

HIV-1 vaccine epitope delivery based on foamy viral hybrid proteins and vectors

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADCVI	Antibody-dependent cellular virus inhibition
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
bnAb	Broadly neutralising antibodies
CA	Capsid protein
CD spectroscopy	Circular dichroism spectroscopy
CMV	Cytomegalovirus
CMV-IE	Cytomegalovirus immediate-early
DTT	Dithiothreitol
Env	Envelope protein
FACS	Fluorescence activated cell sorting
FFV	Feline foamy virus
FeLV	Feline leukemia virus
FP	Fusion peptide
FPPR	Fusion peptide proximal region
IN	Integrase
KoRV	Koala retrovirus
MA	Matrix protein
MSD	Membrane spanning domain
MPER	Membrane proximal external region
nAb	Neutralising antibodies
NC	Nucleocapsid
NHP	Nonhuman Primate
OPD	o-Phenylenediamine dihydrochloride
PFV	Primate foamy virus
PVP	Polyvinylpyrrolidone
PERV	Porcine endogenous retrovirus
qPCR	Quantitative real-time PCR
RT	Reverse transcriptase
SPR	Surface plasmon resonance
SVP	Subviral particle
SU	Surface protein
TCEP	Tris-2-carboxyethyl-phosphine
TM	Transmembrane envelope protein
VLP	Virus like particle

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1. Introduction

Brought into humans through zoonotic transmission (1, 2) HIV-1 and -2, the causative agent of the acquired immunodeficiency syndrome (AIDS) have quickly generated one of the most serious global epidemics. Easily transmitted through sexual contact, by blood/blood products or vertically from mother-to-child, more than 34 million deaths worldwide since its discovery in the early eighties (3-5) and 2.5 million new infections per year can be attributed to HIV-1. Thereby, HIV-1 is not only an issue of the developing countries in which prevalence reaches up to 25% (Botswana, Sub-Saharan Africa, WHO Report, 2012) but also for the industrialised nations where similarly millions are infected (Fig. 1). The ultimate consequences of HIV-1 infection such as reduced life expectancy, vulnerability to opportunistic infections and the development of cancers represent an immense burden to the patient and do impact the performance of health systems and working capacity in severely affected areas. The fight against HIV and its further spread has thus become a major goal of worldwide efforts. Since attempts to develop a preventive vaccine failed till now, research has refocused in the last years on unravelling HIV's biology and studying natural infection to decipher new therapeutic intervention strategies and to improve existing antiretroviral therapy (ART). Remarkable progress has been made in these areas as



Fig. 1 Geographical distribution of HIV-1 infections worldwide. The numbers of adults and children infected with HIV-1 in 2011 are given in dark blue and represent an average of reported HIV-1 infection from different sources (numbers in brackets). Figure adopted from the presentation Global epidemic and health care response 2011 (<http://www.who.int/hiv/data/en/>).

well as in establishing infrastructures for collaboration of scientists around the world to accelerate the transfer of scientific advancements from bench to bedside. Driven forward and coordinated by organisations like WHO, UNAIDS and IAVI and accompanied by a profound financial support (\$2.64 milliards in 2011, Treatment Action Group Report, 2013) the joint efforts and particularly the extensive use of ART have reduced the level of worldwide infections by 20% when compared to 2001 and have minimised AIDS-related deaths from 2.5 million people in 2005 to 1.7 million in 2012 (UNAIDS Global AIDS Report, 2012). However, problems associated with further scale-up of ART such as drug supply, costs, the development of resistance, side effects and the fact that ART cannot cure or prevent HIV infection demonstrate that it is not the magic bullet in the fight against the infection and associated diseases. In contrast, a safe and efficient vaccine able to eradicate HIV-1 would be a major step forward and is therefore urgently needed. The following sections shortly review the current knowledge on HIV biology and vaccine development and provide background information on the strategy to be investigated in this thesis.

1.1 Molecular organisation of HIV-1 and virus replication

In the family of the *Retroviridae*, HIV-1 together with HIV-2, the simian, bovine and the feline immunodeficiency viruses belong to the genus of the lentiviruses which are all characterised by persistent infection with a slow progressing course of disease. Driven by the flanking long terminal repeats (LTRs), the HIV proviral DNA of about 9.5 kb encodes the typical Gag, Pol and Env proteins essential for virion assembly and replication (Fig 2). Furthermore, the transcription of additional auxiliary proteins including Vif, Vpr, Vpu, Ref, Tat and Nef, which are involved in regulatory functions and immune evasion (6, 7) renders HIV a complex retrovirus (Fig. 2). The Gag protein-derived cleavage products, namely Matrix (MA), Capsid (CA) and Nucleocapsid (NC) build up the virion in which two copies of unspliced, positive ssRNA genomes were packaged into the centre of a spherical CA core (Fig 2). This core domain also contains the cleavage products of Pol encompassing the Protease (PR), Integrase (IN) and Reverse transcriptase (RT) enzymes essential for virus maturation and the early steps in virus infection (8). Wrapped onto a second layer of MA proteins, a cell-derived lipid membrane

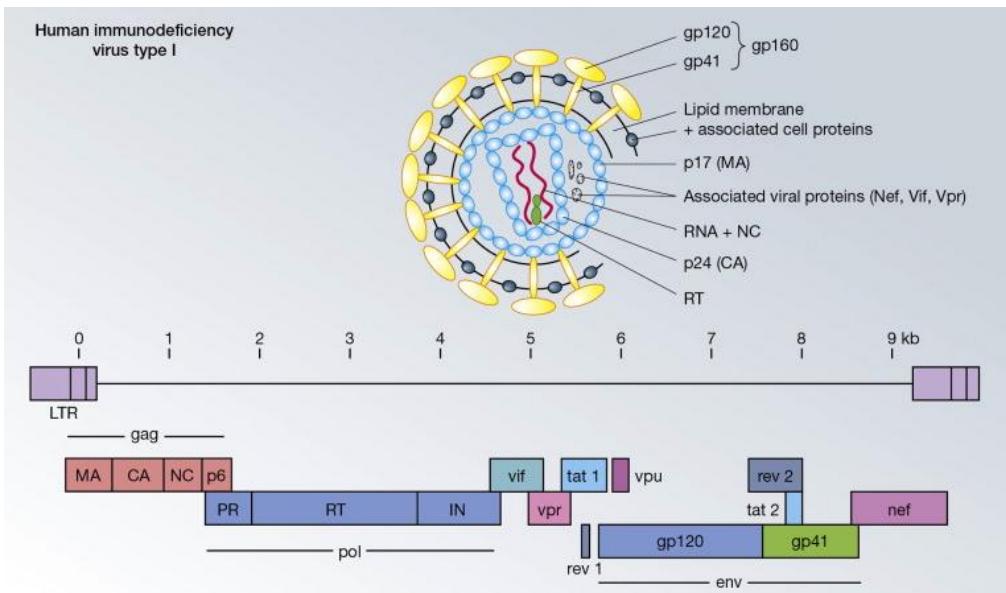


Fig. 2 HIV-1 virus structure and molecular organisation. Upper panel: the individual components of an HIV-1 particle, accessory proteins and enzymes are indicated and labelled on the right. Gp160, gp120 and gp41 correspond to the full-length Env, SU and TM proteins as mentioned in the text. Note that usually only about 15-20 viral spikes can be found on the virion surface. Lower panel: the localisation of HIV-1 encoded proteins within the HIV-1 genome. Figure taken from (9).

harbours around 15 viral spikes consisting of trimers of Env, the 160 kDa glycoprotein which is eventually cleaved into the gp120 surface protein (SU) and the gp41 transmembrane envelope (TM) protein. These components assemble at the plasma membrane and bud as immature, non-infectious particles with 80-120 nm in diameter and finally gain infectivity after Gag precursor processing through the viral PR. For infection, mature HIV-1 virions first encounter cells by non-specific attachment to charged groups on the cell surface such as heparin sulphate proteoglycans (lectins, e.g. DC-SIGN). Although virus entry can be mediated through endocytosis (10), the main route of infection uses sequential binding of the Env SU subunit to the CD4 receptor and CCR-5 or CXCR-4 chemokine co-receptors which are presented on T-helper lymphocytes, monocyte-macrophages, follicular dendritic cells, Langerhans cells in the skin and microglia in the nervous system (11, 12). Additional target cells (NK cells, epithelial cells) might be entered by using alternative co-receptors or the galactosyl ceramide receptor binding domain located in gp41 (13, 14). The binding of SU to the cellular receptors exposes the TM protein which in turn is able to insert its N-terminal, hydrophobic fusion peptide (FP) into the target cell membrane to adopt a pre-hairpin conformation. Subsequently, conformational changes leading to the assembly of the N- and C-terminal helical regions (NHR, CHR) to a coiled-coil (six-helix bundle) brings the viral and the cellular membrane into close proximity and induces lipid mixing and fusion pore formation (Fig. 3). The uncoating process allows the release of the HIV-1 RNA and enzymes

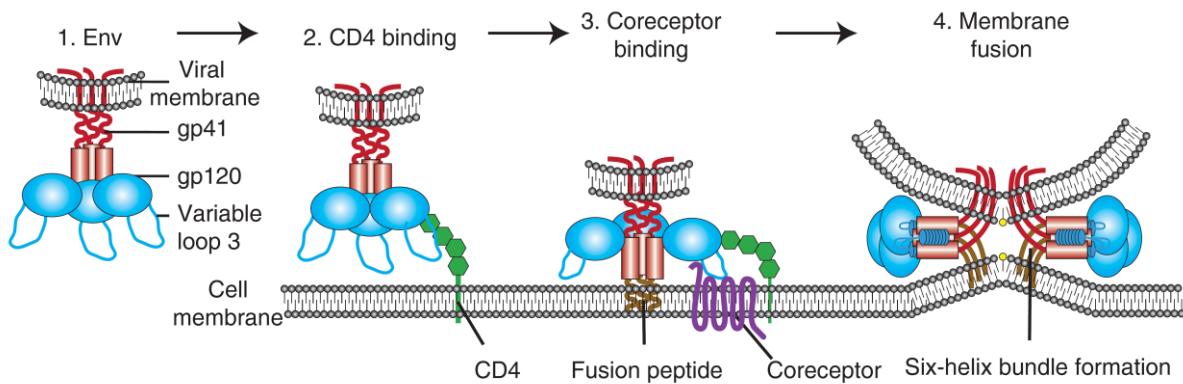


Fig. 3 Model of the Env mediated fusion reaction. (I) Before attachment, the membrane anchored native envelope trimer comprising the SU subunit (blue) and the TM protein with its condensed N- and C-terminal helical regions (NHR, CHR in light and dark red, respectively) is presented on the virion surface. Attachment of the SU protein to CD4 (II) and co-receptor engagement (III) triggers conformational changes in SU that allows the hydrophobic fusion peptide (yellow helices) to insert into the cellular target membrane. Association of NHR and CHR domains to a six-helix bundle eventually leads to approximation of both lipid membranes and cell fusion. Figure from (15).

into the cytoplasm of the infected cell, where a DNA copy of the virus genome is produced by the RT. The linear DNA associated with additional proteins forms the pre-integration complex which is then actively transported into the nucleus for integration into the cellular genome with the help of the IN. Upon transcription of the proviral DNA, another cycle of replication begins (Fig. 4).

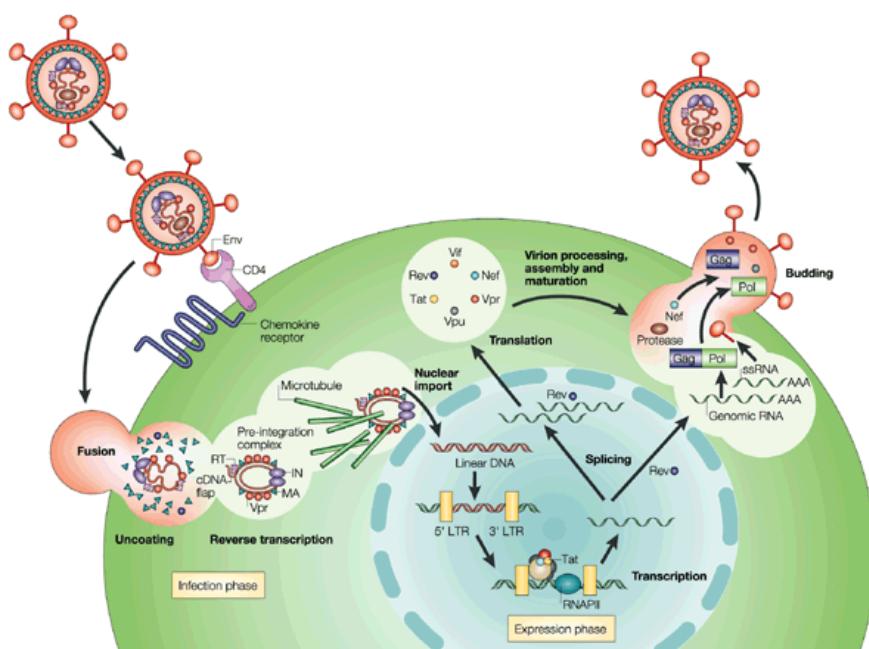


Fig. 4 The replication cycle of HIV-1. After attachment of virus particles (red, left) to CD4 and co-receptors, virus and cellular membranes fuse and the virion content is released into the cytoplasm where cDNA synthesis is accomplished by the Reverse transcriptase (RT). Associated with Integrase (IN), Matrix protein (MA) and the Viral protein R (VPR) the pre-integration complex translocates through the microtubule network into the nucleus where viral DNA is integrated into the genome by IN. Expression of viral genes is achieved through binding of the HIV-1 transactivator (Tat) and transcription by cellular polymerase (RNAPII). Viral structural and accessory proteins along with two copies of unspliced single stranded RNA (ssRNA) then assemble at the plasma membrane to bud as new particle. Figure taken from (16).

1.2 Natural HIV-1 infection and immune responses

After transmission of the virus into a new host, initial infection in the genital mucosa or the gut associated lymphoid tissues (GALT) is established by a small number or even a single founder virus which is best adopted to the new environment (17, 18). The presence of infected cells, predominantly intraepithelial $\alpha 4\beta 7+/CCR5+/CD4+$ resting T-cells (19, 20) together with mucosal chemokines attract plasmacytoid DCs to the site of infection which trigger the influx of additional permissive CD4+ T cells by expressing an array of inflammatory cytokines (21). Within few days, the complex interplay of innate and adaptive immunity along with the error-prone reverse transcription of HIV genomes lead to the rapid formation of viral quasi-species in the eclipse phase, which eventually allows the dissemination of virus throughout the GALT and into the draining lymph nodes (Fig 5). The following acute phase is characterised by an explosive virus expansion, evident by the presence of viral RNA in the blood with levels that can reach 10^6 - 10^7 copies/ml, which allows viral reservoirs to be established. The initial infection is confined by the onset of a more generalised immune response comprising innate and early adaptive immune responses (see below). As an result, a balance between virus turnover and immune

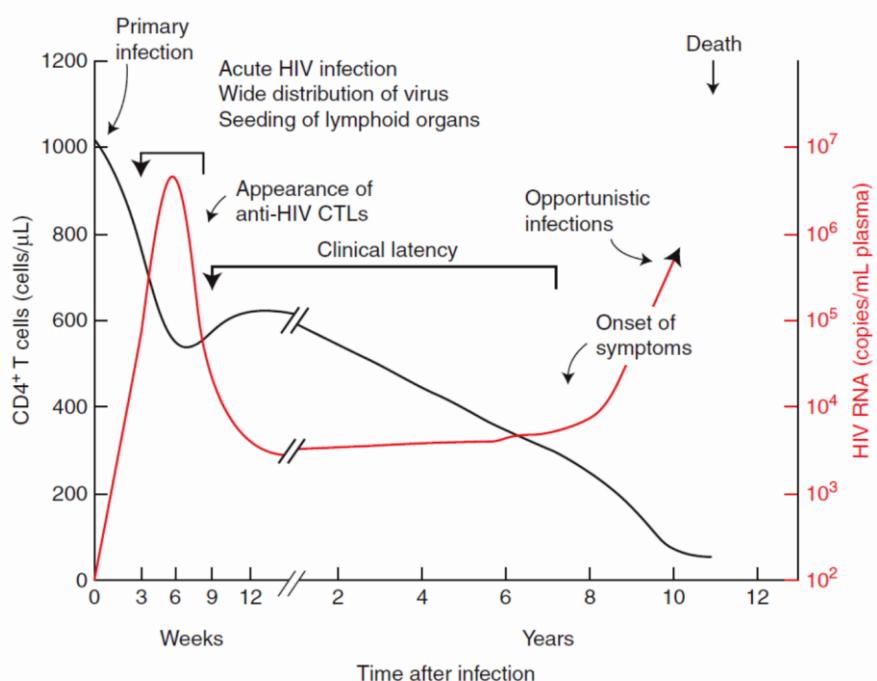


Fig. 5 CD4 T-cell numbers and viral load during different stages of HIV infection. After primary infection a decline of CD4 T cells and drastic increase of plasma viral loads characterise the acute phase (3-7 weeks after infection) in which viral reservoirs are established. Virus spread is condemned by innate immunity and first CTL responses that stabilise at the viral set-point in which clinical latency establishes. Within years, when CD4 cells decline below an individual threshold (~400 cells/µl) the body is incapable to further control virus replication and a rebound of plasma viral RNA becomes obvious. At this stage, the body is highly susceptible to opportunistic infections and other diseases which eventually leads to the death of the patient. Figure taken from Reference (22).

responses leads to a preliminary stabilisation of virus spread and plasma viral loads at the viral set-point and introduces a chronic phase with clinical latency. This asymptomatic phase can last from months to decades, dependent on the genetic background of the individual (23) and viral loads at set-point (24). Throughout this period, a continuous loss of CD4+ T cells is a hallmark of progressive HIV infection (Fig. 5). This has been proposed to be caused by various means (reviewed in (25)), including direct killing of infected cells by HIV-specific CD8+ T cells or antibodies, apoptosis of bystander cells through HIV-1 accessory proteins and abortive infection (26), chronic activation of the immune system resulting in increased T cell turnover as well as the destruction of lymphoid tissues mandatory for restoration of T cell numbers. At the final stages, when CD4+ T cell numbers are very low, the immune system is unable to further control the virus and a rebound of antigenemia and viral loads is observed (Fig. 5). The weakened immune system gets susceptible to diseases and opportunistic infections like pneumonia, dementia, wasting and others, eventually leading to the death of the patient. Notably, this is despite an intensive effort of the immune system to clear the virus (Fig. 6).

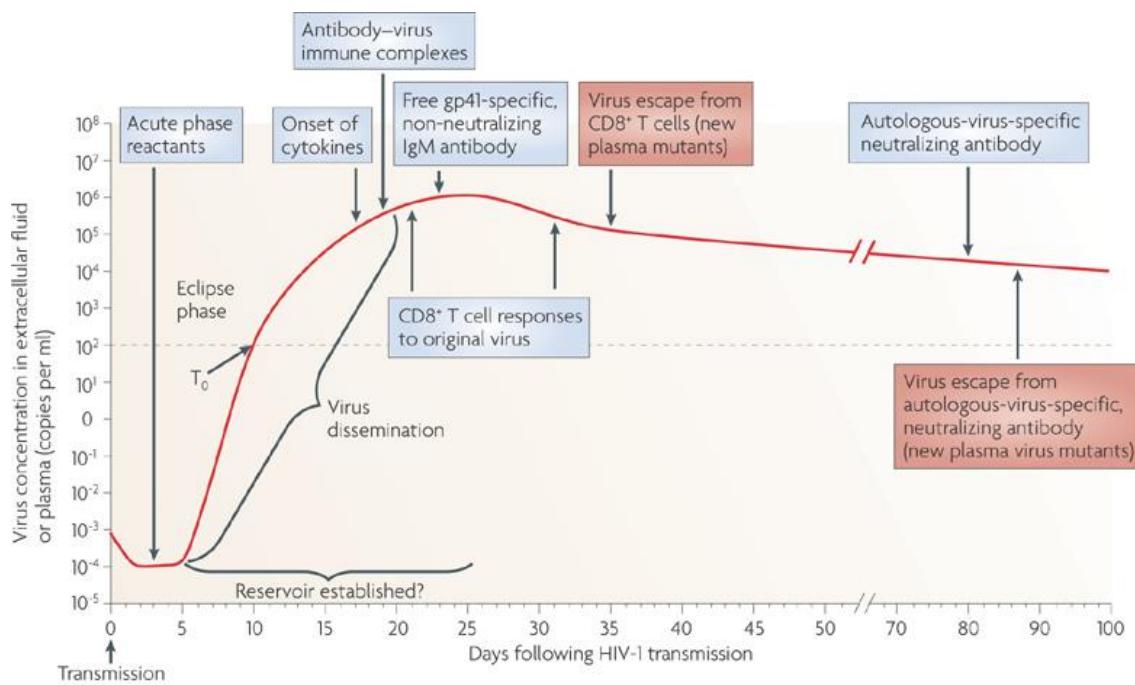


Fig. 6 Immune responses during natural HIV-1 infection. After virus transmission first responses are mediated by innate immunity including acute phase proteins like serum amyloid A and the virus inhibitory peptide VIRIP. Virus dissemination is paralleled by pro-inflammatory and antiviral cytokines within the first three weeks of infection. Subsequently, seroconversion becomes evident by antibodies against structural proteins including Gag and Env. With onset of cellular immunity, first CD8+ cytotoxic T-cells viral loads stabilise at the set-point before viral quasi species arise. First neutralising antibody responses to the founder virus can be detected ~80 days after infection, antibodies with a broadly neutralising phenotype arise later, usually around 2-4 years after initial infection (not shown). Figure from Reference (27).

First responses are mediated by innate immunity comprising NK and dendritic cells, cellular restriction factors like APOBECs, Trim5 α , SAMHD1 and antiviral cytokines such as type I interferons, IL-15 and IL-18 (27). Although these mechanisms are able to constrain viral replication to a certain degree, they lead to an influx of susceptible CD4+ T cells to the foci of infection which serve as new target cells for virus spread. Control of peak viraemia sets in with the raise of CD8+ T cell responses which are directed against Env and Nef in early phases (8-10 days after infection) and to more conserved epitopes in Gag and Pol after 1-2 weeks (28). Humoral responses to HIV directed to the Env protein are limited to low-affinity, non-neutralising antibodies of the IgM and IgG subtype during acute infection (29). Whereas such antibodies were found to be involved in immune complex formation and to support antibody-dependent cellular cytotoxicity (ADCC), they are ineffective in supressing viral loads (29-31). The first neutralising antibodies (nAb) develop after ~80 days of infection (Fig. 6). However, their efficacy is mainly restricted to highly immunogenic, non-conserved regions of autologous/founder virus, from which HIV can readily escape without remarkable loss of viral fitness. Antibodies with a broadly neutralising phenotype (bnAb) develop in about 20 to 30% of infected individuals and are in some cases able to neutralise up to 95% of all circulating HIV strains (32, 33). Their induction has been found to correlate with high viral loads, low CD4+ T cell counts and with the time (2-4 years) the individual is infected (34-36). Taken together, these studies on natural infections demonstrate that the body is principally capable to control virus replication through effective CTL and antibody responses. Triggering such immune responses in healthy individuals should therefore provide a way to control or prevent subsequent HIV infection. The next section briefly summarises attempts to translate these findings for HIV vaccine development.

1.3 HIV vaccine development

Since the first appearance of AIDS in the eighties, the development of a safe and effective HIV-1 vaccine was considered to be essential to avoid further spread of the disease. However, the initial enthusiasm was quickly dampened by the finding that traditional vaccine approaches like live attenuated viruses although providing protection (37, 38), are impractical due to reasonable safety concerns (39, 40). Similarly, attempts to use inactivated virus were disillusioned by the fact that they are modestly

immunogenic, provide protection only against closely related viruses and inactivation procedures may represent a risk-factor or denature antigens in a way that makes them incapable to mount efficient immune responses (41, 42). Since then, further studies have been conducted on various forms of immunogens such as live or non-replicating recombinant vectors expressing HIV antigens, DNA vaccines, subunit vaccines including virus like particles, recombinant Env and gp41 proteins, epitope scaffolds, HIV synthetic peptides, artificial mosaic antigens and combinations thereof (reviewed in (9)). By inducing various degrees of immune responses and protection, several of them have proceeded to Phase I and II clinical trials and four have been tested in large cohorts of healthy volunteers (43). With exception of the most recent RV144 trial in Thailand (16.400 participants, 31% vaccine efficiency (44)), none of the strategies have proven to be able to induce protective immunity, some even enhanced infection rates (45-48). Historically, these vaccine approaches were designed to induce either bnAb or to trigger cellular immunity to control virus replication and decelerate disease progression. As both approaches failed so far when applied alone, it is assumed that an effective vaccine will probably have to stimulate both arms of immunity. However, considering the fact that control of virus replication is unnecessary in presence of a profound bnAb response that prevents infection of any cells and HIV genome integration, triggering humoral immune responses could provide benefits compared to a T cell-based vaccine approach as will be highlighted below.

