum	N3g#1	+	-	-	-	+	+	+
	N3g#2	-	-	-	-	-	-	+
	N3g#3	-	+	+	+	+	-	+
	N3g#4	+	-	-	-	+	+	+

Table 4.2: Summary of neutralization assays performed by David Montefiori´s laboratory (Duke University Medical Center, Durham, USA) performed with guinea pig sera obtained after the fourth immunization. The table describes the assay readout, cells and HIV-1 used for the corresponding assay. (+) and (-) indicate positive and negative result for neutralization. MLV pseudotyped virus was used as negative control in the TZM-bl cell-based neutralization assay, SIV was used as negative control in the A3R5 reporter assay.

Immune sera of the four guinea pigs were also tested at the Montefiori´s laboratory in a TZM-bl neutralization assay (Montefiori, 2009), using four different clade B viruses. In this assay, weak neutralization activity was reported for the immune sera of guinea pig N3g#3 tested against all four clade B viruses, whereas virus inhibition was not observed when tested with the control virus. These results were surprising since the immune serum of N3g#3 showed no neutralizing effect in

the TZM-bl neutralization assay. The weak inhibition of virus infection by immune sera from guinea pigs N3g#1 and N3g#4 might have been due to cytotoxic effects on the cells, although this was also observed in the other assays. Neutralization by immune sera of guinea pigs N3g#2 and N3g#3 was not mediated to antibodies binding to the 2F5 epitope but maybe due to FPPR-binding antibodies or a small population of antibodies binding to a conformational epitope.

Of note, corresponding preimmune sera derived from guinea pigs immunized with N3 were not tested in both neutralization assays performed at Montefiori´s laboratory due to low serum volumes and thus specific neutralization of immune sera could not be objectively proven.

HIV-1 isolate	clade	Accession Number	FPPR Sequence
NL4-3	B	AF324493	AAGSTMGAAS-M-TLTVQARQLLSDIVQQQ
MN.3	B	ADI62634	AAGSTMGAAS-**V**-TLTVQARLLLSGIVQQQ
W61D-TCLA	B	HM215359	AAGSTMGAAS-**I**-TLTVQARQLLSGIVQQQ
SS1196.1	B	AAW64257	AAGSTMGAAS-M-TLTVQARLLLSGIVQQQ
RHPA4259.7	B	JN944944	AAGSTMGAAS-**I**-TLTVQARLLLSGIVQQQ
TRO.11	B	AAW64260	AAGSTMGAAS-**V**-TLTVQARLLLSGIVQQQ
CH58	C	Salazar-Gonzalez et al. 2009	AAGSTMGAAS-M-TLTVQARLLLSGIVQQQ
HIV-1 isolate	**clade**	**Accession Number**	**MPER Sequence**
NL4-3	B	AF324493	E-LL-E-LD-K-W-AS-LWNWF-N-IT-N-WLWYIK
MN.3	B	ADI62634	E-LL-E-LD-K-W-AS-LWNWF-**D**-IT-N-WLWYIK
W61D-TCLA	B	HM215359	E-LL-E-LD-K-W-AS-LWNWF-**S**-IT-N-WLWYIK
SS1196.1	B	AAW64257	E-LL-E-LD-K-W-**EN**-LWNWF-**S**-IT-N-WLWYIK
RHPA4259.7	B	JN944944	E-LL-**A**-LD-K-W-AS-LWSWF-**S**-IT-**H**-WLWYIK
TRO.11	B	AAW64260	E-LL-E-LD-**S**-W-AS-LWNWF-**D**-I**S**-**K**-WLWYIK
CH58	C	Salazar-Gonzalez et al. 2009	**D**-LL-**A**-LD-**S**-W-**KN**-LWNWF-**D**-I**S**-**R**-WLWYIK

Table 4.3: Comparison of FPPR or MPER sequences between different HIV-1 isolates.

The next immunization study was performed in rats with 250 µg N3 and N4 (250 or 500 µg) in order to evaluate the variation in strength of antibody responses between different immunization studies and different antigens. N4 comprised a truncated p15E backbone compared to N3 including the HIV-1 FPPR at the N-terminal end of the protein and MPER located at the C-terminal end. Immune sera of four rats immunized with N3 induced comparable anti-gp41 antibody titers as after previous N3 immunizations of rats, although MPER-specific responses were lower. A moderate MPER-specific antibody response was only observed in the immune serum obtained from animal N3r#7 after immunization with N3. Immune sera obtained from the other N3 immunized rats in the same immunization group contained low titers of MPER binding antibodies, although MPER-specific antibodies of all animals bound to the 2F5 epitope.

Lower anti-MPER antibodies were surprising, since the same immunization regimen, route and adjuvant was used for previous vaccinations. The only difference between the two immunization experiments remained a marginally different purification procedure, using N-Lauroylsarkosine as detergent which is frequently used to isolate transmembrane proteins and was not reported to be immunoinhibitory (Bader et al., 2004; Bilello et al., 2011; Hobb et al., 2009). In addition to that, both bNAbs bound N3 with a higher affinity compared to N4 in SPR analysis, which might explain lower antibody titers after N4 immunization compared to those induced by immunization with N3.

Immunizations with N4 induced similar titers of gp41-specific antibodies compared with antibody titers observed after immunizations with N3. Of note, anti-MPER antibody titers were higher compared to titers observed after N3 immunizations, although the recombinant proteins differed in terms of a 26 amino acid deletion at the N-terminal end of the p15E backbone (3.4.1, Figure 3.8). In addition to that, immunization with 500 µg antigen induced lower anti-gp41 titers than 250 µg, which was maybe due to exceeding the optimal threshold of antigen amount for the rat organism. Furthermore, high antigen dosage can influence affinity of vaccine induced antibodies (Gonzalez-Fernandez and Milstein, 1998)

Nevertheless, immune sera of animals N4r#2, N4r#4 and N4r#7 exhibited a weak inhibition of HIV-1_{NL4-3} infection compared to the corresponding preimmune sera in the TZM-bl neutralization assay. This was defined as false positive for animals N4r#4 and N4r#7, since isolated IgG from these immune sera in additional neutralization tests remained negative for inhibition of virus infection. One immune serum derived from animal N4r#2 after the third immunization (S3) was tested positive in the TZM-bl neutralization assay, as well as in the real-time PCR-based neutralization assay, although IgG isolated from this serum were negative for inhibition of virus infection in the TZM-bl neutralization assay. This effect could have been attributed to a distinct population of antibodies, which did not bind to the Protein G column and was therefore not purified. A cytotoxic effect of the serum on the cells by itself was ruled out, since cytotoxicity of the serum was negatively tested in the real-time PCR-based neutralization assay with simultaneous detection of the GAPDH housekeeping gene and remained negative. Further tests of this serum were not feasible due the fact that the remaining serum was used for the real-time-based neutralization assay. Therefore, a second immunization experiment was performed in rats with the same immunization regimen.

The second immunization regimen with hybrid protein N4 induced similar anti-gp41 titers compared with the previous immunization. Immune serum of one rat obtained after the third immunization showed significant virus inhibition, although this effect was not observed when IgG isolated from this serum were tested in the TZM-bl neutralization assay. This also might be explained by a cytotoxic effect of immune sera on the cells or inability to isolate potential neutralizing antibodies with the Protein G columns. This was not retrospectively tested since the remaining serum was used for IgG purification.

Furthermore, a goat was immunized with N4 in order to obtain enough immune serum to isolate HIV-1 MPER-specific antibodies. The immune serum obtained after the third immunization inhibited infection of TZMbl cells more than 50% and clearly showed a neutralizing effect, although this effect was diminished when testing the immune serum obtained from the goat six weeks later without an additional immunization in between. CHR/MPER-specific antibodies were isolated, but did not inhibit HIV-1_{NL4-3} infection in the TZM-bl neutralization assay. Immune serum of the goat obtained after three and nine weeks after the third immunization was in contrast tested negatively in Christiane Moog´s laboratory (Strasbourg University, France) with a PBMC-based single round assay combined with laboratory-adapted HIV-1 X4 clade B strains (SF162 and QHO) and a macrophage-based neutralization assay tested with HIV-1_{Bal}. In these assays, IC80% and IC90%

values were provided as result for neutralization capacity of the immune sera and might not reflect the presence of very few or weakly neutralizing antibodies in the immune sera obtained after the third immunization. Immune sera were also tested negatively for toxicity in this PBMC-based assay, which supports the fact that the immune serum obtained after the third immunization with N4 specifically inhibited virus infection, although this was not attributed to affinity purified CHR/MPER-specific antibodies. One explanation could be that inhibition of virus infection was due to FPPR-specific or conformational antibodies which did not bind the recombinant CHR protein used for isolation of CHR/MPER-specific antibodies. The neutralizing effect of the immune serum three weeks after the third immunization might also have been due to FPPR-binding antibodies or antibodies against the backbone, mediating inhibition of virus infection by ADCC or ADCVI (Forthal and Moog, 2009) which were not isolated during CHR/MPER-specific antibody purification.