1.4 The envelope proteins of HIV-1 as vaccine antigen for induction of bnAb

The envelope proteins represent the major target of nAb during HIV-1 infection and several bnAb have been isolated from naturally infected individuals (Figure 7, Table 1). By binding to their epitopes, they mask domains within Env that are essential for receptor or co-receptor engagement or inhibit fusion of virions with the target cells. In addition, most of the bnAb can also block virus transmission at the virological synapse or show indirect antiviral activities by permitting killing of infected cells through mechanisms of ADCC and ADCVI and are thus also effective against intracellular HIV-1 (30, 49-52). In the SU subunit, immunogenic regions comprise the CD4 binding site and CD4 receptor binding-induced (CD4i) epitopes as well as variable loops (V2, V3, V4) with varying degrees of glycan contribution (Table 1). Within gp41, nAb are mainly directed to the conserved membrane proximal

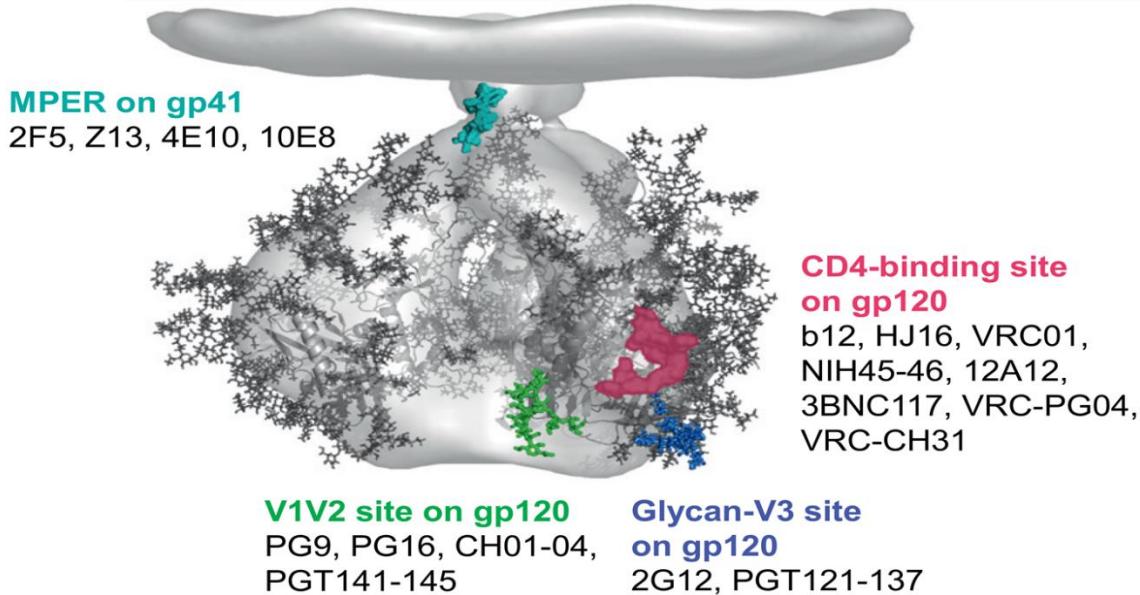


Fig. 7 The HIV-1 envelope spike and its recognition by neutralising antibodies. Names of target sites and their respective localisation within Env are indicated in colour. Antibodies binding to the corresponding regions (black) are listed below. Note that additional sites known to be target of neutralising antibodies and mentioned in the text (e.g NHR, V3 loop antibodies) are not illustrated here. Figure adopted from (53)

external region (MPER) located in close proximity to the transmembrane domain, but nAb targeting the NHR domain have also been described (54, 55). Convincing evidence that such antibodies can prevent HIV-1 acquisition and provide sterilising immunity comes from passive immunisation studies (56-60) and gene therapy approaches inducing the production of bnAb in the transgene host (61, 62). The need for high plasma antibody levels in these reports has been criticised as they would be hard to achieve by an immunisation regimen. Recent data demonstrated, however, that even low levels of bnAb are sufficient to provide protection if antibodies are selected that show excellent breadth and potency (63). The obstacles in using Env or subunits thereof as vaccine antigen lie in their inability to induce high-titre neutralising antibody responses with sufficient breadth (64-66). Most of the HIV-1 bnAb described to date show unique properties such as a high degree of somatic mutation, use of prolonged CDRH3 loops for epitope recognition, need for lipid for efficient binding and self-reactivity, triggering their depletion during B-cell selection (67). Together with their delayed development during natural infection (2-4 years) and the need for high viral loads during the acute phase, this points to a role of increased antigen exposure and affinity maturation as a requirement for the induction of these kind of antibodies. In this work, HIV-1 epitopes should therefore be delivered by a replication-competent vector system to

Table 1. Selected broadly neutralising antibodies and their epitopes within HIV-1 Env

Antibody	Env Subunit	Target	Glycan involved	Reference
2G12	gp120	Glycans	Yes	(68)
b12	gp120	CD4bs	No	(69)
VRC01	gp120	CD4bs	No	(70)
PG9/PG16	gp120	V2/V3 loop	Yes	(71)
PGT121/128	gp120	V3 loop	Yes	(72)
D5	gp41	NHR	No	(54)
HK20	gp41	NHR	No	(55)
2F5	gp41	MPER	No	(73)
4E10	gp41	MPER	No	(74)
Z13e1	gp41	MPER	No	(75)
10E8	gp41	MPER	No	(76)

allow sustained antigen delivery and immune stimulation (see next section). As HIV-1 epitope, the gp41 MPER domain was selected as it shows a higher degree of conservation and is continuous compared to the variable and more complex, discontinuous and often glycan-dependent epitopes within gp120 (77). Notably, bnAb targeting this domain such as 2F5, 10E8 and 4E10 are characterised by a high degree of somatic mutation and prolonged CDRH3 loops and thus might require extended periods of antibody maturation (67, 77). Despite its lower complexity, the MPER is yet a challenging vaccine target and most attempts to induce MPER-specific bnAb also failed (reviewed in (78)). The structural flexibility of these residues which is mandatory to promote fusion and the lack of knowledge which of these MPER conformations induces the desired antibodies is the major problem for MPER antigen design approaches. Furthermore, although there is evidence for the need of lipids to enhance antibody binding, its requirement for the induction of nAb is largely unknown. Under physiological conditions, the MPER adopts an α -helical structure (79-81) which is altered upon antibody recognition (82-85). In case of 2F5, binding induces an extended β -turn structure in the $^{664}\text{DKW}^{666}$ core residues (82, 84, 85) whereas 4E10 binding stabilises the α -helical character of its epitope (86). In line with this, constraining the MPER into β -turn or α -helical conformations through chemical modifications or suitable epitope scaffolds enhances 2F5 and 4E10 binding, respectively (5, 87-91). Recent investigations supported an interaction of the MPER with the N-terminal fusion peptide proximal region (FPPR, Fig. 8) of gp41 and also showed that peptides corresponding to this domain increased binding of 2F5 to its epitope (92-96). This was attributed to a FPPR-induced orientation of MPER residues coinciding with an increased level of β -turn/ β -sheet conformation in ELDKWA residues and an augmented α -helical content in proximal C-

terminal residues (84, 92, 95). As this structure resembles the conformation reported for the MPER in an antibody bound form, it might represent the initial B-cell trigger for induction of 2F5-like antibodies. As a consequence, some antigens used in this study also included the FPPR domain in order to test if its reported constraining properties can translate into an improved antibody response during immunisation.

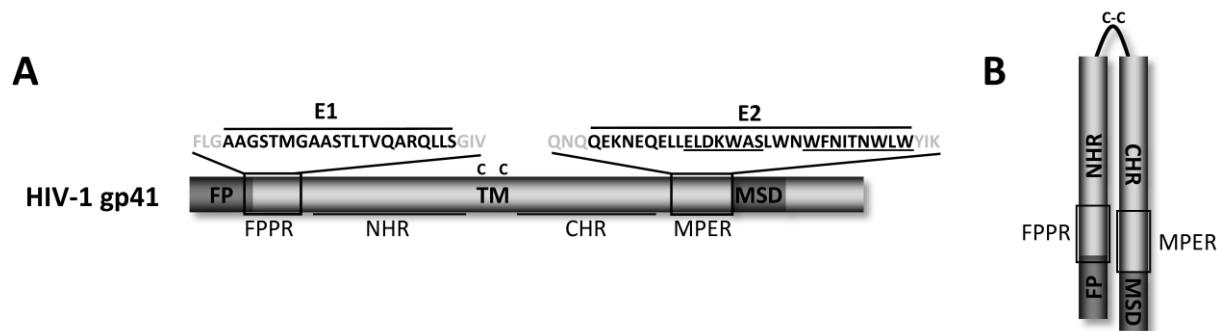


Fig. 8 Schematic presentation of gp41 of HIV-1. (A) Characteristic subdomains are indicated. FP – fusion peptide, FPPR – fusion peptide proximal region, E1- FPPR derived domain, NHR/CHR – N-terminal and C-terminal helical regions, MPER, membrane proximal external region, E2 –MPER derived domain, MSD – membrane spanning domain, C-C – cysteine loop, TM – transmembrane envelope protein. The epitopes of the broadly neutralising antibodies 2F5 (ELDKWAS) and 4E10 (WFNITNWLYIK) within the MPER/E2 domain are underlined **(B)** Model of gp41 in an very early or post-fusional state in which NHR and CHR domains are in close proximity. Note that only one molecule of the actually trimeric complex is shown.

1.5 Foamy viruses

In the group of retroviruses, the spuma- or foamy viruses (FV) constitute the only members of the *Spumaviriniae* sub-family which comprises FVs from various species including primates, non-human primates, cats, cattle, horses and sheep (97). Initially described in the early 50's and later isolated from a human nasopharyngal carcinoma cell line they were named according to the foamy-like appearance of infected cells in culture (98-100). Remarkably, the strong cytopathic effect of FVs observed *in vitro* is in stark contrast to their behaviour in naturally or experimentally infected hosts. Here, no clear association to any pathology or disease could be addressed so far, which is assumed to be an result of at least 30 million years of virus/host co-evolution (101-103). FV were set apart from the orthoretroviruses due to several unique characteristics in their virus biology and replication strategy (104). As all retroviruses they encode the canonical *gag*, *pol* and *env* genes as well as at least two accessory *bel* genes (between env and LTR) giving raise to the Bel-1/Tas (transactivator of spumaviruses) and Bet proteins (Fig. 9). However, differences comprise the translation of Pol from a spliced mRNA transcript instead of synthesis and cleavage of a Gag-Pol fusion protein (105, 106), incomplete cleavage of Pol proteins into individual enzymes (107) and the presence of an internal promoter located at the C-terminal part of

env (108-110). Similar to the Hepadnaviruses, reverse transcription of FV RNA occurs late during replication resulting in DNA-containing viral particles (111-113). Furthermore, FV particle egress is dependent on the presence and interaction with the FV envelope protein as Gag does not contain a membrane targeting signal (114, 115). Notably, this specific Gag/Env interaction currently prevents the use of heterologous envelopes for pseudotyping FV (116). Whereas FV basic research has been conducted for more than two decades now, attempts to exploit their potential for clinical applications has just recently advanced into practice (117-119). FV show several properties that makes them interesting vectors for gene therapy and vaccine purposes as will be outlined in the next section.

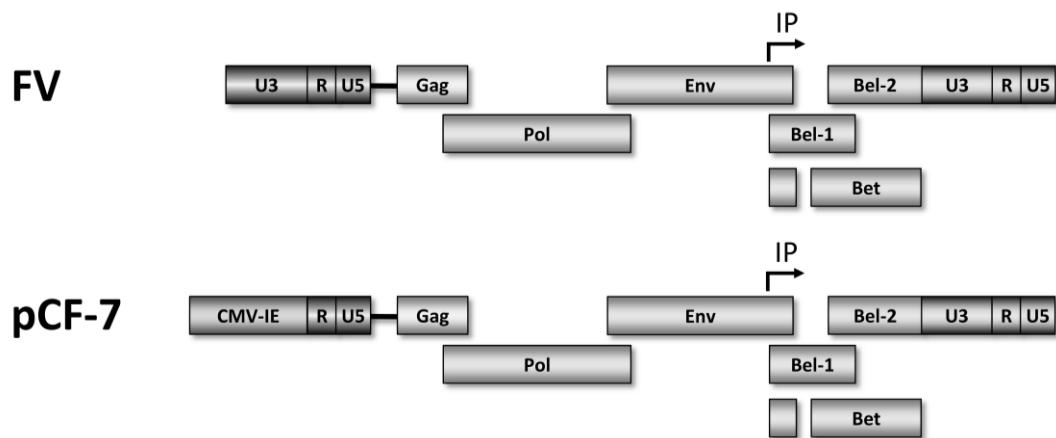


Fig. 9 Molecular organisation of foamy viruses and the infectious FFV clone pCF-7 used in this study. In the infectious FFV clone pCF-7 (bottom), the U5 region of the 5' LTR is replaced by the strong CMV-immediate early promoter (CMV-IE) to trigger high level virus production upon transfection and avoid LTR recombination events during propagation in *E. coli*.

1.6 Replicating foamy viruses as vaccine vectors

The absence of any obvious disease upon natural or experimental FV infection is probably one of the most attractive properties of these viruses for vaccine and gene therapy applications. Therewith, FV vectors show a similar or higher safety profile compared to currently used replication-competent systems (Table 2) that are usually used in highly attenuated forms on the cost of immunogenicity (adenoviruses, measles virus, Sendai virus), have the potential to induce neurotoxicity (vesicular stomatitis virus) or in case of CMV are incompatible for use in pregnant woman, young children and immunocompromised patients (120). Next to such safety concerns, a major drawback of established systems like measles and polio is their extensive use within the human population in the past as well as their high natural prevalence in man in case of herpes and adenoviruses. To avoid issues of vector directed pre-existing immunity and potential reduction of vaccine efficiency, vectors rarely found in humans or viruses of

Table 2. Selected replicating vectors currently used for HIV vaccine development. Modified from (120)

Replication-competent vector	Stage of development	Reference
DNA Viruses		
Human Adenovirus type 4	Preclinical/clinical	(121)
Human Adenovirus type 5	NHP	(122, 123)
Human Cytomegalovirus	Preclinical/clinical	L. Picker Group
Herpes Simplex Virus	NHP	(124)
Vaccinia Virus - Tiantan	Clinical Trial	(125)
Vaccinia virus – NYVAC-KC	NHP	(126, 127)
RNA Viruses (negative strand)		
Canine Distemper Virus	NHP	(128)
Influenza Virus	NHP	(129)
Measles Virus	Clinical Trial	(130-132)
Sendai Virus	Clinical Trail	(133, 134)
Vesicular Stomatitis Virus	Clinical Trail	(135-138)
RNA Viruses (positive strand)		
Poliovirus	NHP	(139, 140)
Semliki Forest Virus chimeras	NHP	(141, 142)
Yellow fever virus	NHP	(143, 144)

NHP- Non human primate studies

non-human origin provide certain benefits (145). In this work efforts were focused on the development of a feline FV (FFV) vector, as this system provides a versatile way to analyse new chimeric vectors in an authentic and fully immunocompetent small animal system for preclinical testing (146). However, for future application in humans additional advantages become noteworthy (Table 3). As mentioned above, the first FV isolate was obtained from a human patient. In fact, humans do not represent a natural host of FV and the human isolate (HFV) was later found to be zoonotically transmitted from chimpanzee and thus renamed to SFVcpz(hu) or PFV (147, 148). Further studies performed to detect FV have demonstrated a low (~2 %) prevalence of primate FV in humans which is limited to certain subgroups with direct contact to infected simians like meat hunters and animal care takers (103, 149-151). Monitoring FV infection in these individuals for more than 20 years suggests that humans represent dead-end hosts with no further spread of virus to relatives or spouses (150, 152-155). Despite its apathogenic character, FV infection is persistent and induces strong humoral and cellular immune responses in the infected host, making them ideal candidates for long-term vaccine antigen delivery and

Table 3. Properties of FV vectors beneficial for gene therapy and vaccine purposes

Property/Advantage	Reference
• Persistent infection with no associated pathogenicity	(118, 149, 156-158)
• Infection induces strong humoral and cellular immune responses	(118, 159-161)
• Preferential integration sites and non-integrating vectors available	(162-166)
• Low prevalence in humans avoids issues of pre-existing immunity	(149, 151, 154, 167)
• Genetic stability and high packaging capacity	(103, 168, 169)
• Active replication in mucosal and lymphoid tissues	(170-173)

immune stimulation. As they mainly replicate in mucosal and lymphoid tissues, they are furthermore also suitable for induction of immune responses directly at the main site of HIV entry (170-173). Still, as all retroviruses FV integrate into the genome of the host cell raising the possibility for insertional mutagenesis or proto-oncogene activation (174). By comparing different vector systems used for gene therapy, it has been found that FV show preferential integration into non-active regions of the genome which reduces the risk for such adverse side effects (162, 165, 166). In line with this, no evidence for clonal expansion of transduced cells was observed after four years of follow-up of dogs treated gene-therapeutically with a FV expressing the canine leukocyte adhesion factor CD18 (117, 175). Alternatively, non-integrating FV vectors that are maintained through episomal replication have also been developed (163). Taken together, these facts encourage the further exploration of these viruses for treating disease including their usability as HIV vaccine vectors.

1.7 The foamy viral envelope protein as HIV-1 vaccine epitope scaffold

FV Env is translated from a single-spliced mRNA encoding the gp130 precursor protein and targeted by its N-terminal signal peptide (envelope leader peptide, ELP) to the endoplasmic reticulum for cleavage by furin-like proteases into a SU (gp80) and TM (gp48) subunit (176). Upon its way through the trans-Golgi network it gets further modified by N-linked glycosylation at 14 out of 15 possible sites in case of PFV (177). Due to a specific interaction of FV ELP with Gag essential for budding and the presence of the internal promoter within Env driving expression of accessory genes, simple replacement of FV Env through heterologous Envs such as HIV gp160 is not feasible (108, 115, 178-180). As a first approach to use FV as carrier for HIV epitopes, the TM proteins of these viruses were considered

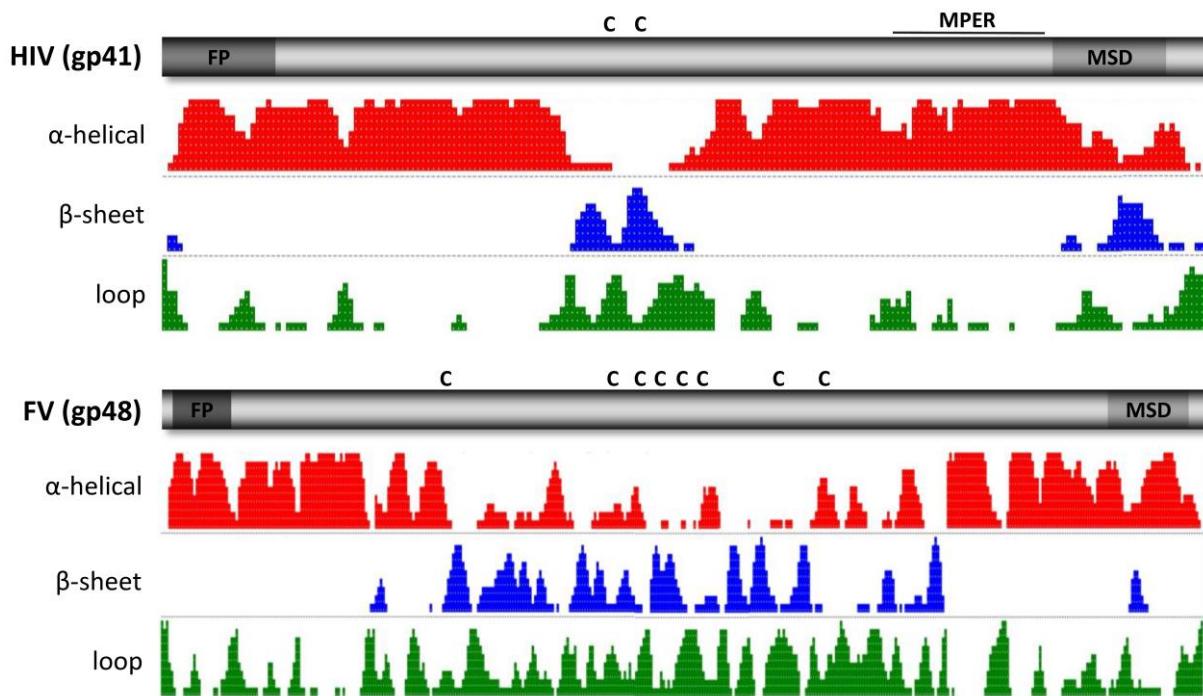


Fig. 10 Comparative secondary structure analysis of the HIV-1 and FV TM proteins. Secondary predictions of gp41 of HIV-1-HXB2 or gp48 of the FFV-FUV isolate were made using the program PROOF and the probabilities of individual residues to form α -helices (red), β -sheets (blue) and loops (green) are depicted. Both proteins show the characteristic TM sub-domains comprising the fusion peptide (FP), N- and C-terminal α -helical regions, a β -sheet and loop rich central element stabilised by cysteines (C) and the membrane spanning domain (MSD) anchoring the protein in the viral membrane. Note that the subsequent cytoplasmic tail is shortened in case of HIV-1 gp41. The membrane proximal external region (MPER) of gp41 which is targeted by several bnAb is also indicated.

useful for several reasons. First, among the retroviruses the overall structural elements encoded in the TM subunit are highly conserved as a result of their similar function in membrane fusion. This holds true for the larger FV TM proteins (Fig. 10), which show similar structural organisation with a hydrophobic fusion peptide, N- and C-terminal α -helical regions connected by an extended β -sheet, loop and cysteine-rich segment, a membrane spanning domain for anchorage in the viral membrane as well as a cytoplasmic tail (181). In previous immunisations studies using various gammaretroviral TM proteins including the porcine endogenous retrovirus (PERV), feline leukaemia virus (FeLV) and Koala retrovirus (KoRV), neutralising antibodies were easily induced and immunogenic regions identified in the FPPR and MPER of these proteins (182-186). Moreover, a partial sequence homology within the MPER epitopes of these viruses and the phylogenetically distant HIV MPER domain was evident (187). These data suggested that the MPER is a conserved target of antibodies during retroviral infection and thus, epitopes targeted by nAb might be also expected to be located in the FV TM protein. Second, as an influence of lipid components on HIV-1 MPER epitope accessibility and structure has been repeatedly reported (188-190), placing HIV epitopes into a FV membrane environment might be

essential for the induction of antibodies with similar properties including neutralisation. Third, the envelope proteins of FVs have been shown to trimerise as the parental Env proteins of HIV-1 (191). Hence, presentation of chimeric Envs in a similar trimeric context could also be anticipated. Fourth, the FV envelope protein is presented on the virion surface in stable hexameric rings at a unusual high density (180), resulting in an abundant presentation of the intended vaccine epitope. Finally, the PFV envelope proteins have been found to bud in the absence of Gag in the form of subviral particles (192-194). As HIV-1 epitopes would be presented here on the particle surface and in a lipid environment as well, such antigens constituted an interesting alternative vaccination platform in case that epitope insertion into the FV backbone is incompatible with virus replication.

1.8 The foamy viral Bet protein as carrier for HIV-1 epitopes

In a second strategy aiming to induce HIV-1 neutralising antibodies, the Bet protein of FFV was employed as carrier protein. FFV Bet is a 43 kDa, cysteine-rich accessory protein encoded downstream of *env* (Fig. 9) and arises from an splicing event fusing N-terminal residues of Bel-1 to the complete Bel-2 open reading frame (195). Bet function has been linked to resistance to superinfection (196), viral latency (197) and plays a key role in counteracting the cellular restriction factor APOBEC3G (198-200). Several reasons rationalised the use of Bet as fusion partner for HIV epitopes. The Bet protein is highly expressed in FV infected cells (159) and a strong target of antibodies during natural and experimental FV infection (161, 201, 202). Furthermore, Bet has been described to be secreted as Bet or Env-Bet fusion protein and to be taken up by neighbouring cells (203-205). Epitopes fused to Bet should therefore benefit from its immunogenicity, will be distributed to neighbouring cells and when secreted, allow the stimulation of surveying immune cells with proteasome-unprocessed conformational epitopes. Although this strategy does not support the presentation of HIV-1 epitopes on the virus surface or their placement in a lipid environment as in case of the FFV TM-based approach, the modification of Bet as an accessory protein is less invasive than changing a structural protein such as Env and thus was more likely to result in hybrid FV vectors with the ability to replicate.

1.9 Aim of this thesis and work flow

The induction of bnAb against HIV-1 by classical immunisation regimens remains an elusive goal. The recent findings that during natural HIV-1 infection such antibodies arise only after years, their induction correlates with high viral loads and the fact that most of the described bnAb show an unusual high degree of somatic mutation supported the view that prolonged antigen exposure and affinity maturation might play essential roles during their development. To investigate this, the objective of this thesis was to establish a novel vaccine epitope delivery platform based on apathogenic, replicating FV that should allow extended antigen delivery and maturation of immune responses to conserved epitopes targeted by HIV-1 bnAb. As later testing of such new chimeric viruses in a small animal system was envisaged, emphasis should be laid on the feline FV model vector to allow a pre-clinical evaluation in cats before transfer to primate FV. Two scaffolding strategies were investigated to deliver the conserved HIV gp41 MPER domain as a well characterised target of several bnAb including 2F5, 4E10 and 10E8. In the first approach comprising the FFV TM protein, the immunological properties of FFV TM were examined by studying the immune response to this antigen in naturally infected cats and TM protein immunised animals. Epitopes identified by this means were used to design FFV/HIV-1 Env hybrid antigens and the compatibility of epitope exchange with envelope function and virus replication was analysed. In the second approach, fusion proteins of Bet with the HIV-1 MPER as well as FPPR-MPER antigens were generated and characterised by biochemical and immunological means to explore the potential of the Bet protein as epitope carrier. In parallel, a set of novel HIV-1 FPPR-MPER antigens was designed with the intention to improve MPER epitope presentation and antigenicity. In a next step, compatibility of subcloning of suitable Bet/HIV-inserts to infectious FFV and the replicative potential of these vectors was tested. Figure 11 summarises the workflow schematically.