With regards to these findings it can also be suggested that reduction of virus infection mediated by rat or goat immune sera was caused by IgM or IgA antibodies which were previously proposed to likewise mediate neutralization (Bukawa et al., 1995; Yang, 2009). IgM or IgA antibodies were not purified with the Protein G column due to specificity for IgG antibodies. Although IgA antibodies are predominantly secreted at mucosal sites and serum concentrations remain relatively low in HIV-1 infected individuals (Alexander and Mestecky, 2007; Mestecky et al., 2004; Wei et al., 2012), gp41-specific IgA were previously demonstrated to effectively neutralize HIV-1 (Bomsel et al., 2011; Tudor et al., 2009) and to protect nonhuman primates against vaginal SHIV challenges. In addition to that, vaccination with gp120 expressed by vaccinia virus induced neutralizing antibodies from the IgA type, already detected 12 days after the second immunization (Moody et al., 2012).
IgM antibodies are early induced in responses to foreign antigen and serum concentrations decrease within days to weeks, which additionally might explain transient effect of the neutralizing capability three weeks after immunization (Yang, 2009). Furthermore, vaccine induced IgM were also previously shown to have antiviral activity (Sheppard et al., 2007) after vaccination with vector-expressed gp120. Since gp120 is relatively easy to access in contrast to gp41 despite heavy glycosylation, gp41-specific neutralization by IgM could have been impaired by steric hindrance of the large antibody subclass by itself.
In addition, evaluation of binding antibody by ELISA and Western blot was performed with polyclonal antibodies. All classes of antigen specific antibodies were detected in the serum and differentiation of antibody classes might have been missed out due to the use of polyclonal secondary antibodies in Western blot analysis and ELISA.

3.3 DNA vaccination of hybrid proteins

DNA delivery-based HIV-1 vaccines comprise certain advantages, such as simplified manufacturing, stability with regards to storage and distribution or low application volumes. Although DNA vaccination is typically performed in order to induce cellular immune responses, a small number of studies described additional stimulation of antibody responses in small mammals, capable of neutralizing HIV-1 (Pissani et al., 2012; Richmond et al., 1998; Wang et al., 1993; Ye et al., 2011). In this immunization regimen, two different expression vectors were used to present

hybrid antigens N1 and N5 in context with the cellular surface and provide *in vivo* folding and to mimic native-like structures and potential trimerization of recombinant hybrid proteins (Figure 4.1). Hybrid protein N1 comprised the FPPR, MPER and the transmembrane domain of HIV-1 since comparison of MPER immunogenicity was impaired when using a non-HIV-derived transmembrane domains. Furthermore, both antigens comprised the HIV-1 MPER which should have been presented in a lipid context since the presence of the membrane influences the presentation of neutralizing epitopes (Montero et al., 2012).

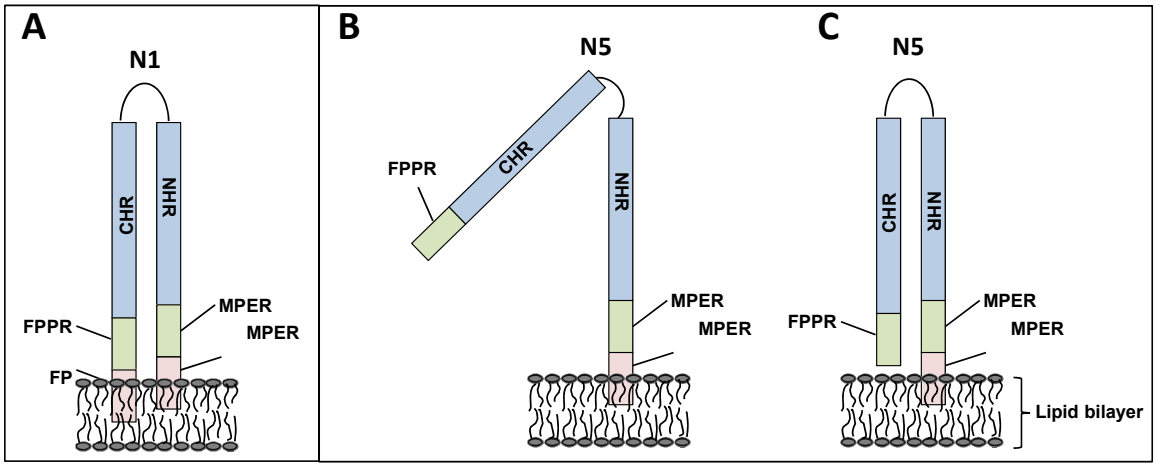


Figure 4.1: Schematic illustration of hypothetical conformations engaged by hybrid antigens N1 and N5, presented on the cellular surface after DNA vaccination. (A) and (C) represent the model of N1 and N5 in a hairpin-like conformation, (A) induced by insertion of the lipophilic fusion peptide (FP) and the transmembrane domain into the lipid bilayer (A) and/or (C) promoted by interaction of MPER and FPPR due to absence of the FP.

Despite membrane-bound presentation of both hybrid proteins and accessibility of the MPER as demonstrated by laser-scanning microscopy with fluorescent labeled 2F5 and 4E10, gp41-specific antibodies specifically binding to prokaryotically produced recombinant gp41 were not detected after DNA vaccination. In addition to that, immune sera of mice also failed to bind to lysate of HIV-1_{NL4-3} infected 293T cells. Importantly, immune sera obtained after immunization with purified hybrid proteins, recognizing purified antigen did not react with lysate of HIV-1_{NL4-3} infected 293T cells. This was surprising since it was expected that antisera of mice immunized with the DNA vaccine would recognize native Env although maybe denatured to some extend in reducing SDS-PAGE and acidic acid used for the Western blot membrane. Since neutralization assays of preimmune and immune sera obtained after the third DNA vaccination showed unspecific inhibition of virus infection, it was not assumed that HIV-1 specific and conformational antibodies were induced. Immune responses of mice after vaccination with hybrid proteins purified from bacterial expression were low overall (3.6), indicating an insufficient induction of antibody responses against gp41 thoughout the DNA-immunization study in mice. This was surprising, since mice are most frequently used for the induction of antibodies in DNA vaccination experiments (Bower et al., 2004a; Bower et al., 2004b; Hutnick et al., 2011; Law et al., 2007; Ljungberg et al., 2002). In addition to that, strong antibody responses were previously induced by DNA vaccination with both vector systems in the mouse organism (Behrendt et al., 2009; Forsell et al., 2007; Nordstrom et al., 2005). DNA vaccines able to induce HIV-1 neutralizing antibodies comprised mainly gp120-derived

antigens and are often used in DNA/VLP (Buonaguro et al., 2007; Harari et al., 2008; Kamdem Toukam et al., 2012; Ye et al., 2010) or DNA/protein (Bower et al., 2004b; Law et al., 2007; Pissani et al., 2012) immunization regimens. This demonstrates that DNA vaccination provides potent priming in a heterologous vaccination design but immunization with DNA remained insufficiently immunogenic (Ferraro et al., 2011). Subsequent immunizations with the corresponding purified antigen might have boosted induction of antibodies in this study, although changed the focus on presented antibody epitopes due different antigen expression.

3.4 Structure-based antigen design

Extensive investigation of HIV-1 Env structures and conformations, Env-derived peptides or distinct structural changes within Env caused by binding of bNAb have given insights on potential *in-vivo* situations during virus entry. One major step was the publication of the HIV-1 gp41 crystal structure including FPPR and MPER (Buzon et al., 2010). Gp41 refolds into a thermostabile six-helix-bundle (SHB) formation (Lu et al., 1995) with a coiled-coil interaction of the NHR and CHR, which is required for cellular and viral membrane fusion (Buzon et al., 2010; Melikyan et al., 2000). Although the NHR forms the inner homotrimeric subunit within the SHB, the FPPR is positioned towards outside of the SHB (Buzon et al., 2010). Accessible positioning of the FPPR in the recombinant hybrid proteins used for this immunization studies support predominant anti-FPPR antibody responses observed in immune sera despite unknown orientation of the FPPR in the hybrid proteins.

The crystal structure of gp41 in SHB conformation (Buzon et al., 2010) supports previous observations of hydrophobic FPPR and MPER interaction (Noah et al., 2008) which was shown to increase 2F5 affinity to its epitope (Fiebig et al., 2009). Moreover, interaction of FPPR and MPER might form a conformational epitope which is required to induce MPER-specific bNAbs (Denner, 2011).

Conformation-based antigen design is currently proposed to be “the” approach to develop a vaccine, capable to induce bNAb. Since the majority of those bNAbs recognize conformational epitopes which are not presented on linear antigens, complex refolding procedures, trimerization or scaffold presentation of proteins or distinct epitopes (Guenaga et al., 2011; Ofek et al., 2010a; Qiu et al., 2011) seem to be the most reliable approach at the moment to mimic *in vivo*-like HIV-1 Env structures. This is often performed with support of computational algorithms to optimize amino acid composition of scaffolds for optimal presentation of epitopes (Correia et al., 2011; Correia et al., 2010). These techniques might also be useful for peptides or proteins designed to present discontinuous epitopes as described for a recently isolated bNAb (Zhang et al., 2012) which neutralized 34% of primary isolates and binds to an epitope overlapping the CD4 binding site and the trimeric NHR of gp41.