HIV Vaccine Epitope Delivery by Foamy Viruses (FV)

FV TM protein-based strategy FV Bet protein-based strategy Novel HIV-1 inserts

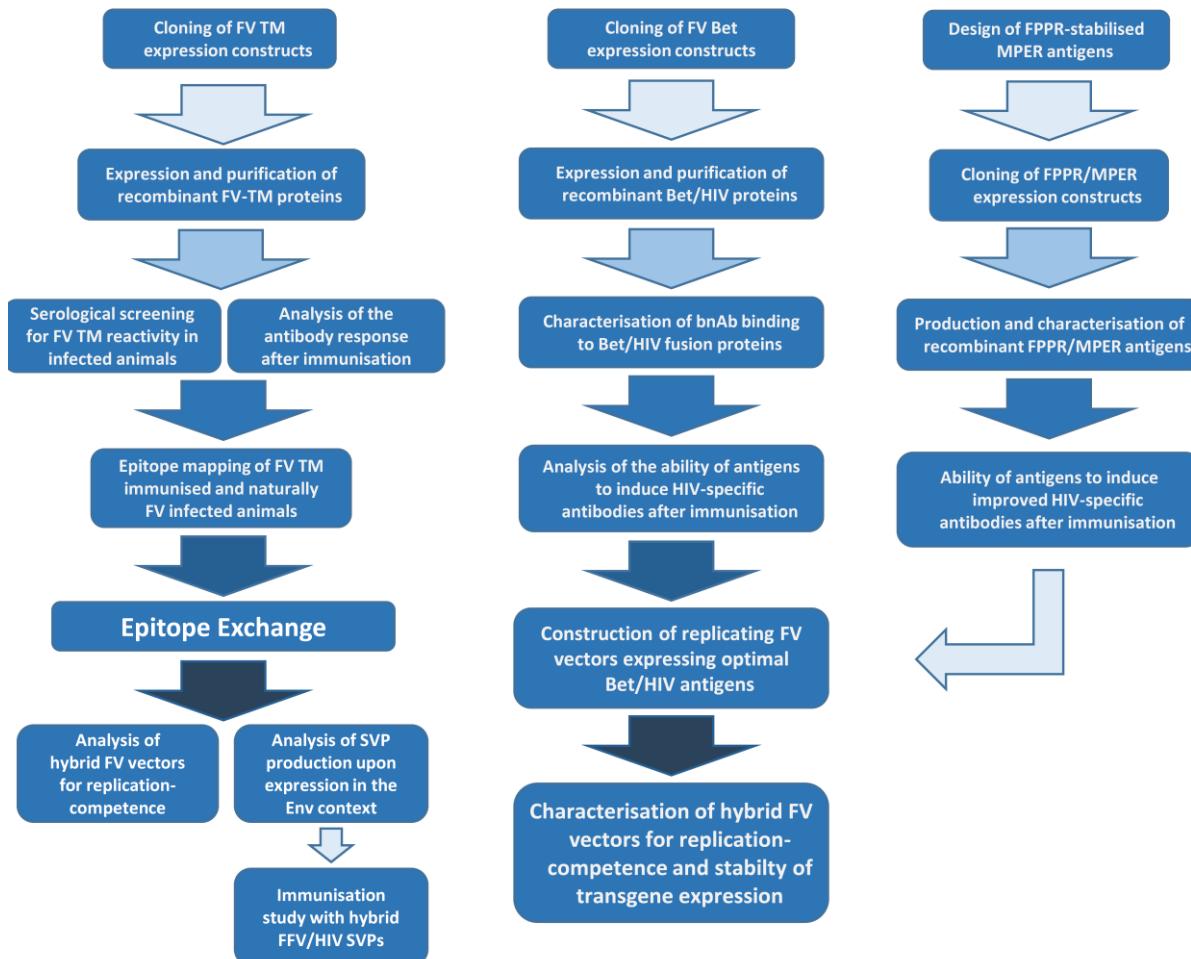


Fig. 11 Schematic overview of the strategies for HIV epitope delivery by FV vectors used in this study. The three independent projects are depicted as separate columns. The FPPR/MPER antigens were developed in parallel and results interlaced into the arm of Bet/HIV fusions.

2. Material and methods

2.1 Materials

2.1.1 Antibiotics

Name	Working concentration	Vendor
Ampicillin	100 µg/ml	Roth
Chloramphenicol	34 µg/ml	Roth
Penicillin/Streptomycin	100 U/ml, 100 µg/ml	PAA
Kanamycin	25 µg/ml	Fluka
Gentamycin/G418	10 µg/ml	Biochrom

2.1.2 Antibodies and antisera

Primary Antibodies/ Serum	Vendor/Source	Dilution
Mouse Anti-RGS-His	Qiagen, Germany	1:3000
Mouse Anti-Penta-His	Qiagen, Germany	1:3000
Goat Anti-GST	GE Healthcare, Germany	1:5000
Mouse Anti-β-Actin	Sigma-Aldrich	1:5000
2F5	Polymun Scientific, Austria	0.2-50 µg/ml
4E10	Polymun Scientific, Austria	0.2-50 µg/ml
Goat Anti-FFV-TM	Mühle <i>et al.</i> , 2011	1:2000
Rat Anti-Bet	Mühle <i>et al.</i> , 2013	1:3000
Rat Anti-HIV-E1	Jürgen Kreuzberger, RKI	1:2000
Normal Goat Serum	Sigma-Aldrich	3-10%

Secondary antibodies	Vendor/Source	Dilution
Rabbit Anti-Rat, Anti-Goat, Anti-Human, Anti-Mouse-IgG-HRP	Dako	1:3000
Goat Anti-Hamster-IgG-HRP	LifeSpan Biosciences	1:2000
Goat Anti-Cat-IgG-Fc-HRP	Bethyl Laboratories	1:3000
Goat Anti-Rat, Anti-Mouse, Anti-Cat, Anti-Hamster-IgG DyLight649	Jackson ImmunoResearch	1:500
Rabbit Anti-Goat-IgG DyLight649	Jackson ImmunoResearch	1:500
Goat Anti-Rat-IgG-FITC	SantaCruz Biotechnology	1:500

2.1.3 Buffers

Standard buffers and stock solutions

PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ , pH 7.2
PBS-T	PBS, 0.05% Tween 20
TBS	50 mM Tris.HCl, 125 mM NaCl, pH 7.5
Proteinase K solution	1 ml 10x PCR buffer, 100 µl Proteinase K, 8.9 ml ddH ₂ O

Buffers for SDS-PAGE and Western blotting

SDS gel buffer	3 M Tris, 0.3% SDS, pH 8.4 adjusted with HCl
Native gel buffer	3 M Tris, pH 8.4 adjusted with HCl
Anode buffer	200 mM Tris, pH 8.9
Cathode buffer	100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.25
Transfer buffer	50 mM Tris, 40 mM Glycine, 20% Methanol, 0.03 % SDS
4x SDS Sample Buffer	300 mM Tris, 40% Glycerine, 10% SDS, 0.04% Bromphenol blue, pH 8.2; added freshly: 500 mM β-mercaptoethanol
Ponceau S	0.1 % Ponceau S, 5 % Acetic acid
Stripping buffer	20 mM Tris.HCl, 2% SDS, 100 mM β-mercaptoethanol, pH 6.7
Coomassie Staining solution	0.25 % Brilliant Blue 250G, 10 % Glacial acetic acid, 45 % Methanol
Destaining solution	10% Acetic acid, 45 % Methanol

Buffers for preparation of chemical competent cells

TFBI	100 mM RbCl, 50 mM MnCl ₂ , 30 mM potassium acetate, 10 mM CaCl ₂ , 15% Glycerol, pH 5.8, sterile-filtered
TFBII	10 mM MOPS, 10 mM RbCl, 75 mM CaCl ₂ , 15% Glycerol, pH 6.8 with KOH, sterile-filtered

Solutions for X-Gal staining

Fixing solution	PBS, 2% PFA, 0.2% Glutaraldehyde
X-Gal staining solution	5 mM K ₃ [Fe(CN) ₆], 5 mM K ₄ [Fe(CN) ₆], 2 mM MgCl ₂ , 1 mg/ml X-Gal diluted in PBS

2.1.4 Chemicals

With exception of the chemicals listed below, all reagents mentioned in this work were obtained from the Carl Roth GmbH and of highest quality necessary for the intended use.

Name	Vendor
Adenosin triphosphate (ATP)	Thermo Scientific
Benzonase	Novagen
n-octyl-beta-D-glucopyranoside (Beta OG)	Qiagen
Big Dye	ABI
Brijj 35	Sigma-Aldrich
CHAPS	Sigma-Aldrich
DMEM	Gibco
dNTP mix, 10 mM	Thermo Scientific
ECL Solution	Thermo Scientific
EDTA	Serva
Ethidium bromide	Sigma-Aldrich
Fetal Calf Serum (FCS)	Biochrom
L-Glutamin	Invitrogen
HEPES	Biochrom
Hydrogen peroxide	Merck
Lysozyme	Sigma-Aldrich
Non-fat dry milk powder	Sucofin
Nonidet P-40 (NP-40)	Sigma-Aldrich
Polyethylenimine, linear 25 kDa (PEI)	Polysciences, Inc
Proteinase K	Qiagen
Nuclease Free Water	Promega
RPMI 1640	Gibco
o-phenylenediamine dichloride (OPD)	Sigma-Aldrich
Trypsin	Invitrogen
X-Gal	Promega

2.1.5 Commercial reagents/kits

Name	Vendor
BCA Protein Assay Kit	Thermo Scientific
Bradford protein assay dye	Bio-Rad
Blood and Tissue Genomic DNA Extraction Kit	Qiagen
Invisorb Spin Plasmid Mini Kit Two	Invitek
Invisorb Spin DNA Extraction Kit	Invitek
Plasmid Midi/Maxi-Kit, EndoFree	Qiagen
MSB Spin PCRapace Kit	Invitek

2.1.6 Instruments and equipment

Name	Vendor
Accu Jet Pipette boy	NeoLab
Avanti J-20XP Centrifuge	Beckmann-Coulter
Äkta Explorer 10s FPLC	GE Healthcare
Axiovert 200	Zeiss
Biacore X100	GE Healthcare
ChemoCam Imager	Intas
Centrifuge 5415D	Eppendorf
Centrifuge 5805R	Eppendorf
Discovery Comfort Multichannel pipettes	Abimed
ELISA-Reader	Tecan/ThermoScientific
ELISA Washer	Tecan
SkanWasher300	Skatron Instruments
EliSpot reader	AID
FACSCalibur	BD Biosciences
GloMax-Multi Microplate Reader	Promega
Guava easyCyte 5HT Flow Cytometer	Millipore
Helios GeneGun	BioRad
Mastercycler pro S	Eppendorf
MxPro3005 qPCR System	Agilent Technologies
Ovation Electronic Pipettes	VistaLab Technologies
NanoDrop ND-1000 Spectrophotometer	PeqLab
SDS-PAGE system (Standard)	Höfer/Biometra
SDS-PAGE system (HT)	CBS Scientific
Sonificator	Branson

2.1.7 Software

Name	Vendor/Reference
ChemoStar gel documentation	Intas
Cell^F Imaging Software	Olympus
LaserGene Sequence Analysis Suite	DNAStar
FlowJo	Treestar, Inc
PredictProtein	Rost <i>et al.</i> , (2004)

2.1.8 Primer and oligonucleotides

Primer Name	Sequence
FFV Bet E1 rev	5'-tataagcttagctcagcagctgacgtgc-3'
FFV Bet E2 fwd	5'-tcagggttcagctgggtcgtggcgccagggtggaggccaggaaaaaaacgaacaggaactgctggaactggat-3'
FFV Bet E2 rev	5'-tataagcttaccacagccagttgtaatatt-3'
FFV BetSeq1 fwd	5'-cgccgcgtacacttactaaaac-3'
FFV BetSeq1 rev	5'-cccaaaggggatcatgttcag-3'
NS005 fwd	5'-aaaatttatttgcatttgagcg-3'
NS006 rev	5'-ccagatggaggctcgaggta-3'
1.2 fwd	5'-caagaaaagaatgaacaagaattattgga-3'
1.2 rev	5'-ttccattgtcatccagctc-3'
1.3 rev	5'-cattgcattccagctcagc-3'
2.1 fwd	5'-gaattacaggccctggaggc-3'
2.1 rev	5'-agacaataattgtctggcctgtac-3'
3.1 fwd	5'-ttacaggccctggaggc-3'
Primer I	5'-ataaccggtacacccaagacggatcctactcg-3'
Primer II	5'-ctagtgttgtgttgtgtatggcttagcttcagagtcaatgtactcgatgt-3'
Primer III	5'-gttagccatcaccatcaccatcaactagaaagcctgaatttacctggattatgca-3'
Primer IV	5'-atatgcattgcggaaagtgttcttcttcttaggttc-3'
Primer V	5'-atagcttagcgggtcgtggcgccagggtggaggc-3'
Primer VI	5'-caggtaaattcaggctttaccacagccaatttgttatg-3'
Primer VII	5'-tggctgtgtaaaaagcctgaatttacctggat-3'
Primer VIII	5'-atagcttagcgggtcgtggcgccagggtggaggccaagaaaagaatgaacaagaatta-3'

2.1.9 Primer and probes for quantitative real-time PCR

Primer Name	Sequence
MM007*	5'-caaattgtcgctggagaat-3'
MM008*	5'-gcccaaaaaaccattggataa-3'
MM009 (Probe)	5'-[6FAM]-tgcatacgatgtacaaaatcaacactcgatgg-[BHQ1]-3'
feline GAPDH fwd	5'-caaattccacggcacagtc-3'
feline GAPDH rev	5'-tactcagcaccagcatcacc-3'
feline GAPDH (Probe)	5'-[HEX]-ggctgagaacggaaacttgtcatcaatg-3'
PFV fwd	5'-cttcaaccttgctgaatg-3'
PFV rev	5'-taatacaggctataagggt-3'
PFV (Probe)	5'-[6FAM]-ttgaaattcagtactccttatcaccc-[BHQ1]-3'

2.1.10 Plasmid vectors and molecular clones

Plasmid	Description	Source/Reference
pQE-30 Xa	Prokaryotic expression vector for expression of N-terminal His-tagged proteins under control of the T5 promoter. Cleavage of His-tag possible through Factor Xa cleavage site. Used for production of FFV Bet and Bet/HIV-1 hybrids as well as FPPR/MPER loop antigens.	Qiagen, Germany
pGEX-KG	Prokaryotic expression vector for expression of N-terminal GST-tagged proteins under control of the pTac promoter. Encodes the LacI ^q repressor and a Factor Xa cleavage site for removal of the GST fusion partner. Used for expression of FFV and PFV TM proteins.	M. Eschricht, RKI (206)
pGST-feA3Z2b	Prokaryotic expression vector encoding a GST-tagged form of the feline APOBEC-A3Z2b protein. Used to evaluate Bet protein integrity after refolding.	D. Slavkovic, DKFZ (207)
pNL4.3	Full length, replicating and infectious molecular clone of HIV-1. Used for production of HIV-1 viral supernatants.	NIH Reagent Program (208)
pCF-7	Full length, replicating and infectious molecular clone of FFV in which the 5' LTR was exchanged by a CMV promoter to increase genetic stability in bacteria. Used for production of FFV viral supernatants and subcloning of HIV-1 sequences in frame to Bet.	M. Löchelt, DKFZ (209)
pEGFP-N3	Eukaryotic expression plasmid encoding enhanced GFP under control of the CMV promotor. Used during transfections to estimate transfection efficiency.	Clonetech, USA
pHSRV13	Full length, replicating and infectious molecular clone of the PFV (SFVCpz). Used to produce supernatants of PFV.	M. Löchelt, DKFZ (210)
pBC12-Env	Eukaryotic expression vector encoding the wildtype FFV-FUV Env protein under control of the CMV promoter.	M. Löchelt, DKFZ (211)
pBC12-EnvG2	Based on pBC12-Env. Encodes the FFV-FUV Env protein silently mutated in the Env splice donor and acceptor sites to prevent splicing of the MSD domain. Contains additional restriction sites: BspE1, eight nucleotides upstream of the splice donor site; SgrAI, 18 nucleotides downstream of the splice donor. BlpI and Bst1107I sites flanking the FPPR region. Used for introduction of HIV-1 MPER and FPPR and production of wildtype subviral particles.	A. Bleiholder, DKFZ
pBC12-Bet	Eukaryotic expression vector encoding the full length FFV Bet protein under control of the CMV promoter. Used as DNA template for cloning of prokaryotic Bet expression vectors.	D. Slavkovic, DKFZ
pMAT	Synthetic plasmid synthesised by GeneArt. Encodes the longest FPPR/MPER loop construct F0-M0. Used for mutagenesis PCR to obtain truncated loop variants.	GeneArt, Germany

2.1.11 Peptides

Two synthetic peptides synthesised by GeneCust, Luxembourg were used in this study. HIV-1 E1 comprising the FPPR residues N-AAGSTMGAASMTLTVQARLLSKKKK-C and HIV-1 E2 consisting of the MPER sequence N-KKKKEQELLELDKWASLWNWFNITNWL-C. Peptides had a purity of more than 90% and were resuspended to 1 mg/ml in DMSO before further dilution as required.

2.1.12 Protein and DNA ladders

Page Ruler protein ladder, PeqLab, Germany

Page Ruler Prestained protein ladder, PeqLab, Germany

GeneRuler DNA Ladder Mix, ready to use, Thermo Fisher, Germany

GeneRuler 1 kb DNA Ladder, ready to use, Thermo Fisher, Germany

2.1.13 Media for eukaryotic cell culture

DMEM	Dulbecco's Modified Eagle Medium, supplemented with 10% [v/v] FCS, 2 mM L-Glutamine, 10 mM HEPES and 5 mM Penicillin/Streptomycin.
RPMI 1640	RPMI 1640, supplemented with 10% [v/v] FCS, 2 mM L-Glutamine, 10 mM HEPES and 5 mM Penicillin/Streptomycin.

2.1.14 Eukaryotic cell lines

HEK 293	Human adherent cell line obtained by transformation of human embryonic kidney cells with sheared Adenovirus 5 (212). Propagated in DMEM medium. ATCC Cat# CRL-1573.
HEK 293T	Highly transfectable derivative of the HEK293 cell line stably expressing the Simian Virus 40 (SV40) Large T antigen for episomal maintenance of plasmids containing the SV40 origin of replication (213). Cells were cultured in DMEM medium. ATCC Cat# CRL-11268.
C8166	A lymphotrophic T cell line derived by fusion of primary umbilical cord blood cells with HTLV-1 producing cells from an adult T cell leukaemia lymphoma patient. The cell line carries, however, does not express the HTLV-1 genome. It promotes high level replication of HIV-1 with simultaneous syncytia formation which can be used to assess neutralising capacity of antisera or antibodies. The cell line was obtained through the AIDS Research and Reference Reagent Program Cat# 404, from Dr. Robert Gallo and cultured in RPMI 1640 medium.

TZM-bl	Highly HIV-1, SIV and SHIV permissive indicator cell line derived from HeLa cells, transduced to stably express CD4 and CCR5 receptors as well as reporter genes for firefly luciferase and β-galactosidase under control of the HIV-1 LTR. Upon infection, expression of the transactivator Tat drives expression of reporter genes allowing quantification of infection (214, 215). Propagated in DMEM medium. The cells were obtained through the NIH AIDS Research and Reference Reagent Program (Cat# 8129): TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.
CRFK	Crandell-Reese feline kidney cells, derived from a primary kidney cell culture of a domestic cat (<i>Felis catus</i>). Has high susceptibility for and promotes high level replication of feline viruses including FFV (216). Used for propagation of FFV. Cultured in DMEM medium. The cell line was kindly provided by Martin Löchelt (DKFZ, Heidelberg).
FeFAB	Indicator cells based on the CRFK cell line. Express the β-galactosidase reporter gene under control of the FFV LTR promoter. Upon infection, expression of the transactivator Tas drives expression of the reporter gene allowing quantification of infection. Used for determination of virus titer and FFV neutralisation assays (217). Cultured in DMEM supplemented with 0.5 µg/ml geneticin. The cell line was kindly provided by Martin Löchelt, (DKFZ, Heidelberg)

2.1.15 Media and recipes for prokaryotic cell culture

LB	10 g tryptone, 5 g yeast extract, 170 mM NaCl, pH 7.5
2YT	16 g tryptone, 10 g yeast extract, 100 mM NaCl, pH 7.4
TB	12 g tryptone, 10 g yeast extract, 0.4% glycerol, 15 mM KH ₂ PO ₄ , pH 7.4
SOC	20 g tryptone, 5 g yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄ , 20 mM glucose, pH 7.2

2.1.16 *E. coli* bacterial strains

DH5α	Recombinase and endonuclease deficient bacterial strain for standard cloning applications. Purchased from Invitrogen Life Technologies. Genotype: F-Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-.
Top 10	Recombinase and endonuclease deficient bacterial strain for cloning purposes. Purchased from Invitrogen Life Technologies. Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (Str) endA1 nupG.
XL1-Blue	Recombinase and endonuclease deficient bacterial strain for cloning purposes. Purchased from Agilent Technologies. Genotype: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)].
ER2925	Methylation deficient bacterial strain used for cloning with methylation sensitive restriction sites. Obtained from New England Biolabs. Genotype: ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2.

BL21	Standard expression strain deficient in lon and ompT proteases. Obtained from Novagen. Genotype: F- <i>ompT hsdSB(rB-, mB-) Ion gal dcm</i> (DE3).
SCS-1/ pSE111	Parental SCS-1 strain from Agilent Technologies transformed with the pSE111 plasmid encoding the lacI ^q repressor and the <i>argU</i> gene for a rare arginine tRNA as well as Kanamycin resistance (218). This strain was kindly provided by Jürgen Kreuzberger (RKI, Berlin).
Overexpress C41, C43	Enhanced BL21 derivatives for expression of toxic and membrane proteins. Purchased from Lucigen, USA. Genotype as BL21, with unknown mutations.
Rosetta 2	Enhanced BL21 derivative encoding seven rare t-RNAs on the plasmid pRARE2 to avoid codon bias with eukaryotic genes. Purchased from Novagen. Genotype as BL21.
Shuffle T7 Express	Enhanced BL21 derivative with mutations in reduction pathways to allow disulphide formation in the cytoplasm of <i>E. coli</i> . Constitutively expresses the chaperone DsbC isomerase for disulphide reshuffling. Purchased from New England Biolabs. Genotype: MiniF lysY (CamR) / fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal λatt::pNEB3-r1-cDsbC (SpecR, lacIq) ΔtrxB sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10 --TetS) endA1 Δgor Δ(mcrC-mrr)114::IS10.

2.2 Molecular biological methods

2.2.1 Isolation of plasmid and genomic DNA

For purification of up to 50 µg of plasmid DNA the Invisorb Spin Plasmid Mini Kit Two with 2-4 ml overnight cultures of bacteria was used. Large scale preparations of 100 to 500 µg DNA was performed with Midi- and MaxiPrep columns with cells pelleted from culture volumes of 100 or 400 ml, respectively. In case of DNA used for Gene Gun immunisation experiments, utilising the EndoFree Plasmid Maxi Kit assured removal of endotoxins from the sample. In all cases, DNA was extracted from bacterial cloning strains to obtain the highest DNA quality possible. Isolation of genomic DNA from infected cells was performed using the Blood and Tissue DNA Extraction Kit following the manufacturer-provided protocol.

2.2.2 Agarose gel electrophoresis and DNA extraction

Agarose gel electrophoresis was applied for various analytical and preparative tasks. In concentrations adapted to the individual experiment gels were made by dissolving agarose in 1x TAE-Buffer and boiling until a homogenous solution developed. After cooling and addition of ethidium bromide the entire mix was poured in a horizontal gel chamber. When polymerised, DNA samples were diluted with DNA loading buffer and separated by applying a current of 120 Volts. Gels were documented using a UV transilluminator system and ChemoStar software. To extract DNA, fragments of interest were excised from the gel under UV exposure, molten in solubilisation buffer at 52°C and bound to micro-columns supplied in the Invisorb Spin DNA Extraction Kit . After wash and ethanol removal, DNA was eluted using 30-50 µl of nuclease free water.

2.2.3 Restriction digestion and DNA modifications

Restriction digestion was performed with FastDigest enzymes obtained from Thermo Scientific, Germany. DNA concentration in analytical as well as preparative digestions was adjusted to 0.05 µg/µl and 1-5 units of the required enzyme(s) per microgram DNA to assure optimal reaction conditions. Dephosphorylation of digested vector or phosphorylation of oligonucleotides and blunt-ended PCR

inserts was achieved with shrimp alkaline phosphatase or T4 polynucleotide kinase following the manufacturer provided protocols (Thermo Scientific, Germany). Similarly, 5' fill-in reactions for subsequent blunt-end cloning was carried out with Klenow-Fragment polymerase as recommended by the supplier (Thermo Scientific, Germany).

2.2.4 Preparation of chemical competent cells

Competent cells were prepared using cultures grown in LB medium without antibiotics overnight and a Qiagen protocol (Ref). Briefly, stationary bacteria were diluted 1:100 into fresh LB medium and grown to an OD₆₀₀ of 0.6. After pelleting (4000 g, 5 min, 4°C), cells were resuspended in ice-cold buffer TFB1 and incubated on ice for 90 min. TFB1 buffer was removed subsequently by centrifugation (4000 g, 5 min, 4°C) and decanting and the pellet resuspended with cold buffer TFB2. Cells were then immediately aliquoted into sterile PCR tubes, snap-frozen in liquid nitrogen and stored at -80°C until use.

2.2.5 Ligation and transformation of chemical competent bacteria

Solutions containing appropriately prepared vector and DNA inserts at a molar ratio of 1:1 to 1:3 (100-200 ng DNA in total) were supplied with 10x T4 ligase buffer and 1 µl T4 ligase in 20 µl reaction volumes and incubated at RT for 1 h or alternatively overnight at 16°C. 5 µl of the reaction mixture was taken for transformation of 50 µl chemical competent cells by incubating the DNA/bacteria mixture for 20 min on ice. After heat shock (30 s at 42°C) cells were cooled on ice for 2 min before addition of 300 µl SOC medium and further incubation at 37°C to allow for expression of antibiotic resistance promoting genes. The entire mix was then plated on LB plates containing appropriate antibiotics and grown further at 37°C until colonies appeared.

2.2.6 Preparation of glycerol stocks

Standard glycerol stocks were prepared by shortly mixing bacterial overnight cultures with sterile 50% glycerol at equal volumes followed by snap-freezing in liquid nitrogen. For glycerol stocks of expression clones, overnight bacterial cultures were first inoculated into fresh LB medium and grown until an OD₆₀₀

of 0.6 before glycerol addition and freezing to assure highest viability and an exponential growth phase. All stocks were kept at -80°C for storage.

2.2.7 Colony PCR

To screen for positive clones after ligation and transformation into bacteria, colony PCR was applied. Individual clones were picked using a pipet tip, set down on a numbered agar plate as backup and the residual bacteria resuspended in the PCR reaction mix that contained appropriate primers for insert detection. Initial denaturation was extended to ten minutes to assure disintegration of bacteria and DNA release. A representative set-up for this type of PCR is given in Table 4

Table 4. Master mix for Colony-PCR

Total	15 µl	final concentration	Cycler conditions
10 x HRS2 buffer	1.5 µl	1 x	94°C, 10 min
MgCl ₂ (25 mM)	1.2 µl	2 mM	94°C, 40 s → Denaturation
dNTP's (10 mM)	0.3 µl	0.2 mM	54°C, 30 s → Annealing
3'-Primer (10 µM)	0.3 µl	0.2 µM	72°C, 60 s → Elongation
5'-Primer (10 µM)	0.3 µl	0.2 µM	72°C, 2 min
DNA	1 colony	-	
Segenetic polymerase (2.5 U/µl)	0.2 µl	0.033 Units	4°C, ∞
ddH ₂ O	11.2 µl		

2.2.8 PCR cloning

Most of the expression plasmids described in this work were generated through amplification of target sequences from available plasmids and subsequent subcloning into a target vector. For these purposes, Platinum *Pfx* proof-reading polymerase (Invitrogen, USA) was used to prevent introduction of mutations during PCR. In most cases, a prior PCR optimisation step testing several annealing temperatures and MgSO₄ concentrations was performed to find optimal conditions for high-yield and specific amplification. A standard set-up mixture is given in Table 5.

Table 5. Master mix for proof-reading-PCR

Total	50 µl	final concentration	Cycler conditions
10 x <i>Pfx</i> buffer	5 µl	1 x	94°C, 10 min
MgSO ₄ (50 mM)	1-3 µl	1-3 mM	94°C, 40 s → Denaturation
dNTP's (10 mM)	1.5 µl	0.3 mM	54°C, 30 s → Annealing
3'-Primer (10 µM)	1.5 µl	0.3 µM	68°C, 2 min → Elongation
5'-Primer (10 µM)	1.5 µl	0.3 µM	72°C, 2 min
Template DNA	variable	10-100 ng	
<i>Pfx</i> polymerase (2.5 U/µl)	0.4 µl	0.016 Units	4°C, ∞
ddH ₂ O	ad 50 µl		

To obtain truncated version of the described HIV-1 E1-E2 loop antigens, the initial pMAT vector synthesised by GeneArt was mutated by amplification of the full-plasmid with appropriate primers. The *Pfx* PCR used here was identical to that described above. However, to allow self-linearisation of the obtained amplicons, phosphorylated primers and lower amounts of DNA during ligation were needed.

Table 6 and Table 7 summarise protocols for both methods.

Table 6. Mixture for primer phosphorylation

Primer (100 pmol/µl)	0.9 µl
10 x T4 PNK buffer A	0.9 µl
10 mM ATP	0.9 µl
T4 PNK	0.8 µl
ddH ₂ O	ad 10 µl

Table 7. Self-circularisation of linear DNA

DNA	10-20 ng
10 x T4 ligase buffer	2 µl
T4 ligase (5 U/ml)	1 µl
ddH ₂ O	ad 20 µl

2.2.9 Quantitative real-time PCR for detection of FFV and PFV proviral DNA

A sensitive qPCR assay for detection of proviral FFV DNA from infected CRFK cells was established by designing primers and a fluorescent probe amplifying a short 156 bp fragment from a conserved region in the *pol* reading frame (nt 2478-2633, NC001871). Reaction conditions were optimised to reach highest sensitivity by short amplification times. When FFV plasmid-spiked cell lysates were used as template, the detection limit of this qPCR reached down to 10 FFV copies and was linear over a several logs of plasmid concentrations (Fig. 12). The sensitivity in FFV neutralisation assays for which this qPCR was developed for (section 2.5.5) was lower (~20 copies, not shown), probably due to the presence of inhibiting substances in cell lysates. In a separate qPCR, feline GAPDH was amplified as reference

gene with an amplicon size of 120 bp using the same cycling conditions as for FFV. The qPCR for detection of PFV provirus was developed and kindly provided by Rico Blochmann (RKI, Berlin). Reaction set-up and cycling conditions for both PCRs are given in Table 8 and Table 9.

Table 8. Master mix for FFV qPCR

Total	25 µl	final concentration
10 x PCR-buffer	2.5 µl	1 x
MgCl ₂ (25 mM)	1 µl	1 mM
dNTP's (10 mM)	0.6 µl	0.25 mM
3'-Primer FFV (10 µM)	0.9 µl	0.3 µM
5'-Primer FFV (10 µM)	0.9 µl	0.3 µM
Probe FFV pol (10 µM)	0.5 µl	0.15 µM
CRFK cell lysate	3 µl	-
AmpliTaq Gold (1 U/µl)	0.25 µl	0.25 Units
ddH ₂ O	15.4 µl	

Cycler conditions

95°C, 10 min
 95°C, 30 s → Denaturation
 54°C, 40 s → Annealing
 68°C, 1 min → Elongation
 4°C, ∞

$\times 50$

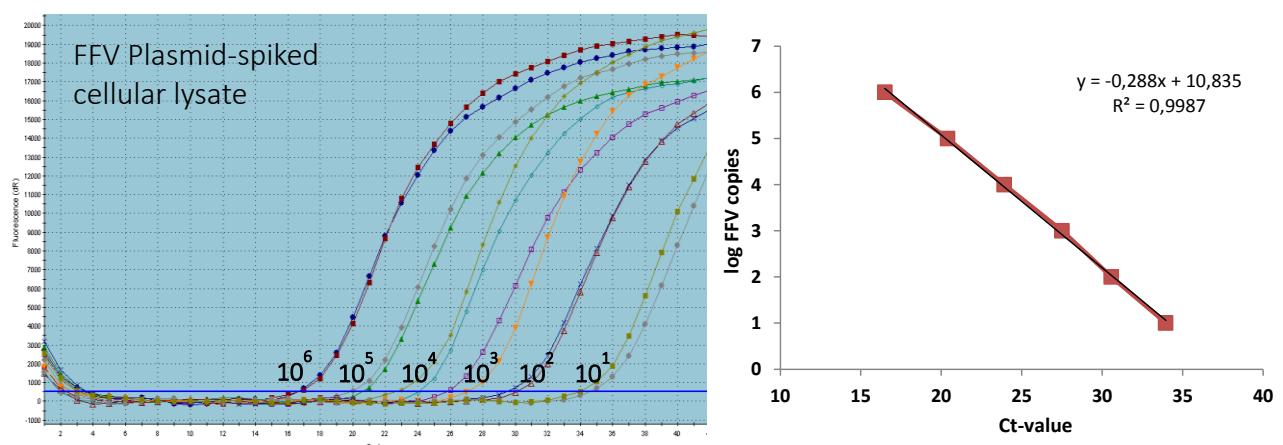


Fig. 12. Efficiency of the qPCR for detection of FFV proviral DNA. Left panel: Amplification plots of a FFV qPCR assay performed with the 3 µl of cell lysate from non-infected CRFK cell spiked with serially diluted pCF-7 plasmid DNA corresponding to the number of genome copies as indicated. Measurements were performed here in duplicates. Right panel: Plotting of FFV genome copies versus Ct values calculated from amplification plots shows linear amplification of FFV DNA over several logs. The regression function and the regression coefficient is given.

Table 9. Master mix for PFV qPCR

Total	25 µl	final concentration
10 x PCR-buffer	2.5 µl	1 x
MgCl ₂ (25 mM)	2 µl	1 mM
dNTP's (10 mM)	0.6 µl 50x	0.25 mM
3'-Primer PFV (10 µM)	0.5 µl	0.3 µM
5'-Primer PFV (10 µM)	0.5 µl	0.3 µM
Probe PFV pol (10 µM)	0.5 µl	0.15 µM
BHK cell lysate	3 µl	-
AmpliTaq Gold (1 U/µl)	0.2 µl	0.2 Units
ddH ₂ O	15.4 µl	

Cycler conditions

95°C, 8 min
 95°C, 1 min → Denaturation
 58°C, 1 min → Annealing
 72°C, 15 s → Elongation
 4°C, ∞

$\times 50$

2.3 Protein chemical methods

2.3.1 Determination of protein concentration

Protein concentration was determined by using (i) the absorbance of the solution at 280 nm together with the molar extinction coefficient or correction factors for 0.1% solutions, both calculated from the primary amino acid sequence by ProtParam (<http://web.expasy.org/protparam>) (ii) a commercial microplate BCA assay by incubating samples along with a BSA standard with BCA solution for 30 min at 37°C and read-out at 562 nm (iii) a commercial Bradford assay by mixing samples or BSA standard with Bradford solution followed by spectrometric reading at 595 nm. Selection of the protein assay was dependent on the amount of sample available and the presence of substances that might interfere with absorbance or assay sensitivity (DTT, Urea, etc).

2.3.2 SDS-PAGE and native PAGE

For separation of proteins, polyacrylamide electrophoresis was performed as described previously (219). Briefly, separation gels in concentrations depended on the size of proteins to be separated (8-12%) were prepared and poured in a vertical gel chamber and overlaid with ethanol. After polymerisation the ethanol was discarded, a stacking gel (4%) poured on top of the separation gel and a comb added for later addition of the samples. After mixing with sample buffer, proteins were boiled shortly at 90°C for 5 min before loading onto the gel. For Native PAGE, SDS in gel and running buffers was omitted, samples resuspended in native loading buffer containing no 2-mercaptoethanol (Bio-Rad, Germany) and loaded without prior boiling.

2.3.3 Coomassie blue staining

Gels from SDS-PAGE or native PAGE were stained by incubation in Coomassie blue solution for 20 min. Successive dye was recovered, the gel shortly rinsed in distilled water as a first wash and then destained in destaining solution until background disappeared and protein bands became visible.

2.3.4 Protein expression optimisation and solubility testing

To optimise protein production, expression vectors were transformed into selected bacterial strains, and grown overnight at 37°C. From each plate, two colonies were selected and grown as day cultures until an OD₆₀₀ of 0.6 before storing as glycerol stocks. From these stocks overnight cultures were grown in LB medium. On the next day, each well of a 96 deep well plate containing 1 ml of either LB, 2YT, TB or SOC medium and antibiotics was inoculated with 100 µl of the stationary overnight to reach a final OD₆₀₀ of 0.2 and grown for 3 h at 37°C. For induction, cultures were adjusted to 0.1 and 1 mM IPTG in parallel to non-induced controls. Empty control vectors were induced with the highest IPTG concentration only. After additional 3 h incubation at indicated temperatures, cells were pelleted by centrifugation for 15 min at 3700 rpm in an Eppendorf plate centrifuge, lysed by three successive freeze/thaw cycles in liquid nitrogen and subsequently resuspended in 100 µl lysis buffer (PBS, 0.1 mg/ml lysozyme, 50 U/ml benzonase). For overall expression analysis 10 µl of the total cell lysate was analysed by SDS-PAGE and Coomassie blue staining. To assess the levels of soluble expressed protein, total lysates were subjected to sonification (2 x 5 pulses with 40% DC, level 4), centrifuged at 16.000 g, 4°C for 20 minutes and supernatants analysed for the presence of the respective protein.

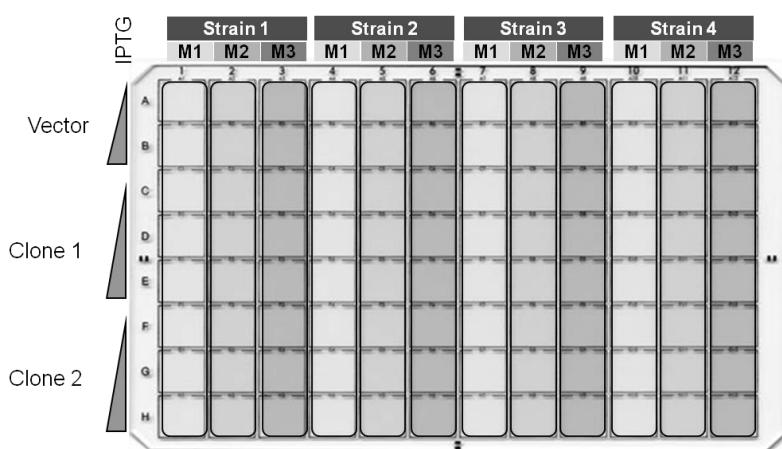


Fig 13. Strategy to optimise protein expression in 96 deep well plates. Four bacterial strains (strains 1–4), three growth media (M1, M2, M3), three IPTG concentrations (grey arrows) and two transformants (clone 1, clone 2) along with the empty vector as control were used. One plate was intended for every temperature to be investigated. The design also allowed easy transfer to SDS-PAGE by multichannel pipettes. Figure taken from Mühlé et al., 2011 (220).

2.3.5 Large scale expression in *E. coli*

After suitable parameters were found by expression optimisation, conditions were scaled-up accordingly for large-scale production (Table 10). Production was performed in 5 L flasks containing 2 L of medium and inoculated with ~100 ml overnight culture grown in LB medium to achieve a final OD₆₀₀ of 0.2.

Table 10. Expression and purification conditions used for large-scale production of indicated proteins

Construct	Expression strain	IPTG conc.	Growing temperature	Growing time	Growing media	Purification
pGEX-FFV-TMop-His	Rosetta 2	1 mM	37°C	3 h	TB	denaturing
pGEX-PFV-TM-His	Rosetta 2	1 mM	37°C	3 h	TB	denaturing
pQE-Bet	SCS-1	0.5 mM	20°C	2 h	TB	non-denaturing
pQE-Bet	SCS-1	0.5 mM	37°C	3 h	2YT	denaturing
pQE-Bet-E1, pQE-Bet-E2, pQE-Bet-E1-E2	SCS-1	0.5 mM	37°C	3 h	2YT	denaturing
pGEX-feA32Zb	BL21	0.2 mM	18°C	18 h	LB	non-denaturing
pQE-F0-M0 to pQE-F2-M2	SCS-1	1 mM	37°C	3 h	TB	denaturing

2.3.6 Detergent screening

To test if detergents could solubilise insoluble proteins or prevent their co-precipitation with *E. coli* membrane fragments, non-ionic and ionic detergents were used for a detergent screening. Detergents were added at 1x or 5x of their critical micelle concentration directly to the cell lysis buffer before sonification or to the pelleted material after cell disruption and centrifugation. Incubation times were at least 2 h after mixing to assure complete extraction. Subsequently, lysates were spun (16.000 g, 4°C) to test for the solubilised target protein in the supernatant by SDS-PAGE or Western blotting.

2.3.7 Purification of recombinant proteins

Purification of FFV and PFV TM proteins by on-column refolding or using a sarcosyl extraction and refolding protocol has been already described in great detail (220, 221). Similarly, purification of Bet and Bet/HIV-1 hybrid proteins has been described elsewhere (222). Production of FPPR/MPER loop antigens followed the sarcosyl extraction and refolding protocol established for the FV TM proteins. Purification of GST and GST-feA32Zb using glutathione beads (GE Healthcare, Germany) was performed using the manufacturer protocol with subsequent gel filtration in PBS for final purification. Typical yields were 15 and 4-5 mg/L for GST and GST- feA32Zb, respectively and purity reached ~80-90% as judged by SDS-PAGE analysis.

2.3.8 Refolding screening for FFV Bet

Denatured Bet protein from affinity chromatography at 2 mg/ml was reduced with 20 mM TCEP for 1 h at RT and complete reduction monitored by analytical gel filtration. After desalting to buffer D (8 M Urea, 50 mM Tris, 300 mM NaCl, pH 8.5) with a HiPrep 26/10 desalting column (GE Healthcare, Germany), refolding was immediately initiated by 1:20 dilution in 50 mM Tris, pH 8.5 or in the same buffer supplied with various co-solvents (0.5 M L-arginine, 20% Glycerol, 0.3% PEG 6000, 0.3 M Sucrose, 0.05% N-laurylsarcosine). Additionally, individual buffers were adjusted to different concentrations of reduced and oxidised forms of Glutathione (0/0, 3/0.3, 3/0.5, 3/1, 3/3, 1/3, 0.5/3, 0.3/3 mM GSH/GSSG). Microplates were then incubated for 48 h at 4°C and samples spun at 16.000 g for 20 min before analysing supernatants for APOBEC ligand binding. Therefore, Bet refolded under the various conditions was further diluted 1:20 in PBS and 500 ng captured on 96 well His-Select HS Ni-NTA plates (Sigma Aldrich, Germany). Plates were washed with PBS-T (PBS, 0.05% Tween 20) and then incubated for 2 h at RT with 500 ng of purified GST or GST-feA3Z2b (207) diluted in PBS-T containing 0.05% BSA. After 3 washing steps, plates were probed with an goat anti-GST antibody detected by an anti-goat-IgG-HRP conjugate, each applied for 30 min at RT and diluted in PBS-T containing 0.05% BSA. Plates were developed with a colorimetric OPD substrate (1 mg/ml in PBS with 0.2% H₂O₂) for 10 min. The reaction was stopped with 5 N H₂SO₄ and absorbance at 492 nm measured in an ELISA reader (Tecan, USA).

2.3.9 Protein desalting and buffer exchange

After purification of recombinant proteins, transfer to a final formulation buffer, in most cases PBS, was achieved by FPLC-assisted desalting on HiTrap 5 ml (GE Healthcare, Germany) or preparative HiTrap 26/10 desalting columns. Alternatively, dialysis tubes (Serva, Germany) with a MWCO suitable for the respective protein were loaded with sample and incubated overnight in a large vessel (200-500x sample volume) and the buffer changed on the next day again to ensure complete buffer exchange.

2.3.10 Gel filtration

Final purifications or analysis of the oligomeric state of proteins was performed on Superdex 75 or Superdex 200 HR 10/300 columns (GE Healthcare, Germany). As protein standards BSA (66 kDa), Ovalbumin (43 kDa) and Lysozyme (17 kDa) were used for column calibration and run under identical conditions (elution buffer, flow rate, sample volume) as the sample to be analysed.

2.3.11 CD spectroscopy

Purified proteins were measured at the Leibniz Institute for Molecular Medicine (FMP) in a Jasco 720 spectropolarimeter in 0.01 cm quartz cuvettes with 0.1 nm dot pitch and 50 nm/min scanning speed in the range of 260-190 nm. Ellipticity θ was calculated with obtained CD data in millidegrees, mean residue weights of 114.4, 111.3, 113.5 and 111.9 g/mol and protein concentrations of 0.21, 0.28, 0.20 and 0.27 mg/ml for the GST-TM and TM proteins of FFV, and the GST-TM and TM proteins of PFV, respectively. In case of the FFV Bet protein mean residue weights of 114.6 g/mol and a protein concentration of 0.3 mg/ml was used. Spectra were means of ten individual measurements performed at room temperature and subtracted by the mean spectrum obtained with buffer only. For estimation of secondary structures, CD data were analysed with SELCON3, CDSSTR and CONTINLL programs which are part of the CDpro software package (223, 224) using the SMP56 protein reference set which includes spectra of 40 soluble and 13 membrane proteins and which is optimised for interpretation of spectra in the range of 190-240 nm (225, 226). Amino acid based secondary structure predictions were obtained with the PROFsec algorithm included in the PredictProtein package (227).

2.3.12 Surface plasmon resonance

Surface plasmon resonance experiments were performed on a Biacore X100 device with anti-human IgG-Fc specific antibodies immobilised on CM5 chips (GE Healthcare, Germany). 2F5 or 4E10 in 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0 were captured at 300-500 response units (RU) on flow cell 2 and three increasing concentrations of fusion proteins diluted in 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0 were injected on both flow cells. After regeneration with 10 mM glycine, pH 2.0 and antibody recapturing, blank resonance was measured by three injections of dilution buffer. Sensograms were reference cell subtracted and blank normalised values from at least two repeated experiments. Curves were fitted using the 1:1 binding algorithm and quality of fitting was checked by assuring K_D values in the range of measured protein concentrations, plausible R_{max} values, small χ^2 values as well as residuals within recommended borders.

2.4 Immunological methods

2.4.1 Western blotting and membrane stripping

PVDF membranes (Milipore, USA) were activated in methanol and transfer buffer and placed on a filter paper, followed by the SDS gel and another layer of filter paper. The sandwich was assembled in a semi-dry blotting chamber (BioRad, Germany). After transfer for 25 min at 20 V, the sandwich was removed from the device and even transfer confirmed by staining with Ponceau S solution and destaining with distilled water. Afterwards, blocking of unspecific binding sites was achieved by incubation with 5% dry-milk dissolved in PBS-T for 1 h at room temperature or overnight at 4°C. Primary antibodies prepared in 2.5% milk in PBS-T at dilutions as indicated were incubated on membranes for 1 h at room temperature, washed (3x5 min PBS-T) and detected by suitable HRP-coupled secondary antibodies (30 min). In cases where primary antibodies from different species had to be detected simultaneously, HRP coupled to Protein A or G (Invitrogen, USA) was used for detection. After additional washing, blots were developed using ECL substrate and light sensitive films or a digital imager (Intas, Germany). For

reprobing of membranes, they were incubated in stripping buffer for 1 h at 60°C under rotation, rinsed shortly in PBS-T to remove any residual stripping buffer and blocked and probed as above.

2.4.2 ELISA

If not stated otherwise, purified proteins or synthetic peptides were coated onto ELISA plates at 200 ng/well by dilution in water or PBS. After attachment overnight at 37°C or 4°C in cases where conformational binding should be investigated, plates were blocked with 5% BSA in PBS-T for 1 h at 37°C and washed once with PBS-T using a ELISA washer. Primary antibodies were added at dilutions as indicated and allowed to bind for 1 h at 37°C. After three washing cycles, suitable HRP-labelled secondary antibodies were added for 30 min at 37°C. Unbound antibody was removed by seven successive washing steps and the plates developed with a colorimetric OPD substrate as before.

2.4.3 Epitope mapping

Two different types of epitope mapping techniques were applied during this thesis. Initially, PepSpot nitrocellulose membranes spotted with the intended peptides and later on PepStar glass chips that bind the peptides through an N-terminal spacer/linker on the surface were used. As the latter one are probed with fluorescent secondary antibodies, higher sensitivity compared to PepSpot HRP/ECL detection system could be achieved. Furthermore, peptides on the glass chips could be spotted in much higher density, which in collaboration with JPT allowed the development of microarrays that simultaneously map up to 21 sera. In case of the PepSpot membrane the FFV TM ectodomain (amino acids 563-944 Uniprot Acc. Nr O56861) were spotted as 15mer peptides overlapping by 13 amino acids. On the microarrays, the complete FFV-FUV Env protein (15mer peptides, 11 residues overlap, residues 1-982) was printed twice per sub-array. Furthermore, these chips contained the extended HIV-1 HXB2 FPPR domain comprising amino acids 512-559 and the extended MPER domain comprising amino acids 644-691 for mapping of HIV-1 gp41 directed responses. Both systems were used with the JPT-provided protocols.