Despite these extensive investigations regarding structure-based antigen design for HIV-1 vaccine design, potential structures engaged by antigens might be disrupted after presentation of degraded antigens together with MHC Class II on antigen presenting cells. Classical stimulation of B-cells is predominantly T-cell dependent, relying on antigen processing and presentation in context with

MHC class II molecules. After vaccination with proteins or peptides, the antigen is internalized for peptide presentation in context with MHC class II molecules by antigen presenting cells. Antigens are endocytosed into antigen presenting cells and cleaved with the help of fusion with acidic lysosomes. Peptides loaded on MHC class II molecules were described to be 12 to 25 amino acids long (Sercarz and Maverakis, 2003). Although a peptide comprising 20 amino acids still can adopt certain conformations, it (i) may form other conformations as presented in the whole protein or (i) distinct conformations might be disrupted by lysosomal proteases and the acidic environment. The peptide presented in context with the MHC class II molecule is recognized by T-helper cells which are subsequently activated. Those activated T-helper cells then provide the stimulatory signal to activate the B-cell (Parker, 1993). Despite this mechanism, B-cells can also be activated without T-cell help by antigens which comprise distinct specificities. Type 1 of T cell-independent antigens, such as LPS or CpG, elicit polyclonal B cell activation via Toll-like receptors (Obukhanych and Nussenzweig, 2006). Activation of the B-cell are also triggered by T-cell independent antigens of Type 2, which are able to crosslink the Ig receptors on the B-cell surface and results in subsequent differentiation into plasma cells, switching from IgM production to IgG/IgA (MacLennan et al., 2003). Thus, only T-cell independent activation of B-cells might induce antibody responses which are specific for certain conformations and evaluated before immunization, although these IgG responses were described as not as robust as those for T-dependent antigens (MacLennan et al., 2003). Since protein aggregates were previously described to activate B-cells by crosslinking the B-cell receptor (Rosenberg, 2006), vaccination with the recombinant hybrid antigens might have triggered this pathway.

In addition to the difficulties regarding initial antigen presentation, the development of potent neutralizing immune responses requires about one to three years and breadth of these antibody responses is correlated with time after primary infection (Sather et al., 2008). Thus it can be suggested, that prolonged and consistent antigen stimulation is required for affinity maturation (Stamatatos et al., 2009; Walker and Burton, 2010) since bNAbs comprise about 10% to 30% of somatic mutations in the heavy chain (Saphire et al., 2001; Walker et al., 2009; Wardemann and Nussenzweig, 2007). Of note, early B-cell ancestors have low reactivity to HIV-1 Env (Bonsignori et al., 2011; Mouquet et al., 2010; Pancera et al., 2010), although a recent study proposed binding of the founder virus by the predicted, unmutated ancestor cell (Haynes et al., 2012b; Ma et al., 2011). The prolonged antigen stimulation to achieve sufficient affinity maturation of corresponding antibodies might be enabled by constant expression of the antigen by a replication competent vector, although this approach will be difficult to implement for human use due to safety issues.

3.5 Are MPER-specific bNAb autoreactive?

Broadly neutralizing antibodies develop in about 2% of HIV-1 infected individuals after at least several months (Euler and Schuitemaker, 2012; Mikell et al., 2011; Sather et al., 2008). One of the main reasons why such antibodies rarely appear in infected individuals was controversially discussed to be due to elimination of self-reactive B-cells by natural B-cell tolerance mechanisms,

especially to lipids (Alam et al., 2007; Haynes, 2005; Matyas et al., 2009a; Sanchez-Martinez et al., 2006b; Scherer et al., 2007).
B-cells reactive to self-antigen with or without extended CDRH3 loop appear frequently (about 70-75%) and are eliminated or inactivated by a number of physiological mechanisms down to a percentage of about 20%, which circulate in the blood (Meffre et al., 2001; Shiokawa et al., 1999; Wardemann and Nussenzweig, 2007). Thus, the repertoire of mature and naïve B-cells, able to respond to antigens mimicking auto-antigenic peptides may be in combination with lipids remains relatively small. Furthermore, the process of antigen-induced somatic hypermutation for the development of high-affinity antibodies can also lead to self-responsive B-cells (Mietzner et al., 2008; Tiller et al., 2007). Naïve B-cell clones express the same Ig molecule with the same variable region on the membrane, which is used as antigen receptor. After binding to the corresponding antigen, the B-cell undergoes activation which is also described as clonal selection, predominantly after receiving the additional signal from a T-helper cell. If a naïve B-cell did not recognize a distinct antigen, it dies within a few days, which explains the high turnover of circulating B-cell populations. In addition to this mechanism, somatic hypermutation increases the affinity of produced antibodies after primary activation of the B-cell (Euler and Schuitemaker, 2012) which might then be depleted from the repertoire.

One particular characteristic of bNAb was defined as an unusual extended complementary determining region in the heavy chain (CDRH3) which is induced by this somatic hypermutation. In case of gp120 specific bNAb, for instance PG9, comprise a hammerhead-like extended CDRH3 which “dives” through the glycan shield for interaction and binds to the stem of the surface envelope protein (Ringe et al., 2012). PGT 141 also comprises an extended CDRH3 loop and a beta-hairpin at the tip to reach the protein beneath its glycan shielded surface, although reactivity to self-antigens was not detected (Walker et al., 2011). Thus, gp120-specific bNAbs have found a way to circumvent viral defence mechanism of glycosylation by direct interaction, but do not exhibit polyreactivity in the first place. On the other hand, the gp120-specific bNAb 1b12 also bound to a number of lipid antigens, such as cardiolipin or phosphatidylserin, despite the fact that the epitope of 1B12 is not located in close proximity to the lipid surface of the virion (Haynes et al., 2005).

When focussing on MPER-specific bNAb, 2F5 and 4E10 also were reported to exhibit polyreactivity and to react with self-antigens. The unusually long CDRH3 loop was described for both bNAb with regards to this issue (Cardoso et al., 2005; Ofek et al., 2010b), which was proposed to enable the antibody´s access to the hidden epitope located in the MPER. The mechanism of primary interaction with the lipid membrane and subsequent engagement with the MPER binding site seems reasonable in order to diffuse into the viral membrane with the aromatic residues in the long CDRH3 loop and to access the binding site in a better way (Alam et al., 2007; Buzon et al., 2010). Of note, MPER-specific bNAbs with a long CDRH3 loop undergo negative selection, which was previously demonstrated for 2F5 (Verkoczy et al., 2010) and mutations in the CDRH3 loop abrogated neutralization of HIV-1 and lipid binding (Alam et al., 2007).
In contrast to these findings, the bNAb D5 does not comprehend an extended CDH3, although neutralization capacity also seems limited (Miller et al., 2005; Sabin et al., 2010). The recently

identified bNAb CAP206-CH12 displays similarities with other MPER specific antibodies. The antibody comprises an extended CDR3 loop but was surprisingly not shown to be polyreactive (Morris et al., 2011).
Taken toghether, there are indications that bNAb comprise distinct structural abnormalities, such as extended CDRH3 loops and polyspecific reactions, although these structural and behavioral abnormalities can not be applied to all bNAbs (Huang et al., 2012).

Although the induction of such polyreactive bNAb during HIV-1 infection demonstrates that such antibodies can develop, it cannot be excluded that B-cells reacting with the recombinant hybrid antigens or epitopes from these proteins were depleted from the repertoire by B-cell tolerance mechanisms. Although antisera derived after immunizations with hybrid antigens did not show significant inhibition of HIV-1 infection, those sera were screened for the induction of autoantibodies, binding to lipid antigens: cardiolipin and sphingomyelin.
None of the rat immune sera derived after the fourth immunization with hybrid antigens N1, N2 or N3 reacted with cardiolipin or with sphingomyelin. Particularly with regard to the fact that some immune sera of rats immunized with N3 recognized the 2F5 epitope and 2F5 did not bind both lipids tested, this result was as expected. The findings concerning the interaction of 2F5 with lipids are controversially discussed (Haynes, 2005; Matyas et al., 2009a; Sanchez-Martinez et al., 2006a; Sanchez-Martinez et al., 2006b), although binding of 2F5 to sphingomyelin and cardiolipin was most likely due to large amount of coated lipid and high antibody concentration in the assay (Haynes, 2005).
The immune serum of one guinea pig immunized with N3 (N3g#3), which inhibited HIV-1 clade B primary isolate infection in the TZM-bl neutralization assay to some extent as tested by Montefiori´s laboratory, did not bind lipid antigens.