2.5 Cell culture methods

2.5.1 Transfection of cells using polyethylenimine (PEI)

For transfection of cells with plasmid DNA, the indicated cell type was seeded 24-48 h in advance and supplied with fresh medium directly before treatment. In a 1/10 culture volume, plasmid DNA was dissolved in serum-free medium and sterile PEI stock solution (2 µg/µl in PBS) added to yield a DNA/PEI ratio of 1:4. After short mixing, the solution was incubated for 15 min at room temperature to allow complexes to form. The mixture was then transferred dropwise to the cells by pipetting and evenly distributed by gently shaking.

2.5.2 Production of viral supernatants

For production of FFV or HIV-1 viral supernatants, 293T cells were transfected with infectious molecular clones of FFV (pCF-7) or HIV-1 (pNL-4.3), respectively. Two and three days post transfection, medium was aspirated and spun briefly at 500 g for 10 min to remove any remaining cells. Cleared supernatants were sterile filtered through 0.45 µm filter and then aliquoted and snap-frozen in liquid nitrogen for storage at -80°C. Since a strong reduction of viral titres was observed by freezing FFV particles compared to storage at 4°C where only a minor decrease in infectivity was observed even after 6 months, short-term storage of FFV was usually performed at 4°C.

2.5.3 Virus titration

For virus titration, aliquots were brought to 37°C in a water bath, serially diluted by twofold dilution and 50 µl of virus added in triplicates to indicator cells seeded one day in advance in 96 well plates. In case of FFV, CRFK or CRFK based FeFAB cells were used dependent on the read-out method applied. Two days after infection, FFV DNA or marker gene expression was measured either by quantitative qPCR (see above) or staining with X-gal solution. For the latter one, cells were washed once and fixed for 15 min at room temperature. Fixing solution was removed by two washes with PBS and X-gal

solution added until colour development (usually within 0.5 to 2 h). Infected cells were counted with an ELISPOT reader using the following instrument settings: Intensity MAX: 100, Intensity MIN: 10, Gradient MAX: 80, Gradient MIN: 16, Size MAX: 35, Size MIN: 2, Emphasis: Tiny. HIV-1 titration was performed identically on TZM-bl cells. Virus titre was calculated based on the dilution and input volume used. Supernatants titrated by qRT-PCR were adjusted to give an ct value of 25 upon infection. Viral supernatants containing SIVmac239, used for testing specificity of neutralisation were kindly provided by Steve Norley (RKI, Berlin). Titrated supernatants of PFV were kindly provided by Rico Blochmann (RKI, Berlin).

2.5.4 Immunofluorescence and flow cytometry

C8166 cells were infected at a MOI of 0.5 with cell free supernatants obtained from pNL-4.3 transfected 293T cells and further cultivated for 48 h or 72 h for indirect immunofluorescence or FACS analysis, respectively. Non-infected C8166 cells were assayed in parallel to assure the specificity of staining and (not shown). Cells were pelleted and washed twice with PBS, fixed by incubation with 2% paraformaldehyde in PBS for 15 min and then used either directly for FACS staining or attached to poly-lysine coated glass slides, dried and rehydrated in PBS overnight. Samples were blocked with 10% normal goat serum in PBS for 1 h and then antisera or a pre-immune rat serum as control (all 1:100 in 3% goat serum, PBS) applied for 1 h. Cells were washed with PBS followed by incubation with a anti-rat IgG-FITC conjugated secondary antibody, washed again and then either analysed in a FACS Calibur flow cytometer or embedded in DAPI containing Vectashield mounting medium (Vector Laboratories, USA) for analysis by microscopy.

2.5.5 Neutralisation assays

To test for the presence of neutralising antibodies, feline CRFK cells or FeFAB cells for FFV or TZM-bl cells in case of HIV-1 and SIVmac239 were plated one day in advance in 96 well plates (1×10^4 cells per well in 100 µl medium). In a separate plate, serial dilutions of antisera or IgGs starting with 1:10 were prepared in medium (50 µl/well in triplicates) and challenged with 50 µl of the intended virus adjusted to ~200 TCID/50µl. After incubation for 30 min, the serum/virus mixture was given to the cells

and incubated for three days. Dependent on the system used, cells were then either analysed by X-gal staining and colony counting as described above, or by qPCR. For the latter one, plates were washed gently with PBS and frozen overnight to disrupt cells. Lysis was completed by addition of 100 µl of Proteinase K solution and incubation at 56°C for 3 h, with subsequent enzyme inactivation at 95°C for 30 min. For qPCR, 3 µl of cell lysate was pipetted into the qPCR reaction mix. To calculate genome copies, a serially diluted plasmid standard mixed with cell lysate from non-infected cells was run in parallel. Standard controls comprised infected cells, non-infected cells, and, if available, pre-immune sera and positive controls such as bnAb 2F5, 4E10. Dependent on the system used, neutralisation was defined as 50 or 70% reduction in provirus detection or number of X-gal positive stained colonies when compared to infection control or pre-immune serum as indicated.

2.6 Animal experiments

2.6.1 Gene Gun immunisation

DNA immunisations were performed using the Helios Gen Gun System (Biorad, USA). Therefore, plasmid DNA obtained from EndoFree Maxi Kits was precipitated on gold particles. 25 mg of gold (diameter 1-3 µm) was mixed with 0.05 M spermidine followed by vortexing and sonication (5 s each). Subsequently, 70 µg of plasmid DNA in 100 µl were added to this suspension and mixed well. Precipitation of DNA on gold particles was achieved by dropwise addition of 100 µl 1 M CaCl₂ during vortexing. After 10 min incubation the DNA-loaded particles were collected by centrifugation and the supernatant discarded. After three washing steps in 100% ethanol the gold particles were transferred to a 15 ml falcon tube prepared to contain 8.75 µl of a 20 mg/ml PVP solution and 3.491 ml 100 % ethanol and mixed by vortexing and sonication. The solution was drawn into nitrogen-dried Tefzel tubes with a syringe. The tubing was then placed into the tubing prep station and let sit until gold particles started to settle. Ethanol in the supernatant was carefully removed with the syringe and the tubing then rotated once by 180°. Afterwards, gold particles were evenly distributed on the inner side of the tube by continuous rotation for 1 min. Cutting the tubes in about 1 cm long pieces gave about 30 bullets per

tubing, corresponding to ~2 µg DNA per cartridge. For quality control, gold particles from individual bullets were eluted by rinsing in DNA loading buffer and subjected to gel electrophoresis (Fig. 13). For immunisation, cartridges were placed into the Gene Gun device and the pistol connected to a helium bottle with a pressure gauge. Delivery of the DNA/Gold particles into skin was achieved by shooting three times onto the shaved abdomen of the animal with a pressure of 350 psi.

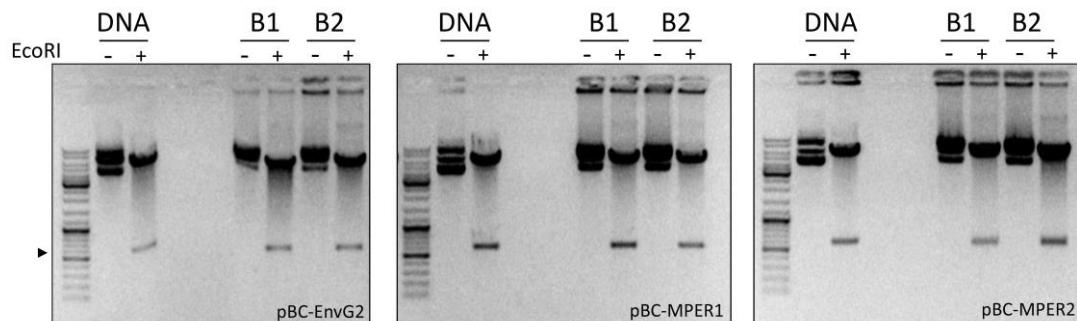


Fig 14. Quality control of Gene Gun ammunition. To verify equal loading onto the cartridges, DNA integrity and to estimate the DNA amount transferred by each shot, original plasmid DNA (1.5 µg, lane DNA) or DNA eluted from two randomly selected bullets (B1, B2) were analysed by agarose gel electrophoresis after (+) or without (-) *EcoRI* control restriction digestion. Representative results of cartridges from the DNA prime/SVP boost immunisation study with FFV Env or Env MPER hybrid antigens are shown. All plasmids show an 658 bp insert after *EcoRI* digestion as expected (black arrow) and are loaded with equal amounts of DNA (~1.5 µg DNA/bullet).

2.6.2 Bleeding and serum preparation

Animals were bled at suitable time points after immunisation by retrobulbar bleeding under anaesthesia. The collected blood samples were incubated for 2 h at room temperature before they were swirled gently with a glass rod to activate the coagulation cascade. Samples were incubated overnight at 4°C to allow clogging to complete. Then, the coagulum was sedimented by centrifugation (3.000 g, 10 min, 4°C) and the serum supernatant recovered in fresh tubes. To remove any potential contamination with erythrocytes, the crude serum was subjected to another centrifugation step (10.000 g, 4 min, 4°C) and again transferred into a new tube. Finally, all sera were decomplemented in a water bath at 56°C for 30 min, spun to remove any precipitated material and aliquoted in fresh tubes for storage at -80°C.

3. Results

3.1 HIV-1 epitope delivery using the foamy viral TM protein

In order to use the TM protein of FV as epitope scaffold, knowledge about immunogenic regions in that protein was essential to assure effective immune responses to HIV-1 epitopes in the later hybrid antigens. In the beginning of this project, no information about the immunogenicity of the TM proteins of FV was available and in contrast to previous studies with gammaretroviral TM proteins, no clear sequence homology between the FV gp48 and the HIV-1 gp41 MPER was evident to allow a conclusive epitope exchange. Thus, a basic question to be answered was if the TM protein of FV is an immunologic target during FV infection and which epitopes were recognised by such potential antibodies. Next to this, it should be investigated which immune responses can be induced when recombinant TM protein is used for immunisation, as this would facilitate later optimisation of epitope insertion and provide control sera for establishment of any TM protein-related assays.

3.1.1 Construction of FFV and PFV TM expression plasmids

For recombinant production of the FFV and PFV TM proteins, constructs suitable for eukaryotic as well as prokaryotic expression were produced. By cloning the TM target sequence into a retroviral backbone for eukaryotic expression it was sought to overcome low protein yields associated with cell line production by the option to generate stable clones. However, although sequence integrity was confirmed in these constructs, no distinct overexpression was evident upon transfection into 293T cells by Western blotting (not shown). In contrast, expression from the prokaryotic expression constructs was easily detectable using the same method. As milligram amounts were needed for the intended immunisation studies, further efforts were focused on production of the TM protein in *E. coli*. A set of expression plasmids were cloned, in which the sequence of the full length FFV and PFV TM protein or the ectodomain, lacking the fusion peptide and membrane spanning domain, was fused to a N- or C-terminal affinity tag. Most of the constructs were rejected from further experimentation as either neglectable expression was observed, protein instability was evident (N-terminal His-tag fusions) or insolubility prevented binding to affinity matrices under non-denaturing conditions (GST- and MBP- tagged fusion

proteins). In case of the FFV TM protein, a codon-optimised ectodomain sequence (kindly provided by Prof. Löchelt, DKFZ) was finally cloned as it gave about five-fold increased expression compared to the wild type sequence. From the various constructs tested, GST-tagged TM proteins showed best expression and including a C-terminal His-tag permitted purification of full-length proteins. Therefore, double-tagged versions of the FFV and PFV TM ectodomains (Fig. 15) were selected for the establishment of suitable expression and purification protocols.

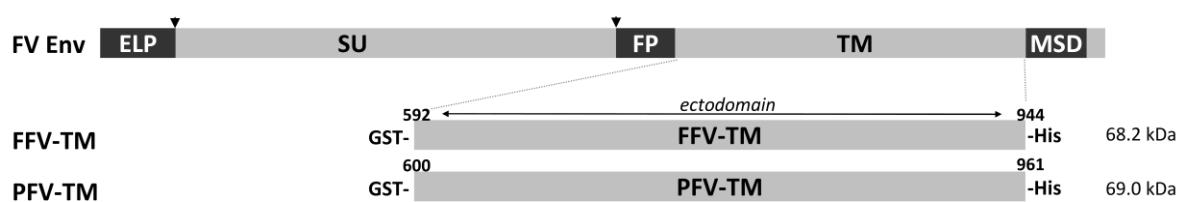


Fig. 15 Structural features of the FV envelope proteins and presentation of resulting recombinant proteins. ELP - envelope leader peptide, SU - surface envelope protein, FP - fusion peptide, TM - transmembrane envelope protein, MSD - membrane spanning domain. Black arrows indicate furin cleavage sites. Amino acid numbering is corresponding to Uniprot entry O56861 for FFV and P14351 for PFV, respectively. The theoretical molecular weights of the recombinant GST-fusions are indicated on the right. Figure adopted from Mühle *et al.*, 2012.

3.1.2 Expression optimisation using a 96 deep well screening assay

Despite being detectable by Western blot, low levels of protein were obtained upon overexpression in the initial BL21 bacterial host under standard expression conditions (LB medium, 37°C, 1 mM IPTG) on the Coomassie level. To overcome this, a 96 deep-well assay was developed for identification of suitable expression conditions by simultaneously testing the most important factors influencing expression, including bacterial strains, media, transformants, IPTG concentrations and temperatures in parallel to the empty, GST-expressing vector as control. For screening, the cloned expression plasmids were transformed into specialised *E. coli* expression strains with differences in their genetic background that could provide benefits for TM protein expression. Shuffle T7 Express cells allow the formation of disulphide bonds in the cytosol of *E. coli* through overexpression of the cytoplasmic disulphide isomerase DsbC (228, 229), C43 cells contain an uncharacterised mutation compensating for expression of toxic and particular membrane proteins (230, 231) and the SCS-1/pSE111 cells are K12 derivatives containing the pSE111 plasmid supplying arginine t-RNAs for improved expression of eukaryotic proteins (218). Similarly, the Rosetta 2 strain supplies tRNAs of seven codons rarely produced in *E. coli*

but has the genetic background of the BL21 strain (232). As shown below, remarkable differences in production levels were found dependent on the conditions applied (Fig. 16). The PFV TM protein was effectively produced in three of four of the selected bacteria with no major influence of the various media or IPTG concentrations. The FFV TM protein was also expressed in three out of four, yet three different bacteria strains and was exclusively detectable in the presence of TB medium, indicating a favourable role of glycerol as carbon source. The results of the screening demonstrated the usefulness of small-scale optimisation and greatly facilitated the subsequent purification process.

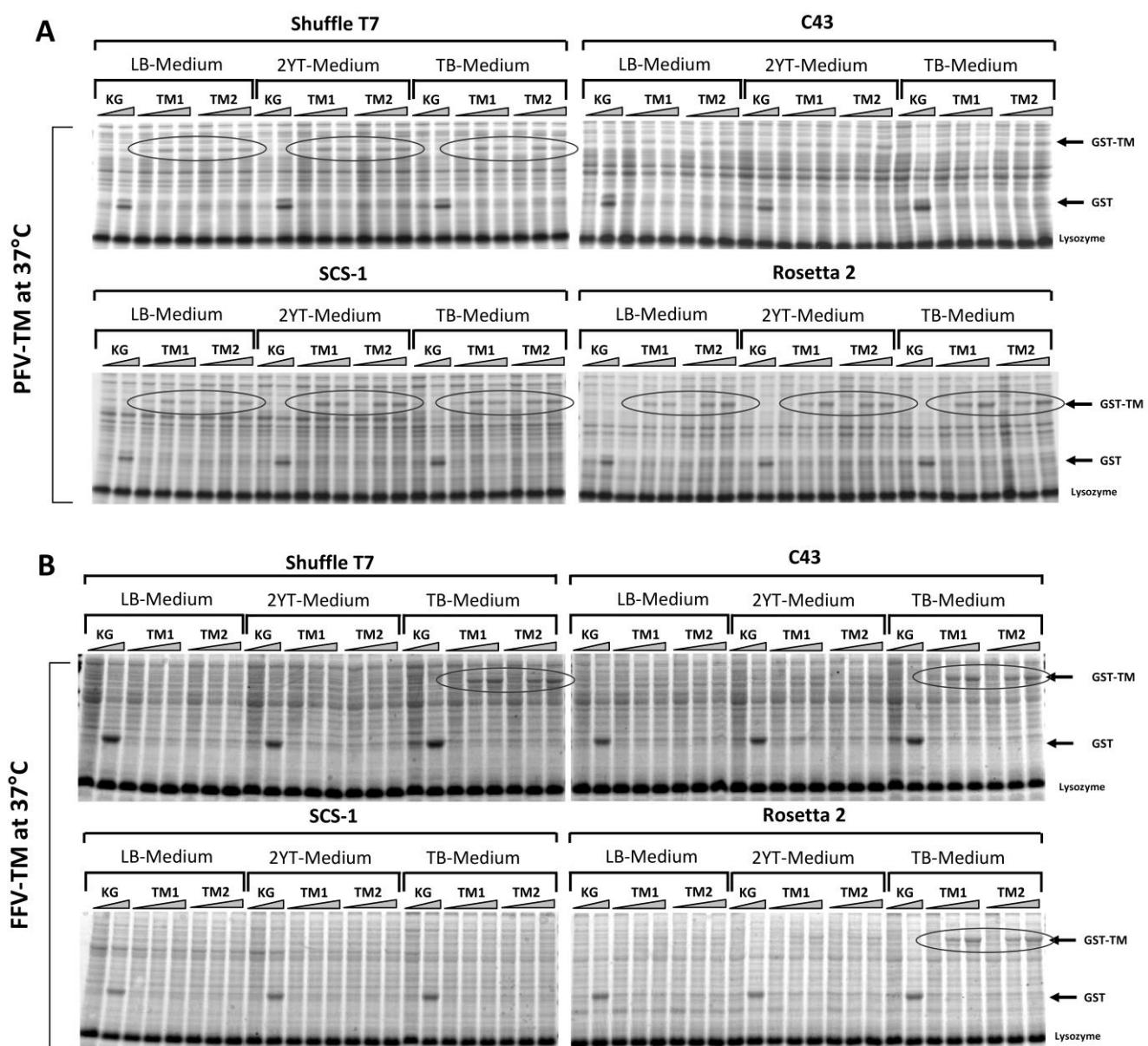


Fig. 16 Influence of the expression conditions on protein production. Bacterial strains Shuffle T7, C43, SCS-1, and Rosetta 2 were transformed with the expression constructs of the TM proteins of FFV and PFV and two transformants (TM1, TM2) along with the empty expression vector (KG) were grown in different media (LB, 2YT, TB). Expression was induced with increasing concentrations of IPTG (grey arrows, corresponding to 0, 0.1 and 1 mM IPTG). Recombinant TM proteins and GST from the parental vector are indicated by black arrows. Conditions allowing high-level protein production are circled. Figure taken from Mühle *et al.*, 2012.

3.1.3 Purification, refolding and characterisation of the produced TM proteins

As a first step to establish a purification protocol for FFV and PFV TM, solubility of the fusion proteins was tested. Both antigens were found in the insoluble fraction, even when fused to the solubility enhancing MBP or GST proteins or when mild non-ionic detergents were used for extraction (data not shown). In consequence, purification was performed under denaturing conditions by including 6 M GuHCl or 8 M Urea in the wash- and elution buffers, respectively. Subsequent dialysis of purified antigens against phosphate buffers resulted in protein precipitation, therefore, the denaturant was removed by a step gradient during Ni-NTA affinity chromatography with elution in PBS buffer containing imidazole (221). Although only low yields of soluble protein was obtained by this way (~0.5 mg/L expression culture), this was sufficient for setting up a FFV TM protein-based ELISA as described below. Alternately, a sarkosyl-based purification protocol was later found to be more practical as no differences in ELISA sensitivity was observed but protein yields increased to 16 to 18 mg/L expression culture for PFV and FFV TM, respectively. In this protocol the inclusion bodies were solubilised by the ionic detergent sarkosyl at high concentrations and then diluted below its critical micelle concentration to allow refolding before purification on Ni-NTA columns. As interference of the 26 kDa GST fusion partner onto the structure or immunogenicity of the antigen might be expected, purified proteins were also treated with thrombin to remove the GST moiety. Analysis of all four proteins by SDS-PAGE, gel filtration and CD spectroscopy showed that they were sufficiently pure, mainly monomeric and with defined secondary structure (Fig. 17). Attempts to crystallise the proteins are currently ongoing in the laboratory of Prof. Dr. Liu (Nankai University, China).

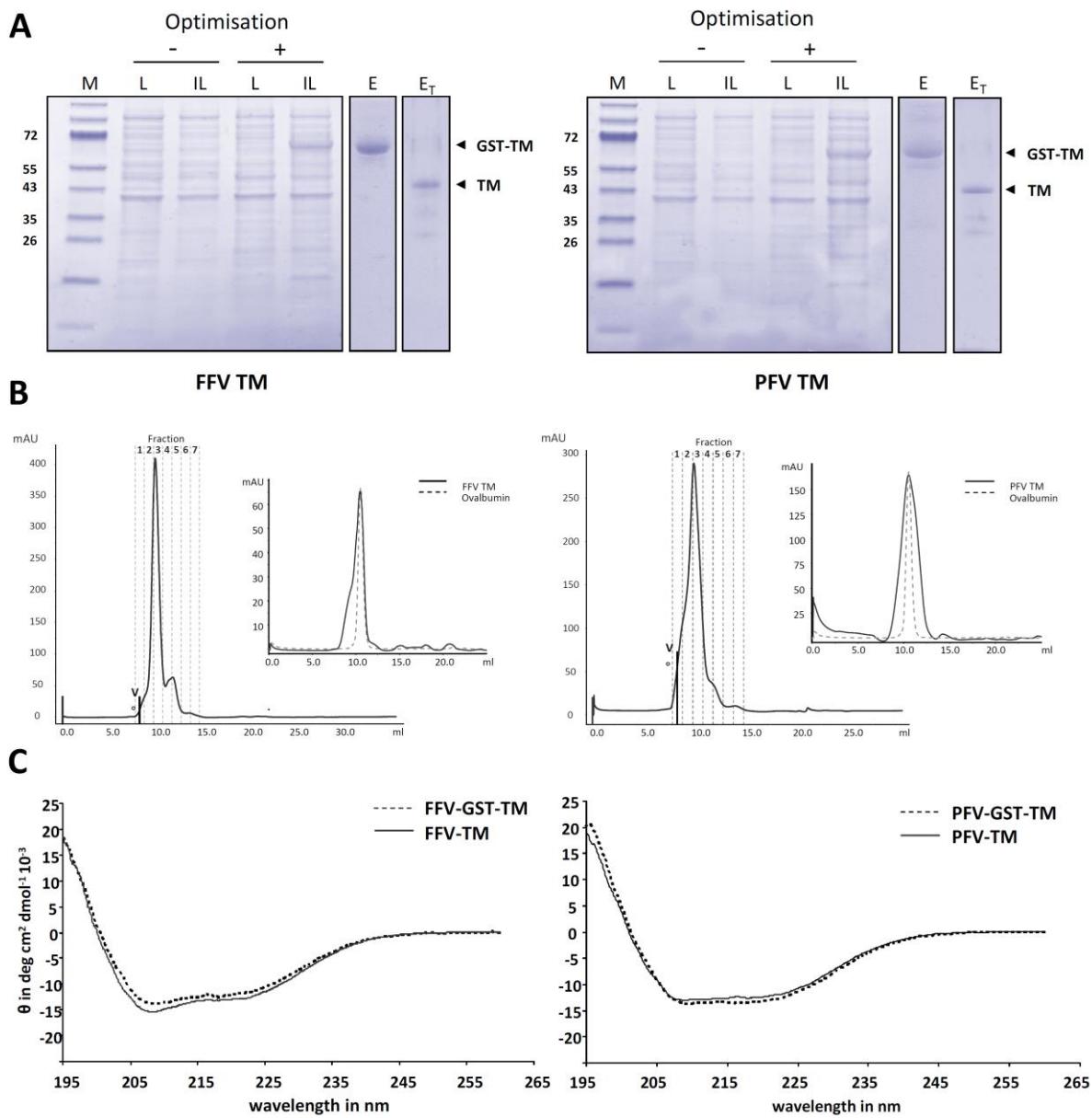


Fig. 17 Purification and characterisation of produced FFV and PFV TM proteins. (A) SDS-PAGE of lysates from non-induced (L) and IPTG-induced (IL) bacteria transformed with FFV TM and PFV TM expression plasmids before (-) and after (+) expression optimisation and proteins after purification. M, Marker; E, Elution fraction after affinity chromatography and gel filtration; E_T, TM proteins after removal of the GST residue through thrombin digestion. (B) Gel filtration of purified proteins with (insets) or without thrombin treatment (large chromatograms) reveals that there are mainly monomeric. V₀, dead-volume of the column. Ovalbumin (43 kDa) was used for calibration (C) CD-spectroscopy of purified FFV and PFV TM proteins with (dashed line) and without (solid line) the GST fusion partner. Chromatograms show that all proteins have defined secondary structure with typical plateaus at 208 and 222 nm corresponding to a dominant alpha helical content. Figure adopted and modified from Mühle *et al.*, 2012.