In contrast to these findings, a MPER-specific bNAb, 10E8, was recently isolated from a HIV-1 infected individual, showing broader neutralization capacity compared to all previous identified MPER binding bNAbs 2F5 and 4E10 (Huang et al., 2012). In addition to that, frequency of antibodies with 10E8-like specificities was described with 8% of all tested sera derived from infected individuals and thus more frequently compared to the frequency of other MPER binding bNAbs (Sather et al., 2008; Sather and Stamatatos, 2010; Shen et al., 2009). 10E8 also comprises an extended CDRH3 loop as proposed for other bNAbs and binds an epitope overlapping the 4E10 binding site (critical amino acids in bold: **NWF**DI**T**NWLWYI**R).** Phosphatidylcholin-cardiolipin liposomes were surprisingly not bound by 10E8, despite the specificity to an epitope located even closer to the viral membrane than the 4E10 binding site. In contrast to the two other MPER-specific bNAb 2F5 and 4E10, the CDR3 loop of the antibody seems to interact directly with the binding site, has a smaller interaction site with the lipid membrane and was thus proposed to be less reactive to lipid surfaces.
With regard to the findings that this bNAb did not bind to lipids and other autoantigens, these distinct properties of 10E8 may refute the hypothesis that MPER-specific are autoreactive due to close proximity of the antigen to the lipid virion surface. This was also supported by recent findings that the reactivity profile of MPER bNAb 2F5, 4E10 and Z13 largely differ from those of

autoreactive antibodies derived from patients with anti-phospholipid-syndrom and lupus erythematosus (Singh et al., 2011).

3.6 Liposomes as vaccine carrier

The vast majority of antigens require T-cell dependent help, only a small number of antigens trigger T-cell independent activation of B-cells. Bacterial polysaccharides, polymeric proteins and LPS are such antigens, comprising repetitive structures which are able to stimulating B-cells to elicit low-affinity germ-line antibody responses in rodents without undergoing affinity maturation (Cerutti et al., 2013). Furthermore, liposomes possess these characteristics of Type 2 T-cell independent antigens to stimulate B-cells through the T-cell independent mechanism (Li et al., 2001). One major advantage of this is potential B-cell activation without $CD4^+$ T-cell help, depending on denseness of conjugated or surface adsorbed antigen (Watson et al., 2012). This provides presentation of conformational epitopes maybe engaged by the liposome-attached antigen in contrast to processes and denatured peptides presented in context with MHC class II molecules.

Liposomes in combination with MPLA were used in these immunization regimens to induce a potent anti-HIV-1 antibody response. The recombinant hybrid protein N1 comprised the TM domain and was thus primarily used for immunization experiments. Surprisingly, immunizations with $N1_{Lip}$ did not induce detectable anti-gp41 antibody responses in the immunized goat and low antibody titers in immunized rats although surface associated antigens were proposed to show potent antibody responses (Watson et al., 2012) with conformational binding properties (Dennison et al., 2011a). Concentrations of $N1_{Lip}$ in both batches were measured prior immunizations and administered at same concentrations as used for immunization regimens with pure recombinant protein. SPR analysis of liposome preparations was planned to evaluate the protein´s orientation on the surface of the liposomes and antigen accessibility. However, SPR analysis could not be performed for binding analysis with mAb 2F5 since both liposome batches formed aggregates in all tested solvents.

Liposome formulations were also prepared by Polymun with hybrid antigens N3 and N4, expecting adsorption on the surface of liposome since both antigens comprised neither FP nor the transmembrane domain. Anti-rgp41 titers after immunization with either $N3_{Lip}$ or $N4_{Lip}$ were higher compared to $N1_{Lip}$ immunizations, whereas it cannot be excluded that distinct proportions of hybrid antigens were not adsorbed to the liposomes and thus more accessible to the immune system. Aggregation and further shielding of potential antibody epitopes might further prohibited antibody responses, or antibodies were maybe only directed against the p15E backbone although p15E-specific antibody responses remained low after primary hybrid protein immunizations, since immune sera after immunization with recombinant hybrid antigens were also tested for reactivity to p15E derived peptides in an additional microarray (data not shown). Orientation of the recombinant protein is not predictable during liposome preparation. The proteins might have been orientated towards the lumen of the liposomes or completely entrapped and were thus not accessible for B-cell recognition and activation despite some disrupted liposomes.

With regard to previous findings that neutralizing antibodies induced by derivatives of gp41 in liposomes induced antibodies that bound to the 2F5 and 4E10 epitopes as well as to lipid epitopes (Karasavvas et al., 2008; Matyas et al., 2009b), antisera obtained after hybrid protein/liposome immunizations were in addition tested for reactivity with lipid antigens cardiolipin and spingomyelin. Immune sera of two guinea pigs immunized with $N1_{Lip}$ bound to cardiolipin but not to sphingomyelin (3.8, Figure 3.36A), which was controversially discussed to be recognized by the bNAb 4E10 (Alam et al., 2007; Haynes, 2005; Haynes et al., 2005; Matyas et al., 2009a; Scherer et al., 2007). Due to the fact that measurable anti-MPER were not observed in those antisera, this recognition was maybe attributed to lipid-specific antibodies induced by liposome immunization but this was unfortunately not evaluated during this study. On the other hand, immune sera obtained after $N3_{Lip}$ or $N4_{Lip}$ immunization unspecifically inhibited HIV-1_{NL4-3} infection (3.6, Figure 3.27B) but did not bind to the tested lipids, showing that virus inhibition was not caused by cardiolipin or sphingomyelin binding antibodies. Despite that, cytotoxic effects of immune sera were partially excluded by the real-time PCR-based neutralization assay. Taken together, immunization of hybrid antigens with liposomes were poorly immunogenic, especially with regards to immunogenicity of the MPER and did not induce neutralizing antibodies. In order to avoid potential anti-lipid antibodies which might exhibit some autoreactivity to human lipids, this approach for vaccination development should be re-evaluated.

3.7 Measuring neutralization with *in-vitro* assays

The efficacy of a vaccine with regards to induction of neutralizing antibodies can only be evaluated by testing immune sera after vaccination in a neutralization assay prior challenge experiments in primates. A wide range of neutralization assays with certain variants are currently applied to detect antibodies in serum responsible for inhibiting HIV infection, mainly differentiating within the used reporter system. Most assays are complex and require from three days to more than a week incubation time, which can be the source of small errors and increased deviation in inter-assay comparability (Heyndrickx et al., 2012; Polonis et al., 2008; Polonis et al., 2009). These variable parameters are preparation of virus, virus dose, target cells and growing density, concentration of antibodies, treatment of sera before the assay (complement deactivation) or freeze/thaw cycles of the tested immune sera. Thus, standardization of neutralization assays is required to allow comparable evaluation of potency and quality of neutralizing antibodies in serum. Two of the most critical parameters in the assay are the choice of target cells and decision for pseudotyped viruses, laboratory adapted HIV-1 strains or primary isolates. This step is critical to evaluate since cell line-based model systems might not reflect the situation *in vivo*, although these assays are easier to standardize (Fenyo et al., 2009) compared to PBMC-based assays. The choice of virus is also challenging since laboratory adapted strains are more sensitive to neutralization compared to primary isolates (Daar et al., 1990; Mascola et al., 1996).

Neutralization of HIV-1_{NL4-3} by immune sera after vaccination was initially measured with a TZM-bl cell-based in these immunization studies. Sensitivity of the this neutralization assay remained high since 2F5 was used as positive control and neutralization of the reference antibody could be measured down to a concentration of 2.5 μg/ml. Measurement of high sensitivity on the other hand

might bear the risk of detecting inhibition of HIV-1 infection which is maybe not antibody mediated. Thus, potential neutralizing sera were further tested in a real-time PCR-based neutralization assay with either stimulated PBMCs or another lymphocyte cell line to address possible cytotoxic effects of immune sera which were not distinguished from antibody-mediated neutralization in the TZM-bl neutralization assay. One of the main disadvantages of PBMC-based neutralization assays might be reproducibility due to non-homogenous stimulation or variability of the donor cells (Trkola et al., 1999).

Of note, a hybrid protein approach comprising p15E and the MPER of gp41 in order induce neutralizing antibodies was proposed years ago by Luo et al. (Luo et al., 2006). In this study, two different antigens were designed, one by substitution of the E2 region in p15E by the MPER of gp41 and the other one by fusion of the MPER to the C-terminal end of p15E. Immunization of rabbits induced neutralizing antibodies in three out of nine animals measured with pseudotyped clade B viruses. Of note, immune sera of rabbits immunized with p15E tested in the neutralization assay also neutralized HIV-1 up to 50%, showing non-HIV-1 specific inhibition of virus infection. In their neutralization assay, 50% inhibition of virus infection was observed with an antibody concentration of 0.03 µg/ml, although our ID50 was measured at 2 µg/ml. It is not described how much pseudotyped virus was used for the assay. Since (i) pseudoviruses were described to be easier to neutralize (Fenyo et al., 2009) and (ii) ID50 values strongly depend on virus type and concentration, both assays are difficult to compare.

The sensitivity of neutralization assays might be the restricting factor since the protective antibody level is unknown, especially at mucosal sites. Passive immunization experiments in non-human primates shed some light on concentrations of bNAb required to protect from HIV-1 challenge. A comparative assay of HIV-1 positive sera and passive immune monkey sera demonstrated that serum of protected monkeys showed a 50% reduction of infection (ID50) at dilutions ranging from 1:125 to 1:300 which seem comparable to the levels detected in sera of infected individuals (Hessell et al., 2009a; Mascola and Montefiori, 2010).