3.1.4 Immunisation studies with FV TM proteins and characterisation of the antisera

To study the humoral responses in animals and to produce TM-specific antisera, the purified recombinant FFV and PFV TM proteins were used to immunise rats and in case of the FFV TM protein also one goat. High levels of binding antibodies were induced in all animals when the sera were titrated against the respective TM antigen (Fig. 18). Analysing the FFV TM sera by epitope mapping using overlapping peptides showed that responses were directed against the FPPR region and the central part, however, no antibodies against the MPER domain were generated. Although high levels of TM-specific antibodies were present, the antisera did not prevent FFV or PFV infection in an indicator cell line or qRT-PCR-based neutralisation assay, even if low serum dilutions (1:10) were used (data not shown).

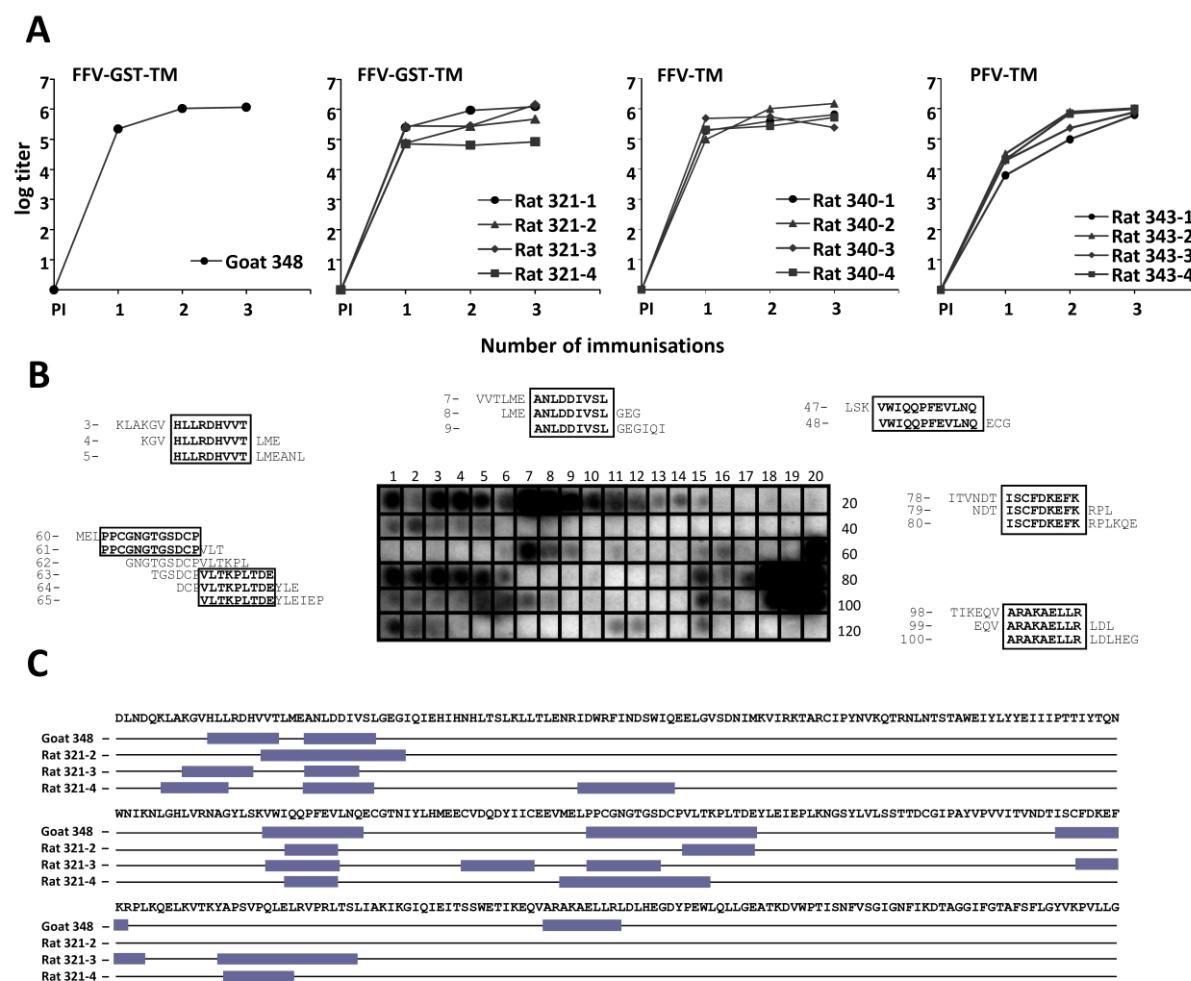


Fig. 18 Characterisation of antibody responses after immunisation with purified TM proteins. (A) Antibody titres after the indicated number of immunisations with FFV TM (goat 348, rats 321), GST-free FFV TM (rats 340) or PFV TM protein (rats 343). Antisera from all animals were titrated in an ELISA using 200 ng recombinant TM proteins as antigen. PI, pre-immune serum. (B) Representative epitope mapping of the FFV TM ectodomain. (C) Summary of epitopes recognised in immunised animals. The amino acids of the FFV TM ectodomain in single letter code is given above the epitopes indicated in blue. Figure modified from Mühle *et al.*, 2011.

3.1.5 Use of the FFV TM protein for serological screening of FFV infection

Through collaboration with the LABOKLIN GmbH, more than 400 cat sera were available to test them for their potential reactivity against the FFV TM protein in a newly established ELISA. From these samples, 262 sera were analysed in parallel with established FFV Gag and Bet ELISAs in the laboratory of Prof. Löchelt to validate the TM ELISA results. Using the purified FFV TM antigen coated to ELISA plates, clear reactivity in a subset of the sera was detectable, which was confirmed to be TM-specific by immunoblotting (Fig. 19). Importantly these reactivity correlated well with that observed by ELISA screening using the FFV Gag antigen and reached higher sensitivity compared to the Bet ELISA, suggesting that the FFV TM protein-based assay is an interesting alternative for detection of FFV infection. By screening all sera in the panel, for the first time the prevalence of FFV infection in Germany was determined to be 39%. By analysing the data in regard to animal characteristics, a clear correlation between age and infection status was found with a significant increase of FFV infection after the first year of birth. Furthermore, the castration status positively influenced the FFV infection rate, but gender-related differences were not observed (Fig. 20).

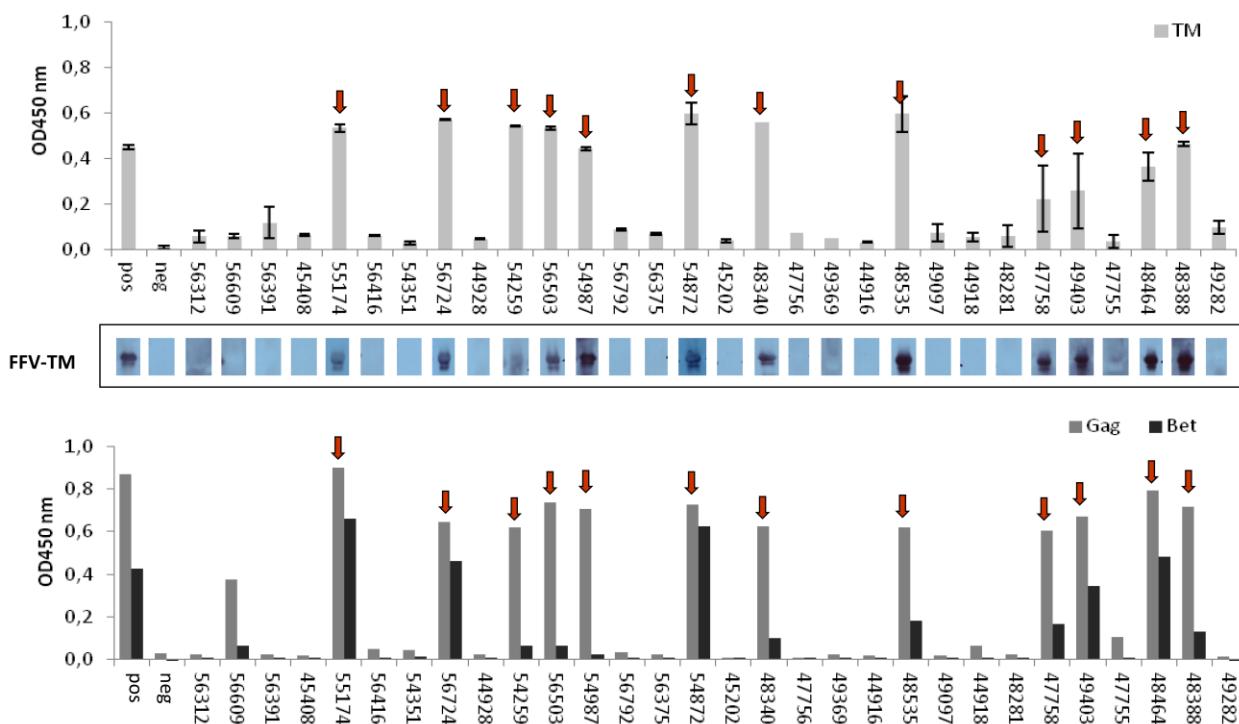


Fig. 19. Serological screening for FFV infection using recombinant FFV TM, Gag and Bet proteins. Purified TM protein (200ng/well) was coated on ELISA plates and incubated either with antiserum of goat 348 (pos) and pre-immune serum (neg) or sera from cats with unknown FFV infection status. Clear reactivity of a subset of sera was evident (upper panel) and reactivity confirmed by FFV TM immunoblot (middle panel). By testing the same sera with established Gag and Bet ELISA (lower panel) a strong correlation between all three assays was found (red arrows). Figure adopted from Mühle *et al.*, 2011. Gag and Bet ELISA data were kindly provided by Anne Bleiholder (DKFZ, Heidelberg).

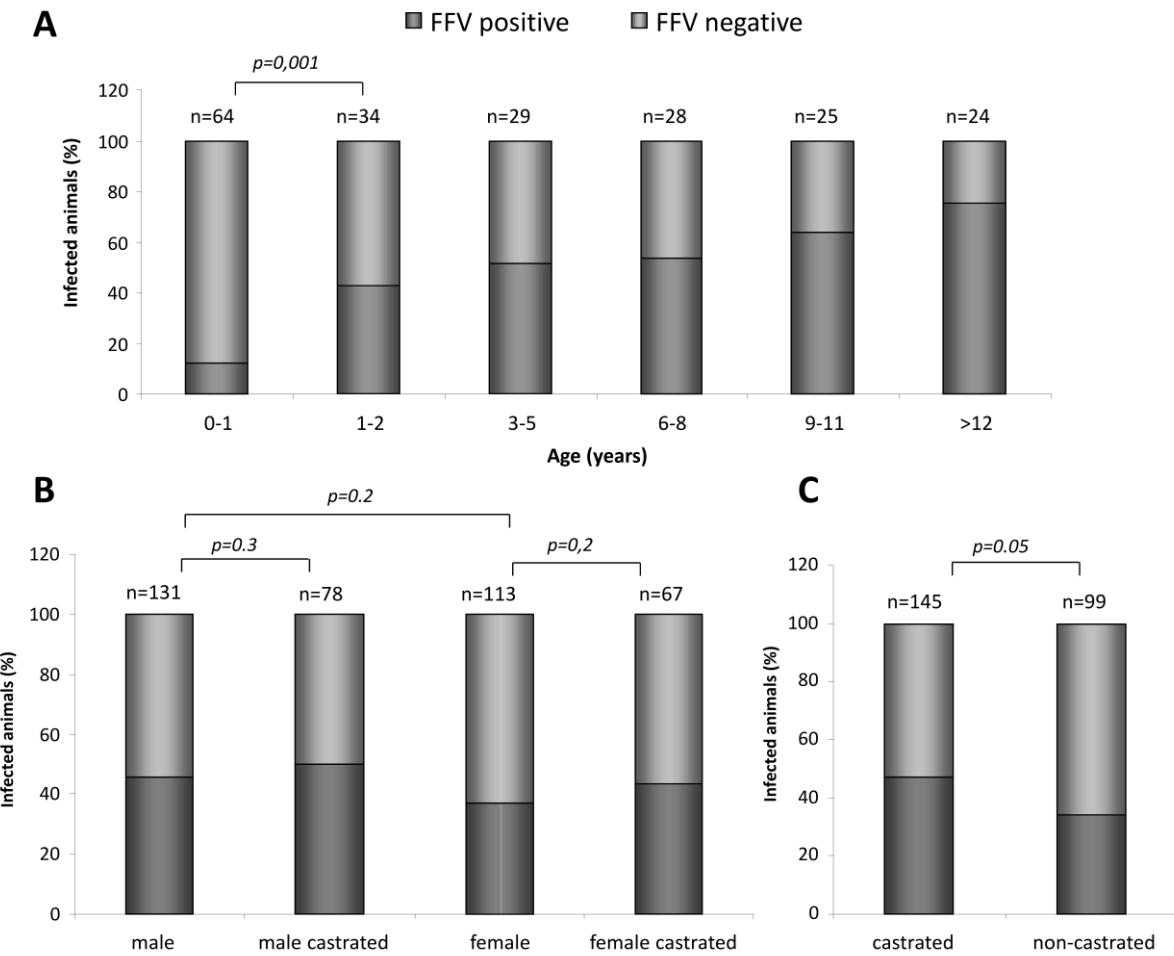


Fig. 20. Prevalence of FFV antibodies in dependence on animal characteristics. Animals were stratified by age, sex, castration status (A, B, C, respectively) and the percentage of the sera positive in a TM protein-based ELISA, the total number of investigated animals and the p-values (Students T-test) are given. Figure taken from Mühle *et al.*, 2011.

3.1.6 Identification of neutralising antisera and epitope mapping of FFV infected cats

After demonstrating that TM-directed antibodies were generated during natural FFV infection, the strength of the TM response and the epitope specificity of these antibodies was analysed. Furthermore, as the TM proteins of retroviruses are often the target of nAb, it was of interest to examine whether a correlation between TM responses and neutralising activity is present. FFV positive sera were therefore titrated against TM protein by ELISA and tested in PCR and FeFAB indicator cell line-based neutralisation assays using the FFV-FUV-7 isolate. For initial screening, serum pools consisting of three sera per pool were studied. In the set of 30 pools, 15 were able to reduce infection to more than 95% at serum dilutions of 1:50 (Fig. 21 A). Assaying these sera individually, 13 sera were found to neutralise FFV-FUV7 but not HIV-1 in a control NT-assay, corresponding to a neutralising antibody response in

about 15% of the infected animals (Fig. 21 B and C). In comparison to the overall TM reactivity measured by titration against recombinant TM protein (average titre of binding antibodies ~1:1000, not shown), no clear correlation between the amount of binding and neutralising antibodies was evident. However, these results demonstrated that the TM protein is an important immunological target during natural FFV infection and rationalised its use as epitope scaffold.

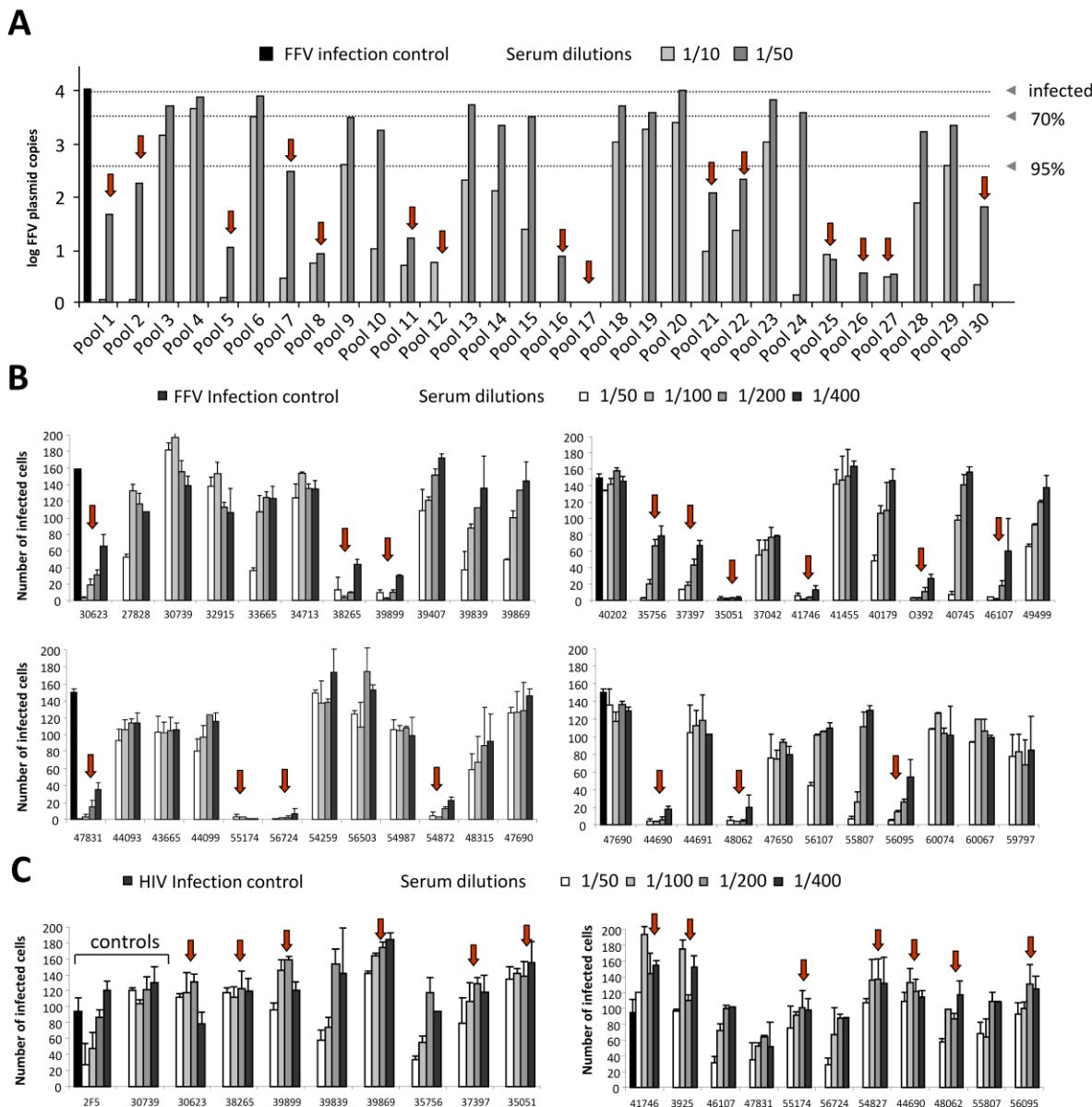


Fig. 21. Identification of FFV neutralising antibodies in sera of naturally infected cats. (A) Serum pools of FFV positive animals were tested in a qRT-PCR based neutralisation assay on CRFK cells at serum dilutions of 1:10 and 1:50 or in absence of serum (infection control). Neutralisation is expressed as reduction of proviral genomic DNA in infected cells, which was quantified with a FFV plasmid standard. (B) Individual analysis of neutralising serum pools identified in (A) in a FeFAB indicator cell based NT-assay at indicated serum dilutions. Several sera show clear reduction of infection even at serum dilutions of 1:400. (C) Determination of the specificity of FFV neutralisation by an HIV-1 NT-assay using TZM-bl indicator cells and titrated pNL-4.3 supernatants. Monoclonal antibody 2F5 was used as positive control at concentrations of 10, 5, 2.5 and 1.25 µg/ml. Specific FFV neutralisation was observed in 13 of the 18 sera tested (red arrows).

Eight of the identified FFV positive sera containing strongly and weakly neutralising antibodies were subjected to epitope mapping on Pepspot membranes as used before for immunised animals. In contrast to the pattern seen before, the antibody responses in the naturally infected cats localised to the FPPR and MPER domain but not to the glycosylated central part of the TM protein (Fig. 22). Interestingly, some of the antisera showed a bipartite recognition of the MPER domain, reminiscent to what has been found for TM-directed antibodies in case of HIV-1 and gammaretroviruses. However, although MPER antibodies were detectable in all of the naturally infected cats, again no obvious correlation with neutralising capacity of the sera was apparent. For better visualisation, a schematic overview comparing the epitopes of immunised and naturally infected animals is given in Figure 22 B.

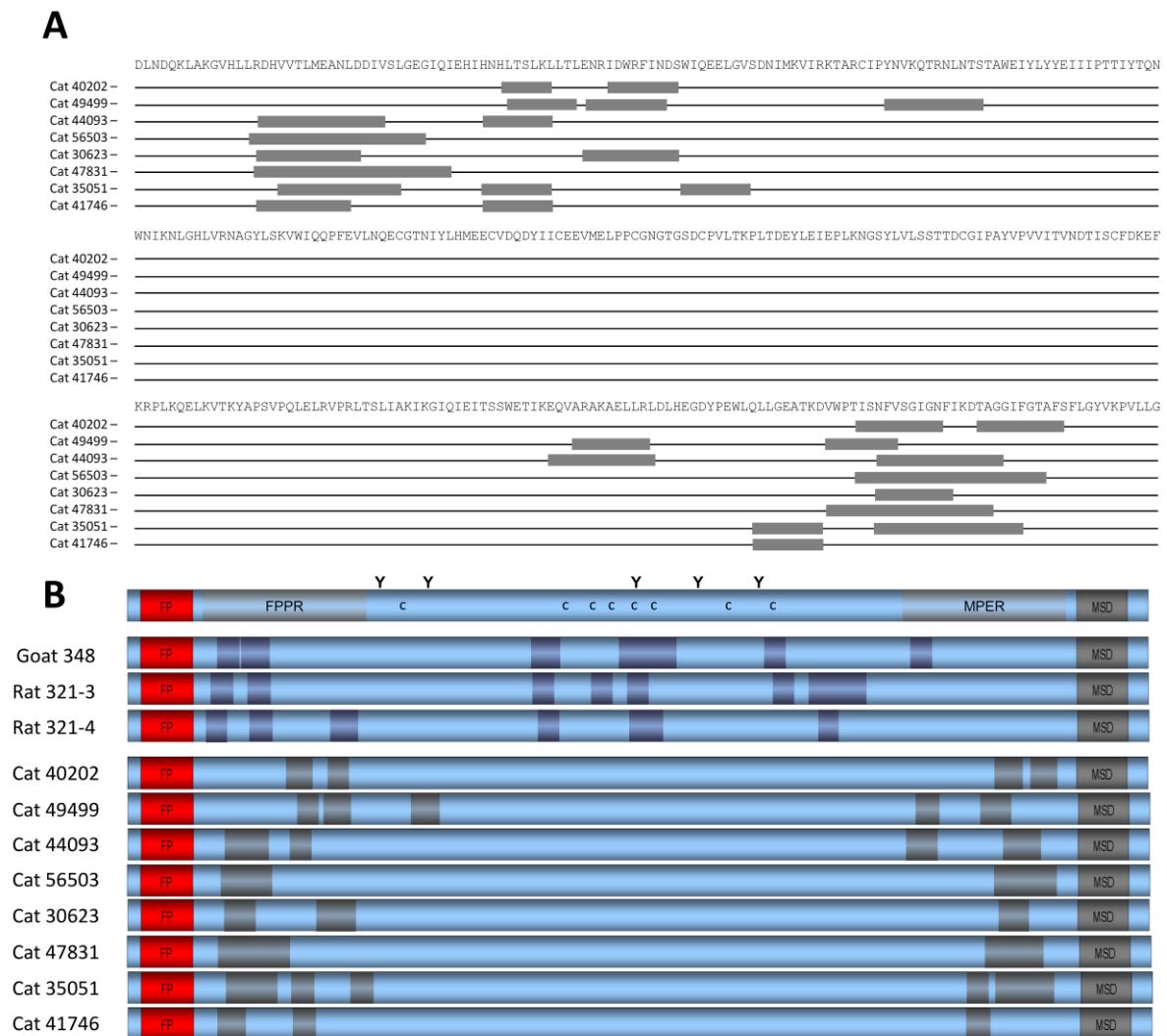


Fig. 22. Epitope mapping of antisera from naturally FFV infected cats. (A) Epitopes recognised by naturally FFV infected cat sera as determined with overlapping peptides from the FFV TM ectodomain. The spotted amino acid sequence is given in single letter code and identified epitopes indicated by grey bars (B) Schematic presentation of the TM protein of FFV showing the location of the fusion peptide (FP), the fusion peptide proximal region (FPPR), the membrane proximal external region (MPER) and the membrane spanning domain (MSD). The location of the predicted glycosylation sites (Y) and the cysteine residues (C) is indicated. For comparison, the localization of the epitopes recognised by the sera from immunised (dark blue) and infected animals (grey) is shown.