A whole new field developed in the recent years in order to detect low titers of neutralizing antibodies in serum or even in mucosal tissue or lavage fluids of vaccinated individuals. The knowledge about protective titers also increased in the last 10 years, thus predominantly focusing on physiological *in-vivo* concentration of virus at mucosal sites and antibodies required for neutralization (Mestecky et al., 2011). The most prominent example for that represents detection of IgGs in vaginal fluids of macaques with neutralizing properties and ADCC activity after immunization with gp41 subunit antigens embedded in virosomes which was already approved for human use (Bomsel et al., 2011).

The different outcomes of 16 neutralization assays using a panel of viruses and different target cells was extensively evaluated by the NeutNet Report (Fenyo et al., 2009), testing sensitivity, standardization and variability when using PBMC- or engineered cell-line-based assays, pseudotyped viruses or primary isolates in 16 reference laboratories. Measuring *in vitro*

neutralization reveal first hints for protection *in vivo* – although this correlation is not always applicable. The main outcome of the study showed that neutralization assays are difficult to standardize. In order to evaluate the effect of a vaccine in model organisms with regard to induction of neutralizing antibodies, different neutralization assays with a panel of viruses and different target cell was recommended (Fenyo et al., 2009; Heyndrickx et al., 2012).

3.8 Prevention from HIV-1 infection

A large number of different approaches to establish a defending system able to prevent HIV-1 infection have been proposed in the recent years. Since the general approach to achieve protection by vaccination with HIV-1 derived proteins, peptides, expressed *in vivo* from DNA-vectors or VLPs were not very successful, rethinking and evaluation of other approaches gained attention in the whole vaccine field.

One example of new approaches thinking outside the box was a recent study performed by Lu and colleagues (Lu et al., 2012). Since initial virus replication after infection potentially depends on activation of $CD4^+$ T-cells in case of X4 virus this replication may be prevented by inhibition of this activation (Andrieu and Lu, 1995; Fauci, 1996). Administration of anti-inflammatory agents prevented macaques from SIV infection after mucosal challenge which was hypothesized to be caused by inhibition of immune activation (Li et al., 2009). With regards to this study, an oral vaccination regimen in Chinese macaques comprising bacteria which favour immune tolerance and inactivated SIV was performed (Lu et al., 2012). The vaccine induced regulatory T-cells, suppressing *in vivo* $CD4^+$ T-cell activation and protected macaques after high dose of intrarectal challenge. These results are remarkable since animals were immunized only once with an antigen which was simply to produce and to deliver.

Another interesting idea to prevent HIV-1 infection was proposed by Balazs and colleagues (Balazs et al., 2012) by immunization of humanized mice with a specialized adeno-associated virus vector, optimized for the expression of human IgG. Mice were immunized with the vector, expressing five different bNAbs (2F5, 4E10, b12, 2G12 or VRC-01). Only mice expressing b12 and VRC-01 were protected after intravenous challenge with HIV-1, mice expressing the other antibodies were partly protected, maybe due to lower antibody expression levels in *vivo*. Measuring antibody concentration expressed after one-time immunization with the antibody-expressing vector demonstrated continuous high level expression of antibodies over twelve weeks, showing long-term effectiveness. With regards to only one vaccination required and relatively persistent production of bNAb over a distinct period of time, this approach seems applicable for further preclinical trials.

Although a vaccine would be the golden goal to prevent transmission of HIV, a new drug came to focus in the past year. Tenofovir in combination with emtricitabine are nucleoside and nucleotide analogue reverse transcriptase inhibitors (NRTIs), blocking the viral reverse transcriptase, respectively. The active component tenofovir disoproxil fumarate in combination with emtricitabine,

trade name Truvada ®, was introduced to the European market in 2005 for antiretroviral therapy but only in combination with other therapeutic agents for the treatment of HIV infection. In addition to this indication, tenofovir was tested as vaginal gel in the CAPRISA 004 trial. The reduction of HIV-1 infection among African women when used before and after sexual exposure (Abdool Karim et al., 2010) was promising, although the efficiency was reduced when the setting was re-tested by the NIH. Truvada ® was also introduced to the US market in July 2012 for the indication of prevention in HIV-negative adults, especially for homosexual or heterosexual sero-discordant couples. Effectiveness of Truvada in humans was reported to be 75% protective in discordant, heterosexual partners with a maximum of 90% protection when the drug was taken on regular bases (Donnell et. al 2012, reported at CROI), which supports the previous studies (Baeten et al., 2012; Mujugira et al., 2011). This indication was controversially discussed in the scientific field and the media. One package of the medication is estimated with 800 Euros and therefore limited to countries which can afford these high costs of treatment and prophylaxes.

With regards to these findings, a combination of vaccine and PrEP might also work synergistically to additionally reduce the risk of HIV-1 infection (Excler et al., 2011). Since vaccines seem to be more effective in challenge regimens using repeated low dose applications of virus, PrEPs can already reduce virus concentration prior contact. Nevertheless, despite large improvements of microbicides, a vaccine capable to prevent infection during a greater period of time is inevitable.

4. Summary

A vaccine against HIV-1 to keep the pandemic in bay is one of the major goals in vaccine development. Although the scientific field of vaccine development is largely supported with extensive fundings, attempts to design a vaccine to induce potent protection only remained successful in the macaque model, but not in human trials. In order to address this aim, different hybrid antigens were designed, comprising sequences of the HIV-1 and PERV transmembrane protein. P15E, the transmembrane protein of PERV was used as scaffold protein, since immunization with the ectodomain of different gammaretroviruses resulted in the induction of neutralizing antibodies in various animal species. These neutralizing sera recognized epitopes located in the MPER and in the FPPR of p15E. Alignments of gp41 and p15E showed a sequence homology within the MPER (F/YEGWFN in the case of gammaretroviruses and NWFNIT, the 4E10 epitope, in case of HIV-1) despite the evolutionary distance between PERV and HIV-1. Based on these findings, both epitope regions of p15E recognized by neutralizing sera were substituted by corresponding sequences derived from gp41 of HIV-1. One gp41 sequence contained the epitopes of 2F5 and 4E10 in the MPER and the other corresponded to the FPPR. Structural interaction of the MPER and the FPPR had been suggested as a reason for an increased binding of 2F5 to its epitope in the presence of peptides corresponding to the FPPR. In addition, a direct interaction of FPPR and MPER sequences during infection and a stabilization of the 6-helix-bundle formation by peptides corresponding to the MPER and FPPR have been demonstrated. These results allow speculations that an interaction of the FPPR with the MPER may be required to induce neutralizing 2F5-like and 4E10-like antibodies.

On the basis of above mentioned considerations, four different recombinant hybrid proteins (N1, N2, N3, N4) were designed, produced by *E. coli* overexpression and purified up to a purity of 95%. These four antigens were used for immunization studies in guinea pigs, rats, goats and one rabbit in a three week immunization regimen. Humoral antibody responses were evaluated after each immunization with focus on anti-gp41 specific binding antibodies and recognition sites of these antibodies. Immune sera were furthermore tested for inhibition of HIV-1 infection.
Immunization studies with recombinant hybrid proteins N1 or N2 induced antibodies predominantly binding to the FPPR. Weak MPER-specific antibody responses were observed in rat sera after immunization with N2, these immune sera did not inhibit HIV-1_{NL4-3} infection. Immunization of guinea pigs, rats and one goat with N3 or N4 also induced antibodies binding to the FPPR of gp41, but also to the 2F5 epitope (ELDKWA) located in the MPER. Immune sera of guinea pigs and rats immunized with N3 did not show any inhibition of virus infection as tested in the TZM-bl neutralization assay. Although immune sera of rats and the goat immunized with N4 also recognized the same epitopes as after immunization studies with N3, immune sera of one rat and the goat showed a weak neutralizing effect after the second boost. However, IgG or CHR/MPER-specific antibodies isolated from these immune sera did not neutralize HIV-1_{NL4-3}.

Nonetheless, the strategy of hybrid proteins used as antigens was successful in focusing the antibody response towards the desired MPER epitope and immune sera after vaccination with N4

showed a weak neutralizing effect. Specific investigations regarding the mechanism of HIV-1 neutralization mediated by antibodies after immunization with p15E may contribute to improvement of hybrid protein design and thus the likelihood to induce potent HIV-1 neutralizing antibodies.

5. Zusammenfassung

Eine der größten Herausforderungen in der Impfstoffentwicklung weltweit ist die Entwicklung eines Impfstoffs gegen HIV-1 um damit die HIV-1 Pandemie einzudämmen. Obwohl hohe Summen für Forschungsmitteln auf diesem Gebiet investiert werden, konnte bis heute kein Antigen und kein effektiver Immunsierungsplan gefunden werden, welche die Infektion mit dem Retrovirus komplett verhindern kann. In dieser Arbeit wurde eine Strategie gewählt, die auf Immunisierungsstudien mit Hybridproteinen basiert. Für diese Studien wurden redombinante Hybridproteine produziert, die aus Sequenzen des transmembranen Hüllprotein von HIV-1 und jenem des porcinen endogenen Retrovirus (PERV) bestehen. Das transmembrane Hüllprotein p15E von PERV wurde hier als Proteingerüst verwendet, da in Immunisierungsstudien mit der Ektodomäne von p15E in verschiedenen Tierspezies die Induktion von neutralisierenden Antikörpern gezeigt werden konnte. Jene PERV neutralisierenden Seren erkannten sowohl Epitope in der fusionspeptid-proximalen Region (FPPR), als auch in der membrane-proximalen externen Region (MPER) des transmembranen Hüllproteins. Alignments von gp41 und p15E zeigten eine Sequenzhomologie in der MPER (F/YEG**WFN** bei PERV und N**WFN**IT im 4E10 Epitop bei HIV-1) trotz unterschiedlicher Evolution beider Retroviren. Aufgrund dieser Sequenzhomologie wurden jene Epitope in der FPPR und MPER von p15E, die von neutralisierenden Seren erkannt wurden, durch korrespondierende Sequenzen in gp41 ersetzt.

Ziel dieser Arbeit war es, Immunisierungsstudien mit diesen Hybridproteinen durchzuführen und die induzierte Antikörperantwort zu charakterisieren. Hierfür wurden vier verschiedene Hybridantigene (N1, N2, N3, N4) konstruiert, in *E. coli* exprimiert und bis zu einer Reinheit von 95% aufgereinigt. Diese vier Antigene wurden für Immunisierungsstudien in Meerschweinchen, Ratten, Kaninchen und Ziegen verwendet, wobei die Tiere in einem 3 Wochenabstand immunisiert wurden. Nach jeder Immunisierung wurden die Antiseren der Versuchstiere auf die Induktion von HIV-1 spezifischen Antikörpern untersucht, sowie die Epitope jener Antiköperpopulationen bestimmt. Zusätzlich wurden die Immunseren auf Neutralisation von HIV-1 untersucht.
Immunsierungen mit N1 oder N2 induzierten eine FPPR-fokussierte Immunantwort in Ratten und Meerschweinchen. MPER-spezifische Antikörper konnten nur in Immunseren von N2 immunisierten Ratten festgestellt werden, jedoch konnte keine HIV-1 neutralisierende Wirkung der Immunseren nach der Immunisierung mit den Hybridproteinen N1 oder N2 nachgewiesen werden. Immunisierungen von Meerschweinchen, Ratten und einer Ziege mit den Hybridantigenen N3 und N4 induzierten FPPR spezifische, sowie MPER bindende Antikörper, die das gleiche Epitop erkannten wie der breitneutralisierende Antikörper 2F5. Immunseren oder isolierte IgGs der Meerschweinchen und Ratten nach Immunisierung mit N3 zeigten keine Inhibition der HIV-1_{NL4-3} Infektion in den TZM-bl und C8166 Zell-basierten Neutralisationsassays. Nur für das Immunserum eines Meerschweinchens konnte eine schwache Neutralisation in dem PBMC-basierten Neutralisationsassay nachgewiesen werden, allerdings nicht für die isolierten IgGs aus diesem Immunserum. Obwohl die Immunseren von Ratten und einer Ziege nach der Immunisierung mit N4 die selben Epitope erkannten wie nach den Immunisierungstudien mit N3 konnte eine virusspezifische, schwache Neutralisation von HIV-1_{NL4-3} durch ein Rattenserum und dem

Ziegenserum nach der dritten Immunisierung mit N4 gezeigt werden. Dieser neutralisierende Effekt wurde allerdings nicht durch IgG oder MPER-spezifischen Antikörpern vermittelt, welche aus dem Serum isoliert wurden.

Obwohl die Seren nach Immunisierung mit N4 nur einen schwachen neutralisierenden Effekt zeigten, stellt der Ansatz von Hybridproteinen einen innovativen Ansatz zur MPER gerichteten Vakzinentwicklung dar. Die genauere Untersuchung des Mechanismus zur Induktion von neutralisierenden Antikörpern durch Immunisierung mit dem Transmembranprotein p15E könnte dabei helfen, weitere Hybridproteine zu optimieren und damit eine stärker neutralisierende Immunantwort zu erlangen.

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7. Publications

Strasz N., Morozov V., Kreutzberger J., Keller M., Eschricht M., Denner J. **Immunization with Hybrid Proteins Containing the Membrane Proximal External Region of HIV-1**. (Submitted to *AIDS Research and Human Retroviruses* 2013)

Poster:

Strasz N., Morozov V., Kreutzberger J., Lau M., Denner J. **Immunisation with the membrane proximal external region of gp41 of HIV-1 grafted into the transmembrane envelope protein of a gammaretrovirus**. (AIDS Vaccine 2012, Boston, USA)

Strasz N., Morozov V., Kreutzberger J., Lau M., Denner J. **Hybrid proteins composed of the membrane proximal external region and the fusion peptide proximal region of gp41 of HIV-1 and the transmembrane envelope protein of the porcine endogenous retrovirus: implications for HIV-1 vaccine design**. (22nd Annual Meeting of the Society for Virology, Essen, Germany)

8. Appendix

Amino acid sequence of Pepspot Membrane and Microarray

HIV-1_{HXB2} gp41 (GenBank: K03455)

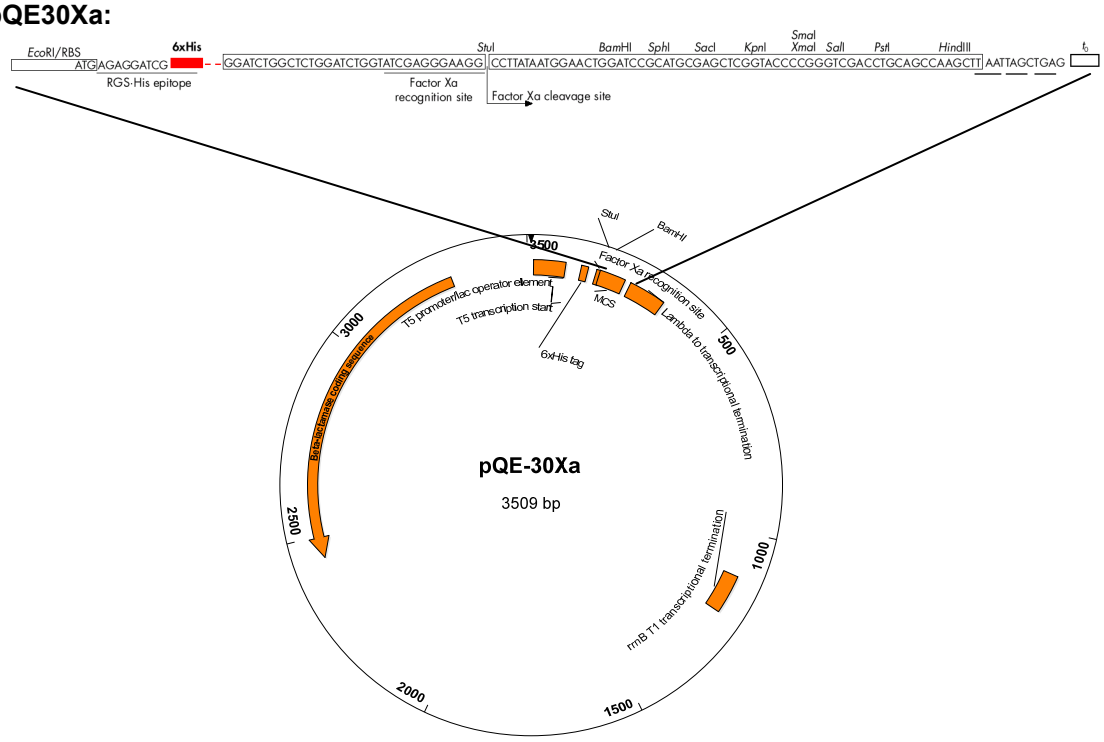
AVGIGALFLGFLGAAGSTMGAASMTLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVE
RYLKDQQLLGIWGCSGKLICTTAVPWNASWSNKSLEQIWNHTTWMEWDREINNYTSLIHSLIEESQNQQEKNE
QELLELDKWASLWNWFNITNWLWYIKL

PERV-A p15E (GenBank: AJ133817)