3.1.7 Exchange of immunogenic epitopes in FFV Env by HIV-1 FPPR and MPER domains

The data presented above indicated that although not clearly linked to neutralisation, the FPPR and MPER domain were immunogenic during natural FFV infection and therefore suitable for insertion of the intended HIV-1 epitopes. Furthermore, as the immunogenic regions in the FFV MPER were located close to the transmembrane domain as the epitopes of 2F5 and 4E10 in gp41, the introduction close to a lipid environment could provide a potential benefit for the induction of nAb. Based on the epitope mapping data, immunogenic consensus sequences in the FPPR and MPER of the FFV TM protein were defined. Since the overall identity to the corresponding HIV-1 sequences on the amino acid level was low, additional bioinformatic parameters including secondary structure, hydrophobicity, surface exposure and spacing to neighbouring functional domains (fusion peptide, membrane spanning domain) were also considered to select insert positions (data not shown). Next, to further increase the probability of finding a position in which the overall protein integrity remains uncompromised, the domains were shifted by three amino acids to the C- or N-terminus, representing a $\frac{3}{4}$ turn in the alpha helical domains. Overall, six HIV-1 FPPR hybrids and two MPER chimeras were designed and in parallel, the corresponding FPPR and MPER domains from PFV were included as controls (Fig. 23). The respective domains were cloned into an optimised full-length Env expression construct (pBC-EnvG2) by Anne

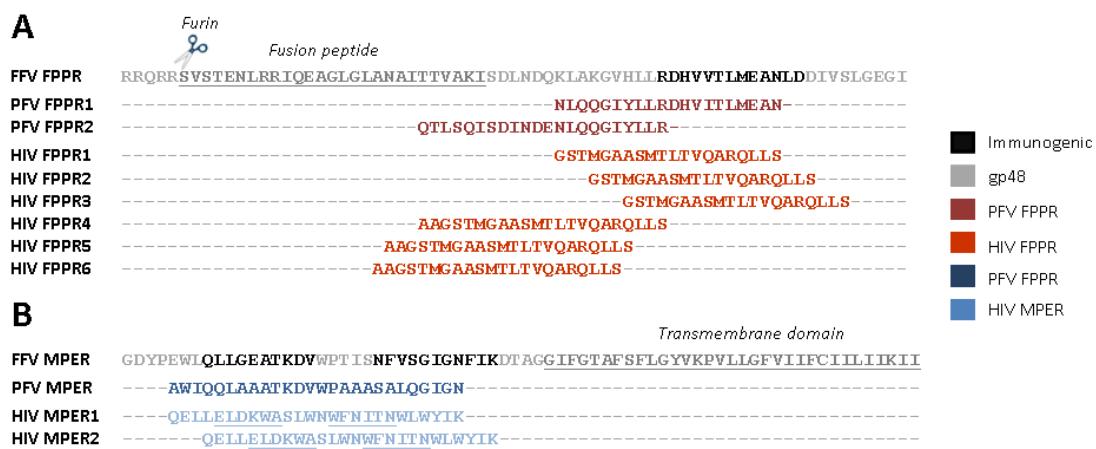


Fig 23. Generation of chimeric FFV Env proteins containing HIV-1 FPPR and MPER epitopes. (A) Insertion of HIV-1 FPPR (red) or PFV FPPR residues (dark red) into selected positions of the wildtype FPPR region of the FFV TM protein. The furin cleavage site (scissors), the fusion peptide (underlined) and the immunogenic domain obtained by epitope mapping of naturally infected cats (bold) are indicated. (B) Insertion of HIV-1 MPER (blue) or PFV MPER residues (dark blue) into the MPER region of the FFV TM protein. The wildtype sequence with immunogenic epitopes derived from epitope mapping (bold) and residues of the membrane spanning domain (underlined) are shown. Residues underlined in the HIV-1 MPER sequences correspond to the sequence recognised by 2F5 (ELDKWA) and 4E10 (WFNITN), respectively.

Bleiholder (DKFZ, Heidelberg). Compared to wildtype Env, the active splice donor and acceptor pair naturally flanking the membrane spanning domain of FFV Env was silently mutated in this construct to prevent the formation of *env* transcripts that lack the transmembrane domain. Therefore, they are more efficiently anchored into the plasma/viral membrane instead of being secreted as soluble Env protein only (A. Bleiholder, unpublished). When the constructed Env chimeras were transfected into 293T cells and analysed by immunoblots using the TM-specific goat serum 348, those having the PFV FPPR or MPER domain as well as hybrids containing the HIV-1 MPER were efficiently expressed and processed by cellular proteases. In contrast, insertion of HIV-1 FPPR sequences resulted in complete loss of furin cleavage and occurrence of the Env precursor protein only (Fig. 24). Interestingly, all of the constructs that were found to be cleaved, could be detected in concentrated cell culture supernatants, indicating that FFV Envs are able to bud in absence of Gag as subviral particles (SVPs) as has been described for PFV Env and was further confirmed for the FFV Env shown here by density gradient ultracentrifugation (A. Bleiholder, unpublished). Further investigations on the FFV/HIV-1 MPER hybrids showed that their sub-cellular localisation as well as their glycosylation pattern was similar to wt Env and that both chimeric proteins were effectively immunoprecipitated by 2F5 and 4E10, suggesting that the HIV-1 MPER epitopes were accessible under non-denaturing conditions (A. Bleiholder, unpublished). However, in contrast to the PFV chimeras, both HIV-1 MPER Envs did not allow marker gene transfer when used in pseudotyping experiments with Env deficient FFV (Fig. 24), proposing an inability of the hybrids to permit fusion of virus particles with the cellular plasma membrane. In consequence, this strategy could not be used to construct replication-competent vectors, however, further efforts were undertaken to examine the usability of the secreted MPER containing SVPs as antigens for induction of HIV-1 specific antibodies.

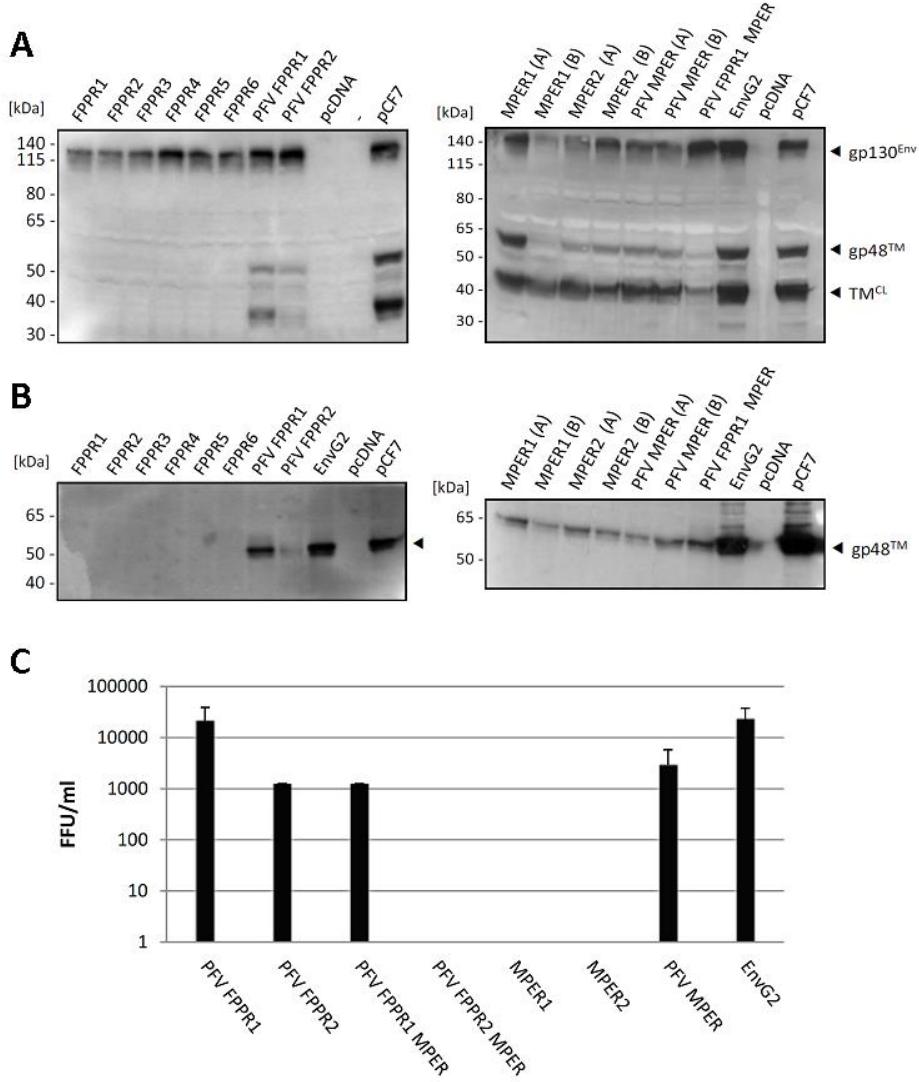


Fig 24. Characterisation of chimeric FFV Env proteins containing HIV-1 FPPR and MPER epitopes. (A) Expression and processing of wildtype FFV Env (pCF-7, EnvG2) and when modified to contain the HIV-1 or PFV FPPR (left) or HIV-1 and PFV MPER (right). 293T cells were transfected with corresponding expression constructs or empty vector (pCDNA) and analysed 2 days post transfection. Clone numbers correspond to the various positions in which the chimeric sequences were introduced. A and B are two individual clones with the same insert. Size of marker proteins, the gp130 Env precursor, the cell lysate associated form of TM (TM^{CL}) and the mature gp48 TM protein are indicated by arrows. Whereas modification of FFV FPPR residues was compatible with Env processing only in case of FFV/PFV chimeras, constructs containing HIV-1 and PFV MPER sequences were both efficiently cleaved. (B) Analysis of secretion of wildtype and chimeric Envs. Supernatants from transfected 293T cells were concentrated by ultracentrifugation and analysed by immunoblot with TM specific goat serum. Envs found to be efficiently cleaved were secreted into the supernatant. (C) Marker-gene transfer (focus forming units, FFU) of supernatants obtained through parallel transfection of Env-deficient, beta-gal expressing UbiLacZ FFV vectors with wildtype (EnvG2) or chimeric Envs containing the HIV MPER, PFV MPER or both PFV FPPR and MPER, respectively. Whereas PFV hybrids produced infectious virus able to infect new cells, no marker gene transfer was observed by pseudotyping the vector with Envs containing the HIV-1 MPER. Data kindly provided by A. Bleiholder (unpublished).

3.1.9 Immunisation studies using chimeric FFV/HIV MPER SVPs

For evaluation of the immunogenicity of the described SVPs in immunisation experiments, large scale expression in cell stacks and purification protocols using cross-flow filtration concentrated supernatants and sequential gradient ultracentrifugation were established by our collaboration partners at the DKFZ

(A. Bleiholder, unpublished). The purified particles were reasonably pure and contained the TM protein as judged by silver staining. However, BSA as a main contaminant could not be separated by the purification protocol applied and overall yields of purified SVPs, in particular that of both HIV MPER hybrids which were less efficiently secreted into the supernatant, were relatively low (Fig. 25).

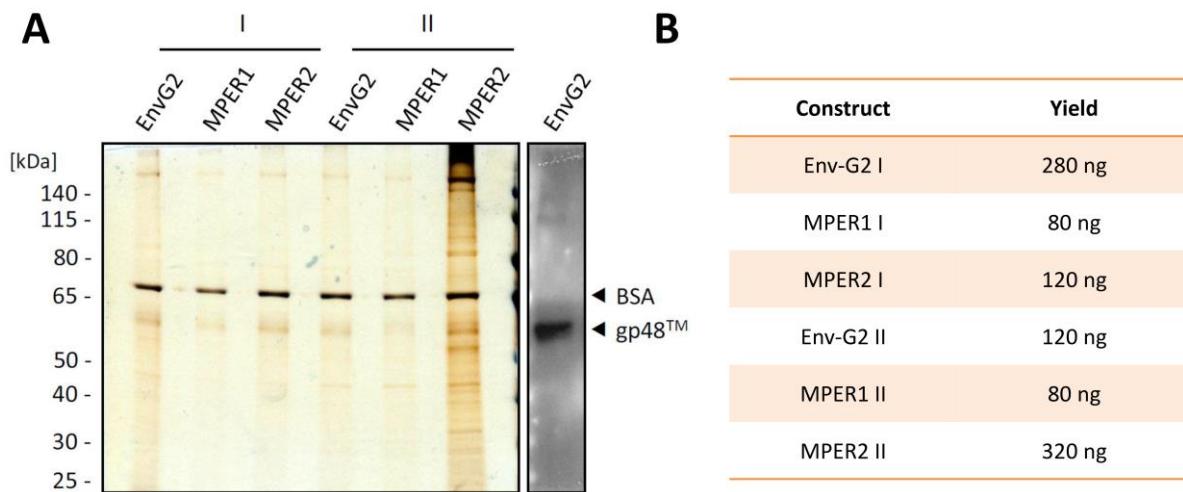


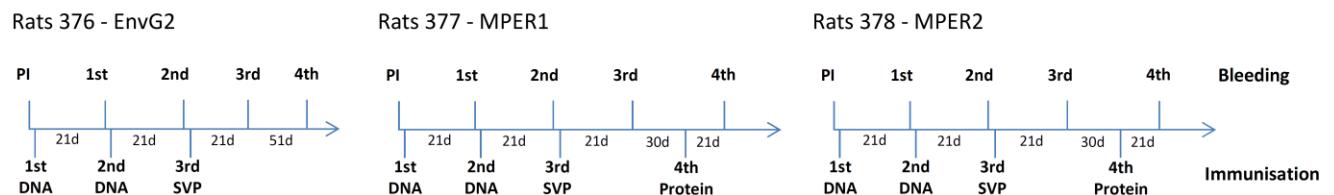
Fig 25. Purification of FFV subviral particles and amounts obtained after large-scale production. (A) Purity of SVPs used for immunisation of rats. 5 µl of SVPs from wildtype FFV Env (EnvG2) or Envs containing the HIV-1 MPER domains (MPER1, MPER2) from two different purification charges (I, II) were subjected to SDS-PAGE and silver staining. Right lane: Immunoblot of EnvG2 SVPs detected with the TM-specific goat serum 348. The glycosylated gp48TM protein is visible as fuzzy band below the BSA contaminant (arrows). Molecular weight marker is indicated on the left. (B) Yields of indicated SVPs in individual charges after large-scale production. The amount of TM protein in SVP preps was estimated by parallel loading of serial dilutions of recombinant FFV-TM protein during immunoblot. Data were kindly provided by Anne Bleiholder (DKFZ, Heidelberg).

To enhance immune responses, a DNA prime, SVP boost protocol was chosen for immunisation experiments in rats. Four animals per group were immunised twice by gene gun bombardment with plasmids pBC-EnvG2 (group 376), pBC-MPER1 (group 377) or pBC-MPER2 (group 378) in a three weeks interval, followed by injection of SVPs pooled from both purification charges three weeks later. In case of rats 377 and 378, further boosting was performed using 250 µg of an recombinant FPPR-MPER antigen (F2-M2, see section 3.3) to enhance HIV-1 MPER directed responses. Subsequently, antisera were titrated in ELISAs against recombinant FFV TM protein to analyse overall immune responses as well as recombinant gp41 purified from bacteria and a synthetic HIV MPER peptide. As shown below, the antibody response induced against the FFV TM protein at serum dilutions of 1:500 was moderate in group 376 which obtained EnvG2 DNA and SVPs and weak in groups immunised with the MPER chimeras until further boosting with recombinant FPPR-MPER antigen (4th injection, Fig. 24A). When the immune responses against gp41 and MPER peptide were analysed, the EnvG2 group

showed as expected no reactivity against these antigens. However, a similar pattern was observed in groups 377 and 378 obtaining the MPER antigens until the 3rd immunisation (2x DNA, 1x SVP boost).

A more robust response against gp41 and MPER peptide was obtained after boosting with the recombinant FPPR-MPER antigen with no differences between groups 377 and 378. Analysing the antisera for neutralising activity, no reduction in the level of HIV-1 infection was observed (not shown).

A



B

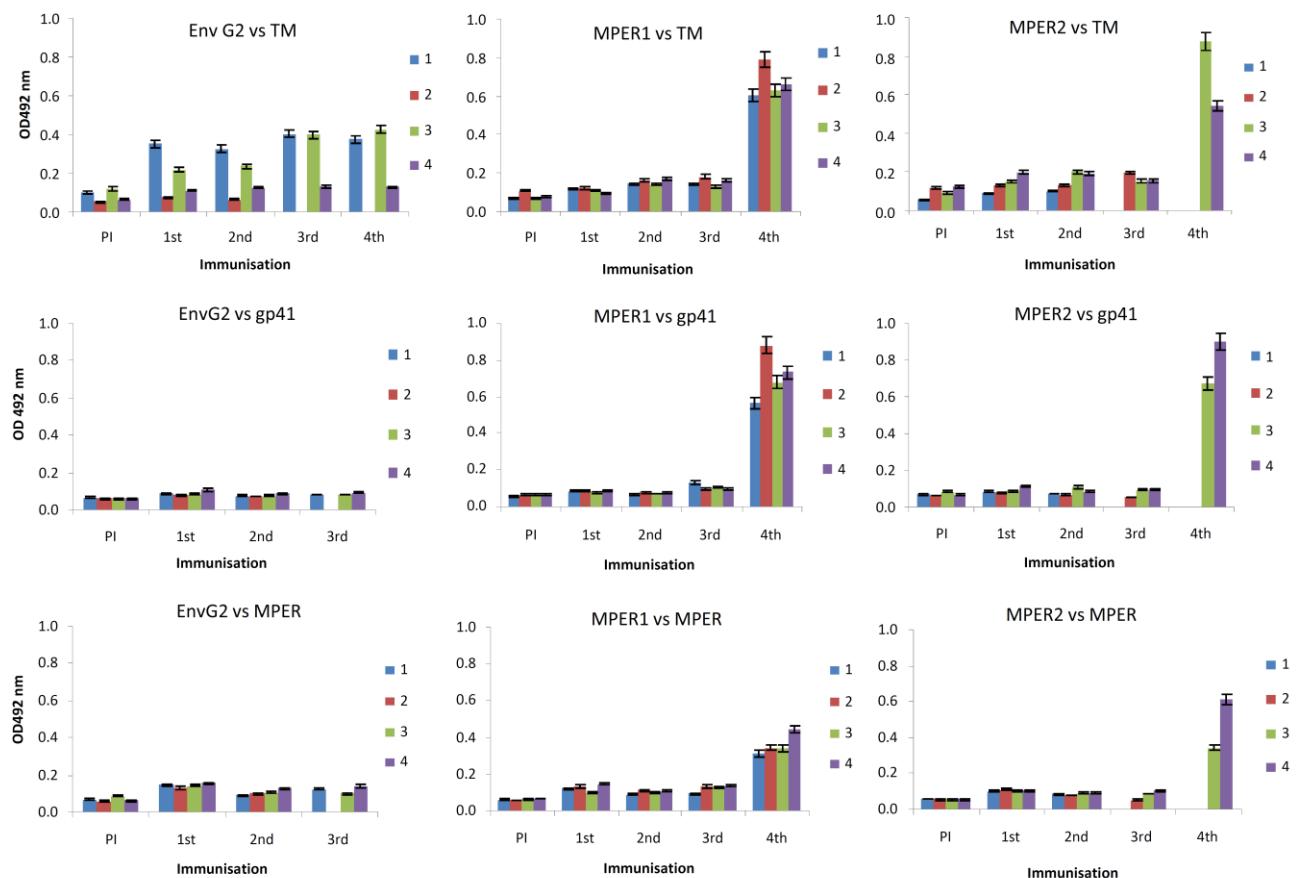


Fig 26. Immunisation studies in rats using FFV Env DNA and SVPs as antigen. (A) Immunisation schedules for rat groups 376, 377 and 378. Rats were immunised twice by Gene Gun immunisation (DNA) with indicated expression plasmids (EnvG2, MPER1, MPER2) followed by boosting with subviral particles (SVP) expressed and purified from 293T cells transfected with the same plasmids. In case of group 377 and 378, further boosting of HIV-1 specific responses was achieved by injection of the recombinant FPPR/MPER antigen F2-M2. PI, 1st, 2nd, 3rd and 4th correspond to pre-immune serum or the number of immunisations and bleedings as indicated. (B) Analysis of rat antisera by ELISA at serum dilutions of 1:500, using recombinant FFV GST-TM protein (TM), gp41 or a synthetic MPER peptide (MPER) as antigen. The number of immunisations and reactivity for individual animals 1-4 (coloured bars) is indicated. No antiserum was available in cases where no bars are shown (groups 376 and 378).

3.2 HIV-1 epitope delivery by FFV Bet fusion proteins

In a second approach aiming to construct replication-competent FV vectors expressing HIV-1 epitopes, the accessory Bet protein of FFV was used as fusion partner. As mentioned earlier, Bet has been described to be immunogenic during FV infection and fusion of a neutralising epitope of feline calicivirus (FCV) to Bet was successfully used to induce partial protection against high-dose FCV challenge in cats (118). In order to characterise epitope accessibility and immunogenicity of the small HIV-1 epitopes when expressed in the Bet context, they were first produced as recombinant proteins and thoroughly analysed. Subsequently, the strategy was transferred into the FFV context.

3.2.1 Design and construction of Bet and Bet/HIV-1 expression plasmids

The basis for production of FFV Bet and Bet/HIV-1 hybrid proteins (Fig. 27) were expression plasmids constructed stepwise by subcloning of the full-length Bet sequence from plasmid pBC12-Bet (kindly provided by M. Löchelt, DKFZ) into the prokaryotic pQE-30 Xa expression vector, followed by the introduction of the intended HIV-1 epitopes. In cloning step one, four additional amino acids (AGAA) were added to the C-terminus of the Bet protein.