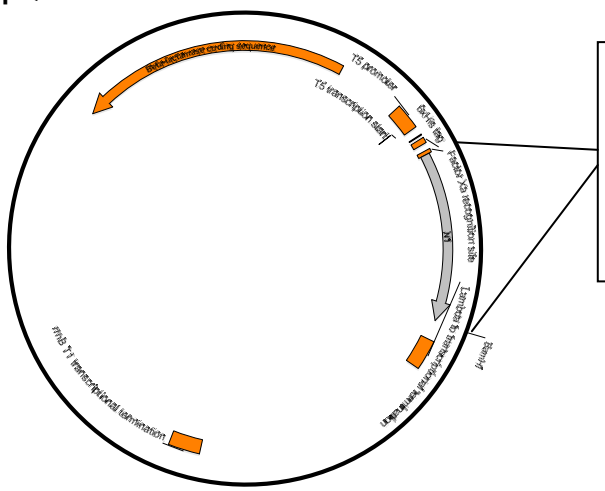
EPISLTLAVMLGLGVAAGVGTGTAALITGPQQLEKGLSNLHRIVTEDLQALEKSVSNLEESLTSLSEVVLQNR
RGLDLLFLKEGGLCVALKEECCFYVDHSGAIRDSMSKLRERLERRRREREADQGWFEGWFNRSPWMT

Maps of hybrid protein expression vectors for *E. coli* SCS1/pSE111

pQE30Xa:



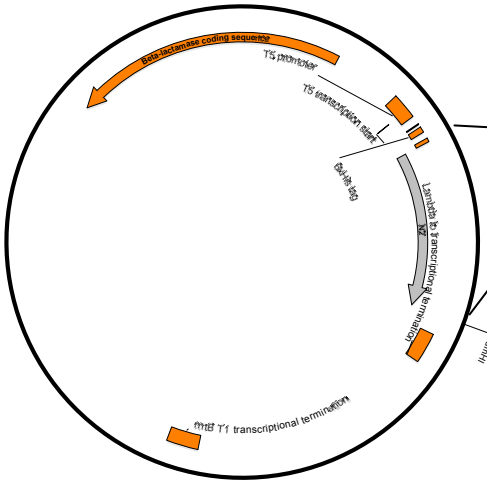
pQE30Xa-N1:



StuI–**N1**–*BamHI*

MRGSHHHHHHGSGSGSGIEGR**AVGIGALFLGFLGAAGS**
TMGAASMTLTVQARQLLSGIVQQQSNLHRIVTEDLQAL
EKSVSNLEESLTSLSEVVLQNRRGLDLLFLKEGGLCVA
LKEECCFYVDHSGAIRDSMNKLRERLEKRRREKETTQG
ELLELDKWASLWNWFNITNWLWYIKLFIMIVGGLVGLR
IVFAV

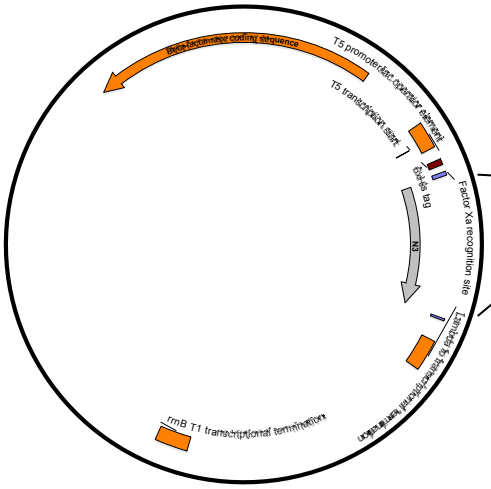
pQE30Xa-N2:



StuI-**N2**-*BamHI*

MRGSHHHHHHGSGSGSGIEGR**EPISLTLAVMLGLGVAA GSTMGAAASMTLTVQARQLLSGIVQQQSNLHRIVTEDLQ ALEKSVSNLEESLTSLSEVVLQNRRGLDLLFLKEGGLC VALKEECCFYVDHSGAIRDSMNKLRERLEKRRREKETT QGELLELDKWASLWNWFNITNWLWYIKLLSALTGPLIV LLLLLTV**

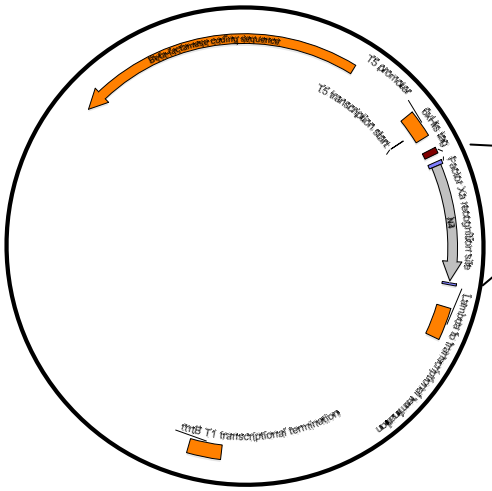
pQE30Xa-N3:



StuI-**N3**-*StuI*

MRGSHHHHHHGSGSGSGIEGR**AAGSTMGAAASMTLTVQA RQLLSGIVQQQSNLHRIVTEDLQALEKSVSNLEESLTS LSEVVLQNRRGLDLLFLKEGGLCVALKEECCFYVDHSG AIRDSMNKLRERLEKRRREKETTQGELLELDKWASLWN WFNITNWLWY**PYNGTGSACELGTPGRPAAKLN

pQE30Xa-N4:

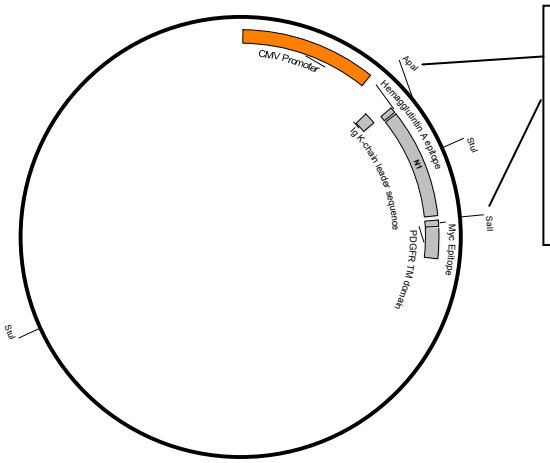


StuI-**N4**-*StuI*

MRGSHHHHHHGSGSGSGIEGR**AAGSTMGAAASMTLTVQA RQLLSGIVQQQSLSEVVLQNRRGLDLLFLKEGGLCVAL KEECCFYVDHSGAIRDSMNKLRERLEKRRREKETTQGE LLELDKWASLWNWFNITNWLWY**PYNGTGSACELGTPGR PAAKLN

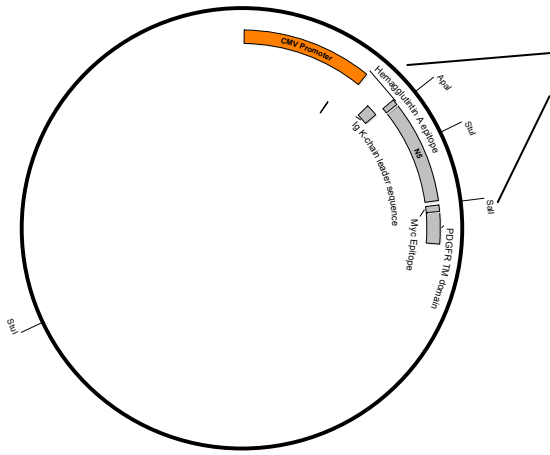
Maps of hybrid protein expression vectors for DNA immunization (pDisplay)

pDisplay-N1:



ApaI-**N1**-SalI
METDTLLLWVLLLWVPGSTGDYPYDVPDYAGAR**AVGIG**
ALFLGFLGAAGSTMGAASMTLTVQARQLLSGIVQQQSN
LHRIVTEDLQALEKSVSNLEESLTSLSEVVLQNRRGLD
LLFLKEGGLCVALKEECCFYVDHSGAIRDSMNKLRERL
EKRRREKETTQGELLELDKWASLWNWFNITNWLWYIKL
FIMIVGGLVGLRIVFAV

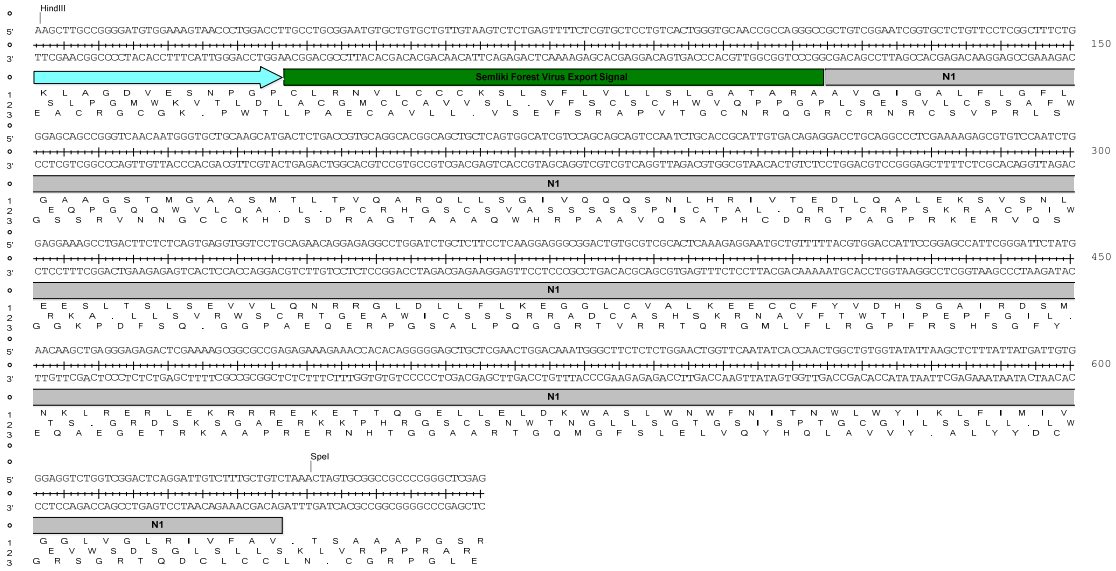
pDisplay-N5:



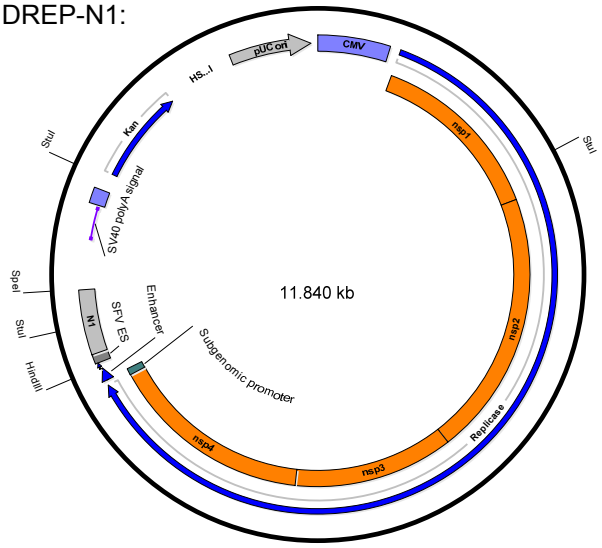
ApaI-**N5**-SalI
METDTLLLWVLLLWVPGSTGDYPYDVPDYAGAR**AAGST**
MGAASMTLTVQARQLLSGIVQQQSNLHRIVTEDLQALE
KSVSNLEESLTSLSEVVLQNRRGLDLLFLKEGGLCVAL
KEECCFYVDHSGAIRDSMNKLRERLEKRRREKETTQGE
LLELDKWASLWNWFNITNWLWYIKLFIMIVGGLVGLRI
VFAV

Maps of hybrid protein expression vectors for DNA immunization (DREP)

Insert N1 ordered from Genescript for cloning into the DNA replicon vector (DREP):



DREP-N1:

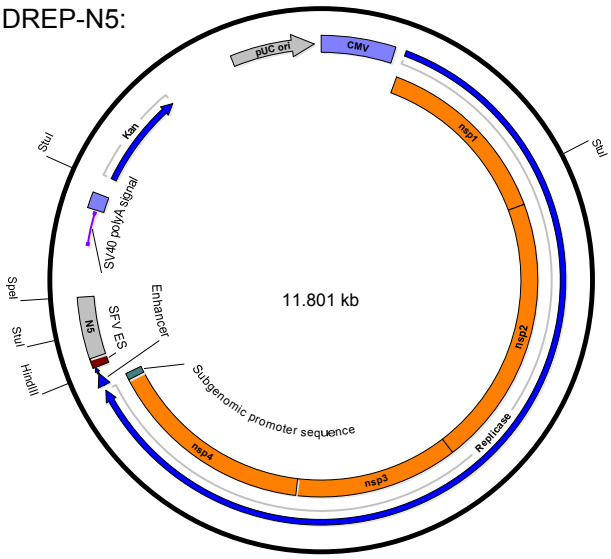


Export signal sequence **Hybrid protein sequence**

HindIII–**N1**–*SpeI*

CLRNVLCCCKSLSFLVLLSLGATARA**AVGIGALFLGFL**
GAAGSTMGAASMTLTVQARQLLSGIVQQQSNLHRIVTE
DLQALEKSVSNLEESLTSLSEVVLQNRRGLDLLFLKEG
GLCVALKEECCFYVDHSGAIRDSMNKLRERLEKRRREK
ETTQGELLELDKWASLWNWFNITNWLWYIKLFIMIVGG
LVGLRIVFAV

DREP-N5:

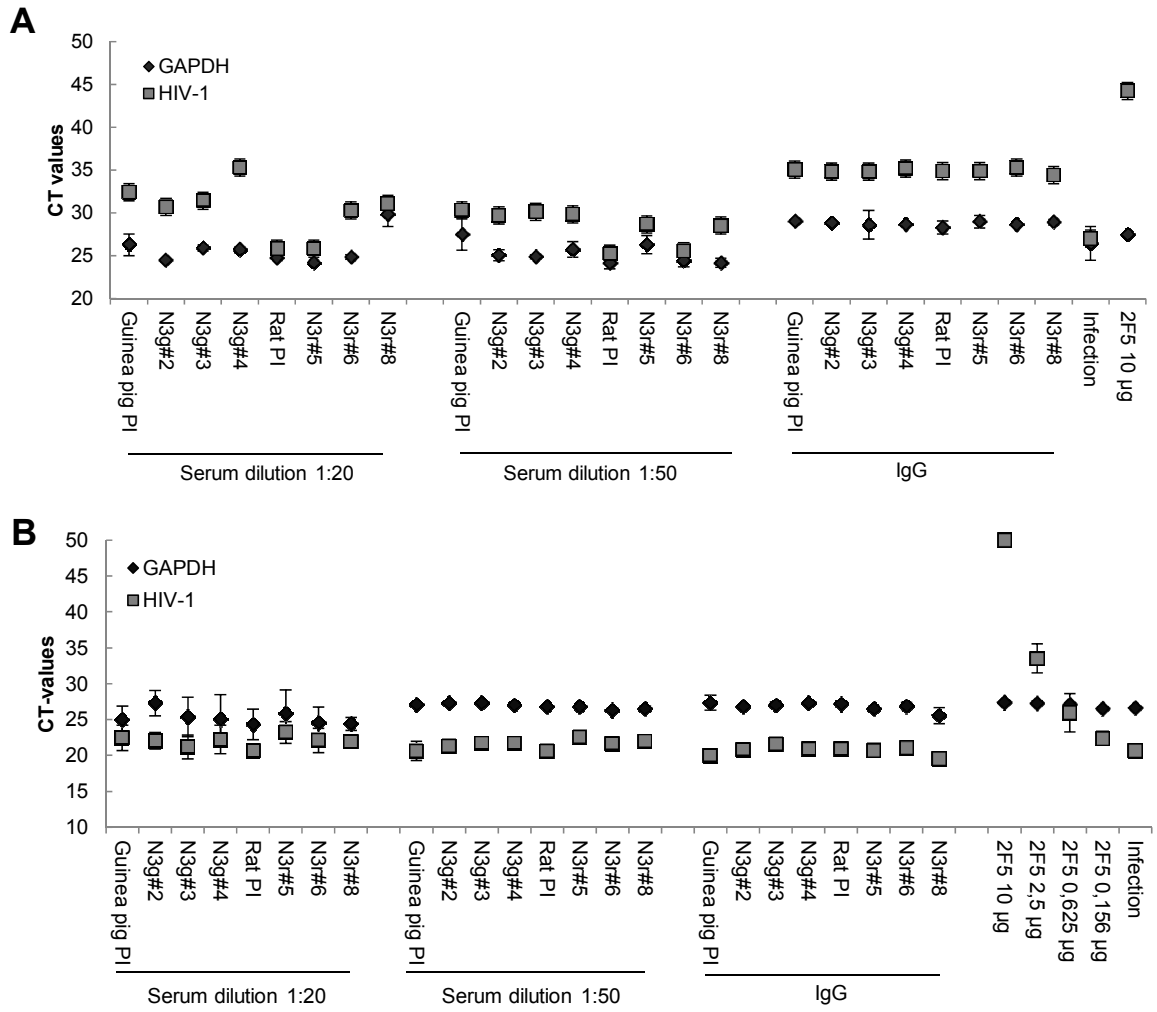


Export signal sequence **Hybrid protein sequence**

HindIII–**N5**–*SpeI*

CLRNVLCCCKSLSFLVLLSLGATARA**AAGSTMGAASMT**
LTVQARQLLSGIVQQQSNLHRIVTEDLQALEKSVSNLE
ESLTSLSEVVLQNRRGLDLLFLKEGGLCVALKEECCFY
VDHSGAIRDSMNKLRERLEKRRREKETTQGELLELDKW
ASLWNWFNITNWLWYIKLFIMIVGGLVGLRIVFAV

Real-time PCR-based neutralization assays with sera from animals immunized with N3



A.1: Neutralization assays based on PHA stimulated PBMC (A) and C8166 cells (B,C) with sera from animals immunised with N3 (A,B) or N4 in different dilutions (1:20 and 1:50) (C). Corresponding preimmune sera (PI) or animals immunized with PBS only (CTRL) were used as negative control sera. Isolated and concentrated IgG from sera of animals immunised with N3 were diluted 1:20. C8166 cells or PBMC infected with HIV-1_{NL4-3} were taken as positive control (infection).

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