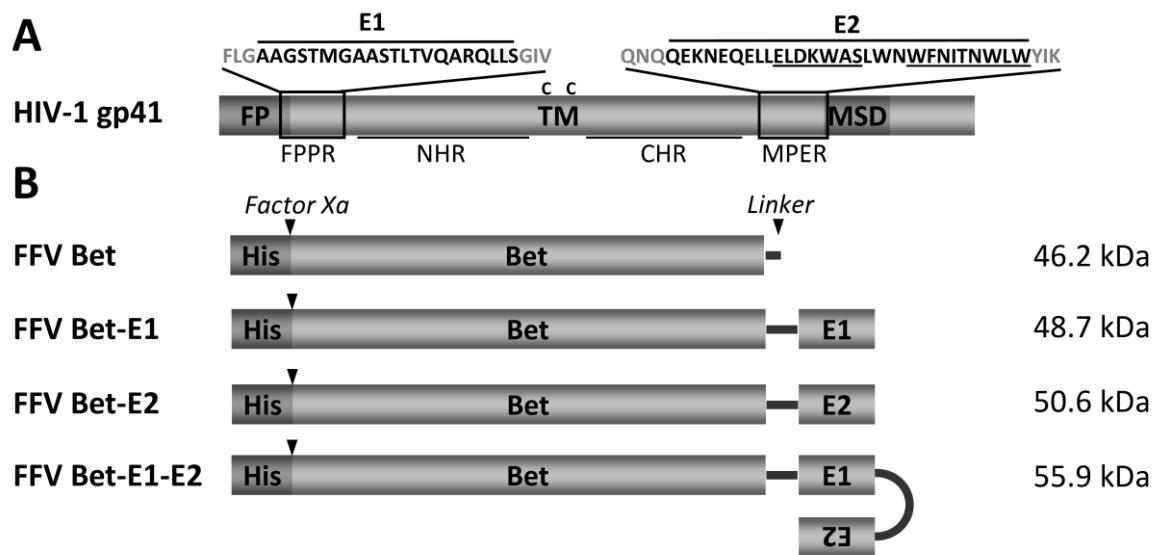


Fig. 27. Schematic presentation of gp41 of HIV-1 (A) and of FFV Bet and the FFV Bet/HIV-1 fusion proteins (B). FP fusion peptide, FPPR fusion peptide proximal region, NHR/CHR N-terminal and C-terminal helical region, MPER membrane proximal external region, MSD membrane spanning domain, C-C cysteine loop, TM transmembrane envelope protein, His histidine tag, Factor Xa factor Xa protease cleavage site, Bet full-length FFV Bet sequence, Linker 4 (FFV Bet) or 15 (all other constructs) amino acid spacer. E1–HIV-1 FPPR-derived domain, E2–HIV-1 MPER-derived domain, E1–E2, HIV-1 E1 and E2 domains connected by a flexible loop sequence. The theoretical molecular mass of all antigens is indicated. Lines below the E2 sequence of gp41 of HIV-1 mark the location of the neutralising epitopes of 2F5 and 4E10. Figure taken and adopted from Mühle et al., (2013a).

encoding a *NaeI* site were introduced just before the Bet stop codon which allowed the subsequent insertion of HIV-1 FPPR and MPER sequences with extra spacer residues (SGSAGAGAGGG), included to spatially separate HIV-1 epitopes from Bet. Additionally, the construct Bet-E1-E2 was designed in which the FPPR and MPER domain was connected through a loop element in order to potentially stabilise interactions that promote enhanced MPER antibody binding (Fig. 28). This construct was based on the de novo designed alpha-turn-alpha ($\alpha\alpha$) peptide (46, 233), in which the N- and C-terminal helices were replaced by HIV-1 FPPR and MPER residues with an amino acid spacing that allows contact of critical residues as found in the crystal structure of gp41 (81). As the loop element and eight flanking amino acids of the N- and C-terminal alpha helical residues were left unaltered, these

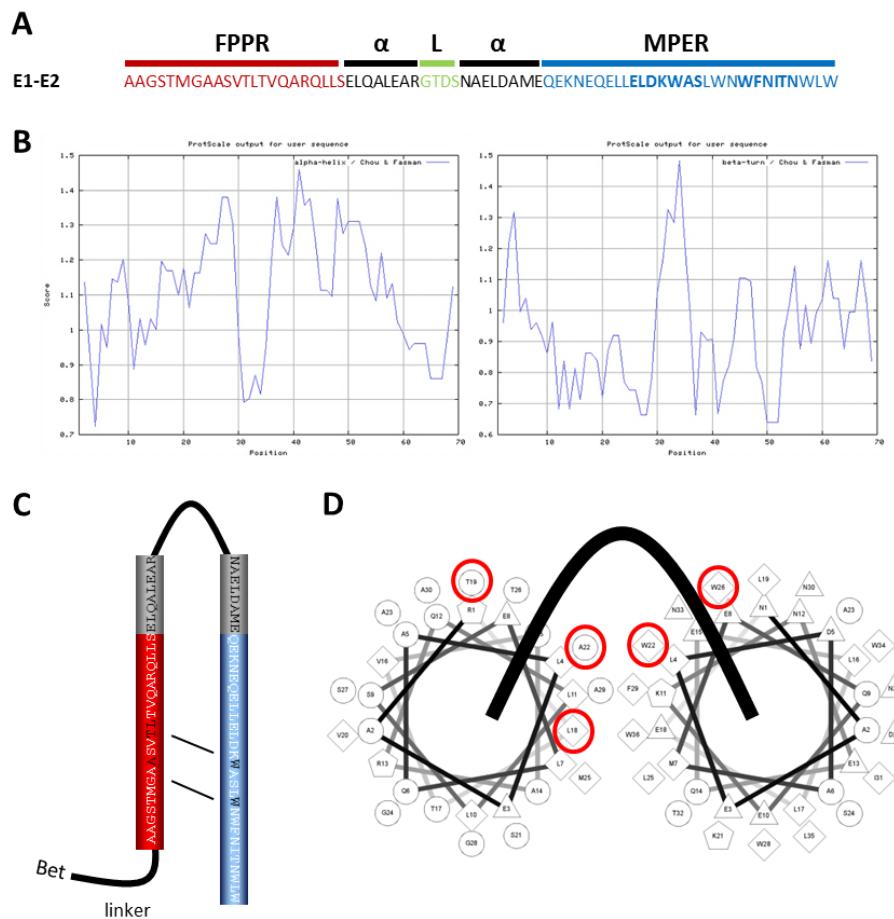


Fig. 28. Design and bioinformatic characterisation of the E1-E2 antigen. (A) Amino acid sequence of E1-E2 and individual domains. The fusion peptide proximal region (FPPR, red), the membrane proximal external region (MPER, blue), the alpha helical flanking regions (α , black) and the β -turn loop element (L, green) are highlighted in colour. (B) Chou-Fasman secondary structure predictions for the E1-E2 antigen, plotting the probability score to form alpha helices (left diagram) or beta-turns (right diagram) against individual amino acids. Note the distinct structural break at amino acids 30-34, representing the loop element. Graphs were obtained with the ProtScale software provided on the expasy proteomics server (<http://web.expasy.org/protscale>). (C) Model of E1-E2 interaction and positioning of FPPR and MPER contacting amino acids. The 15 amino acid linker and the Bet fusion partner are schematically shown (D) Alpha helical wheel projections of both domains with the positioning of critical residues as in (B) indicated by red circles. Projections were made with the help of the Wheel program provided by Don Armstrong (<http://trimer.tamu.edu/cgi-bin/wheel/wheel.pl>). Mühlé et al., 2013a

construct should promote interactions of the FPPR and MPER as found in the wildtype *ata* peptide. Indeed, bioinformatic analysis confirmed the proposed secondary structure and correct positioning of FPPR and MPER residues. Having constructed the described plasmids, suitable expression and purification methods had to be established in a next step. The Bet protein constituted the major part of the fusion proteins and the corresponding expression plasmid was available first. Therefore, protocols were established for Bet and then finally applied also to the hybrid proteins.

3.2.2 Production of FFV Bet and protein characterisation

As a first step, expression screening in deep well plates was performed as described before for the FFV and PFV TM proteins. SCS-1 bacteria and to a lesser extent T7-Shuffle allowed production of the protein at high levels in total lysates (Fig. 29A). Unfortunately, only low amounts of recombinant protein were found in the soluble fraction, which were slightly increased at reduced expression temperatures but did not exceed the Western blot level (Fig 29B). Since the HIV-1 part of the Bet-E1-E2 protein was designed to form a conformational epitope, it was essential that the intended proteins were available in a soluble form to allow formation of such structures. Various attempts were undertaken to achieve soluble expression of Bet, including fusion to GST, MBP and Thioredoxin as solubility-enhancing fusion proteins, use of additional specialised bacteria for expression, supplementation of growth media with Zn²⁺ and DTT or performing expression under a more broad temperature and IPTG profile as in the initial screening (data not shown). None of this factors did improve yields of soluble Bet, pointing to an inability of *E. coli* to manage production of a correctly folded protein, probably due to the large number of 13 cysteines that may contribute to structure stabilisation in native Bet. As a consequence, purification under denaturing conditions using the His-tag was performed (typical yields 35 mg/L) and further efforts focused on establishing a suitable refolding protocol for renaturation.

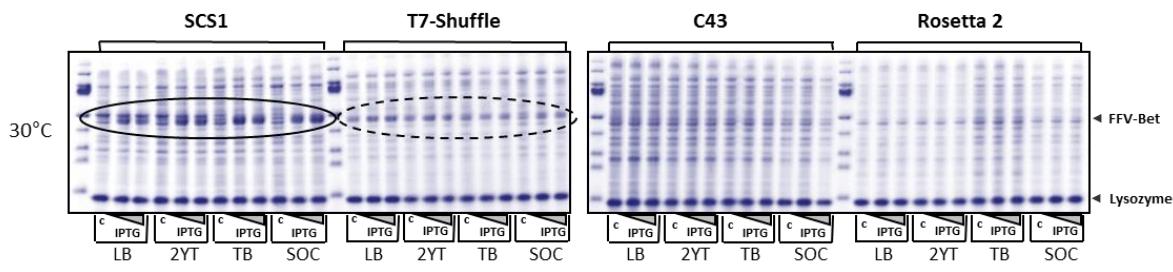
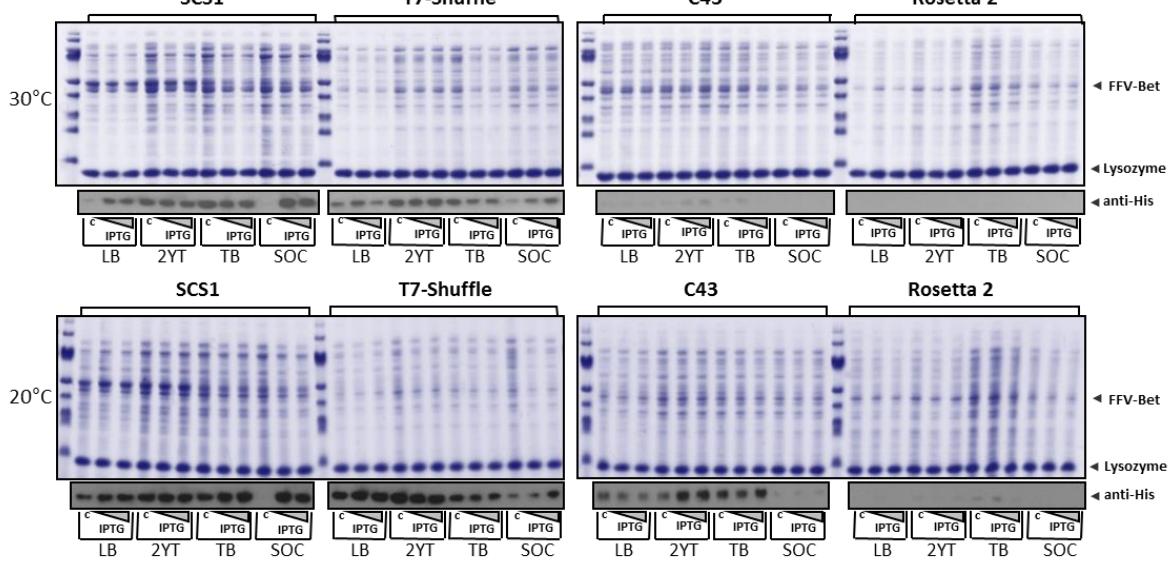
A**B**

Fig. 29. Expression optimisation for FFV Bet. (A) SDS-PAGE analysis of protein production in total lysates with expression strains SCS-1, T7-Shuffle, C43 and Rosetta 2 bacteria at 30°C expression temperature. The type of medium (LB, 2YT, TB, SOC), the amount of IPTG ($c=0$ mM, grey arrows 0.1 and 0.5 mM), Lysozyme used for lysis and the expected size of recombinant FFV Bet is indicated. SCS-1 and to a lower extent T7-Shuffle promote expression (black circles). (B) Expression of FFV Bet in the soluble fraction. Labelling of gels is as in (A) but include results obtained at 20°C (lower panel) and an anti-His blot for detection of low levels of proteins (grey boxes). Whereas lowering the expressing temperature had a positive effect on protein solubility, yields did not exceed the Western blot level.

A refolding screening assay based on 96-well plates was developed to address this issue. Denatured and reduced protein from the Ni-NTA purification step was diluted into Tris buffers with or without co-solvents that aid refolding processes by various mechanisms. Additionally, a redox-system based on different ratios of oxidised and reduced glutathione (GSSG/GSH) was included to allow formation of disulphide bridges that might be necessary to stabilise the soluble form of Bet. After renaturation for two days under the distinct conditions, the Bet protein was captured on 96-well Ni-NTA plates and functional refolding analysed by measuring binding of the Bet ligand feline APOBEC3Z2b, purified as soluble GST-fusion protein. From all conditions tested, the presence of L-Arginine had significant impact on the refolding success (Fig. 30). Furthermore, only in the presence of a redox-system, inter-

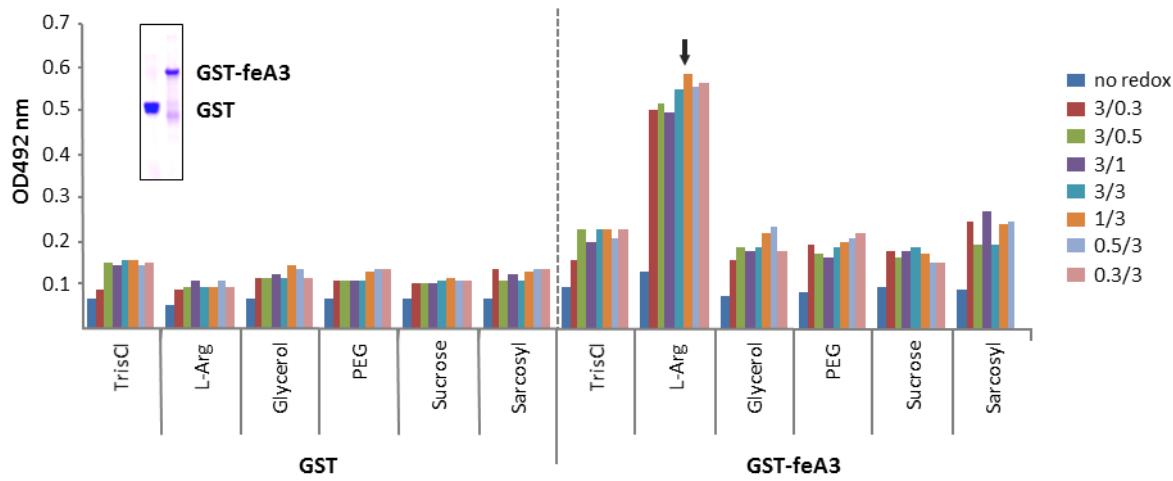


Fig. 30. Screening for suitable refolding conditions for FFV Bet. Denatured and reduced FFV Bet was renatured by rapid dilution into TrisCl-based refolding buffers without or with additional folding enhancers (L-Arg, Glycerol, PEG, Sucrose, Sarcosyl). Buffers were further supplied without or with a redox system based on oxidised and reduced glutathione (GSSG/GSH, see legend for colour code, the concentrations of red/ox glutathione is given in mM). After incubation at 4°C for 2 days and centrifugation, soluble proteins were captured on Ni-NTA plates and probed with 500 ng of purified of GST or feline APOBEC3Z2b (GST-feA3, inset). After washing, bound GST proteins were detected by an HRP-coupled anti-GST antibody and plates developed with a colorimetric OPD substrate. Refolding in the presence of L-Arg was most successful and the presence of a redox system was essential to obtain interaction. Excessive amounts of oxidised glutathione further improved refolding efficiency with ratios of 1/3 mM GSH/GSSG giving highest reactivity (black arrow).

action of both proteins occurred, suggesting that formed disulphide bridges in Bet are essential to allow Bet/APOBEC3 engagement. Excessive amounts of GSSG thereby slightly improved binding activity, which might be explained by formation of a mixed disulphide intermediate with GSSG, which is then slowly replaced by GSH. Similar advantages have been seen during refolding of tissue plasminogen activator (t-PA) containing 17 cysteines and were attributed to a higher solubility of GSSG-coupled refolding intermediates. In respect to these data, large scale refolding of Bet was performed under the identified conditions, the protein was subsequently dialysed against phosphate buffer and concentrated by another Ni-NTA chromatography step using non-denaturing phosphate buffers supplied with imidazol. SDS-PAGE analysis and CD-spectroscopy demonstrated a sufficient purity of purified Bet and the presence of defined secondary structures, which were in good agreement with values predicted from the primary sequence (Fig. 31).

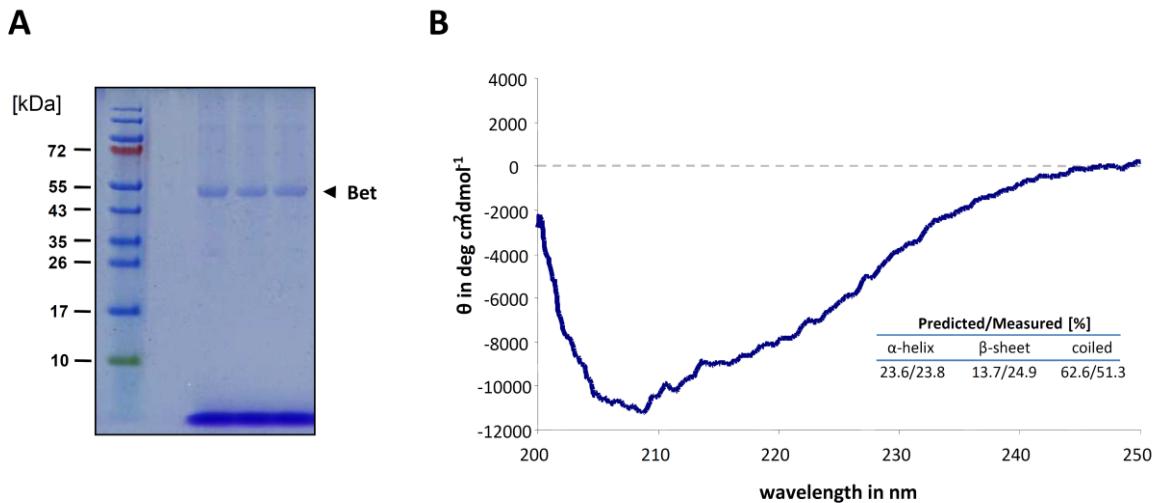


Fig. 31. Purity of FFV Bet and CD analysis of secondary structure in the refolded protein. (A) SDS-PAGE and Coomassie staining of purified FFV Bet. Molecular weights and the Bet protein at its expected size of 46 kDa (arrow) is indicated. The three lanes correspond to Elution fractions 1-3, respectively. (B) CD spectroscopy of renatured FFV Bet measured in a Jasco720 spectropolarimeter. The pattern resembles a typical chromatogram of a mixed alpha-beta protein and measured values correlate well with the secondary structure content predicted from the primary sequence (predicted/measured inset).

3.2.3 Production and characterisation of Bet/HIV-1 hybrid proteins

The protocols established for FFV Bet were successfully transferred to the Bet/HIV-1 hybrid proteins described earlier and all proteins could be obtained at high purity with yields of 11-18 mg/L expression culture. A first characterisation of these proteins by Western blot using 2F5 and 4E10 as well as a FPPR directed antiserum (J. Kreuzberger, unpublished), demonstrated their specific recognition by respective antibodies at the expected sizes and thus antigen and epitope integrity (Fig. 32).

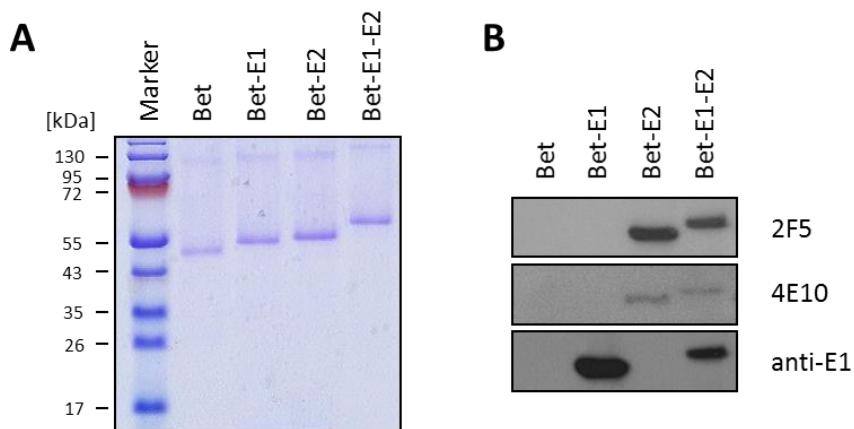


Fig 32. Characterisation of FFV Bet and Bet/HIV-1 fusion proteins. (A) Purity of the produced antigens. Two micrograms of recombinant proteins was loaded for SDS-PAGE and stained by Coomassie blue. The molecular mass of the marker is indicated. (B) Recognition of the produced antigens by the monoclonal antibodies 2F5 and 4E10 and a FPPR-specific antiserum. One hundred and fifty nanograms of recombinant proteins were transferred onto a PVDF membrane and tested with the antibodies 2F5 (0.4 µg/ml), 4E10 (1.2 µg/ml) and an E1-specific rat serum (1:800) to confirm the presence of the corresponding HIV-1 domains. Figure adopted from Mühle *et al.*, 2013a.

To investigate binding of 2F5 and 4E10 also under non-denaturing conditions, antigens were coated on ELISA plates overnight at 4°C and then probed with both antibodies in serial dilutions. In case of 2F5, no significant differences in recognition of the only MPER containing antigen Bet-E2 was observed when compared to proteins that presented MPER and FPPR together, either through mixing (Bet-E1+Bet-E2) or by direct linkage with a loop as in case of the Bet-E1-E2 antigen (Fig. 33). Interestingly, binding to the Bet-E1-E2 antigen was even slightly reduced when compared to Bet-E2 alone. However, in case of 4E10 a clear enhancement of recognition was obvious for the Bet-E1-E2 antigen, whereas the remaining antigens were detected weakly. These data showed that the HIV-1 epitopes are accessible for antibodies under physiological conditions and suggested that the FPPR domain contributed to improved binding of 4E10 but not 2F5 to the Bet-E1-E2 antigen.

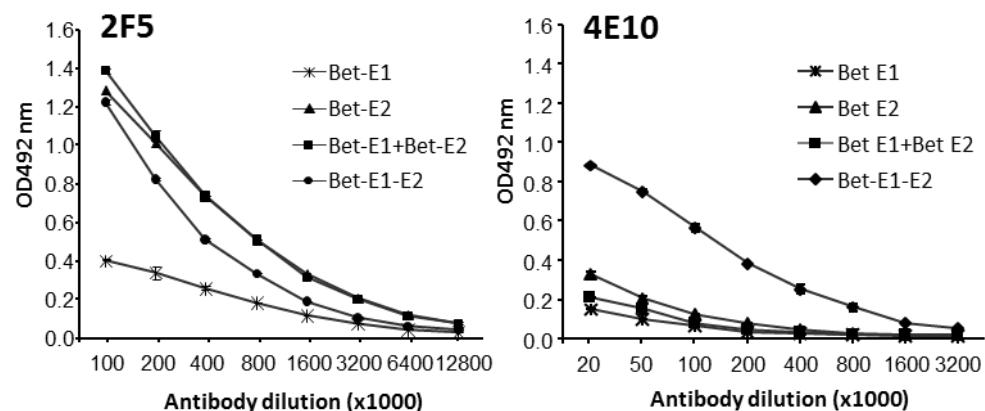


Fig. 33. Recognition of the produced antigens by the monoclonal antibodies 2F5 and 4E10 under non-denaturing conditions in an ELISA. One hundred nanograms of indicated Bet/HIV-1 antigens in phosphate buffer was coated and incubated with 2F5 (11.3 µg/µl) or 4E10 (11.9 µg/µl) serially diluted as indicated, followed by detection with a HRP anti-human IgG antibody and OPD development. Whereas only minor differences in 2F5 binding were detected, threefold increased binding of 4E10 to the FPPR-stabilised Bet-E1-E2 protein was found. Figure taken from Mühlé *et al.*, 2013a.

To study the binding of 2F5 and 4E10 to the hybrid proteins in more detail, SPR experiments were performed with both antibodies captured on Biacore-chips and antigens in solution (Table 11). High affinity binding of 2F5 in the nanomolar range was observed for all proteins containing the MPER epitope. In line with the previous ELISA results, the presence of FPPR residues did not allow increased affinity to the E2 domain and slightly reduced binding due to faster dissociation or slower association rates ($K_D = 5.9$ and 19 nM for Bet-E1+Bet-E2 or Bet-E1-E2 compared to 4.3 nM for Bet-E2). Much

higher protein concentrations were necessary to measure interactions in case of the 4E10 antibody. Due to the precipitation of recombinant Bet/HIV-1 proteins above 1 mg/ml (15-20 µM), no suitable concentration ranges to obtain reliable data for the Bet-E2 antigen and the combination of Bet-E2 and Bet-E1 could be selected. At the highest concentration tested (8 µM), no binding of Bet-E1 and a weak signal (15 RU) for Bet-E2 were observed, suggesting that the K_D values of Bet-E2 were probably in the micromolar range (data not shown). However, when the Bet-E1–E2 antigen was investigated, kinetic constants could be determined, as nanomolar protein concentrations yielded stable data. The dissociation constant of 223 nM resulted from slow target recognition ($k_{on} = 2.1 \times 10^3/\text{Ms}$) and a moderate dissociation rate ($k_{off} = 4.7 \times 10^{-4}/\text{s}$). Thus, the SPR data substantiated the ELISA results and the beneficial effect of the FPPR residues for enhanced 4E10 antibody binding.

TABLE 11. Kinetic constants determined by SPR measurements

Analyt	Ligand	k_{on} [1/Ms]	k_{off} [1/s]	K_D [nM]
Bet-E1	2F5	- ^a	-	-
Bet-E2	2F5	2.0×10^4	8.6×10^{-5}	4.3
Bet-E1+Bet-E2	2F5	1.9×10^4	1.1×10^{-4}	5.9
Bet-E1-E2	2F5	1.1×10^4	2.1×10^{-4}	19.0
Bet-E1	4E10	-	-	-
Bet-E2	4E10	n.d. ^b	n.d.	n.d.
Bet-E1+Bet-E2	4E10	n.d.	n.d.	n.d.
Bet-E1-E2	4E10	2.1×10^3	4.7×10^{-4}	223

^a no interaction observed

^b not determinable due to protein concentration limitations

3.2.4 Immunisation studies with recombinant Bet/HIV-1 fusion proteins

The results above showed that high affinity binding was achieved by fusing the HIV-1 epitopes to the C-terminus of Bet. To test if this translates into efficient immune responses to the HIV-1 parts in vivo and to determine the epitope specificity of such potential antibodies, all hybrid antigens were used for immunisation studies in rats. After three injections (250 µg/animal in CFA/IFA) in a three weeks interval, high levels of antigen-specific antibodies were induced in all animals, with titres reaching levels of 10^6 - 10^7 after the third immunisation when titrated against the administered antigen by ELISA (222). More importantly, antibodies to the respective HIV-1 parts were also elicited at high levels (titres of 10^3 - 10^5) when FPPR and MPER derived synthetic E1 and E2 peptides as well recombinant gp41

produced in bacteria were used as ELISA antigens (Fig. 34). Notably, the FPPR domain was more immunogenic than the MPER domain in all groups. The strongest MPER responses (titre of 8×10^3) was achieved when the Bet-E2 antigen was given alone or together with the Bet-E1 antigen (groups 365, 366), whereas in group 375 (Bet-E1-E2 antigen) E2 reactivity was quite low. The gp41 response was similarly strong within all groups and confirmed to be specific by Western blot (222). These results demonstrated that Bet/HIV-1 hybrids can be successfully used to induce HIV-1 specific antibodies that recognise their corresponding epitopes also in the molecular context of gp41.

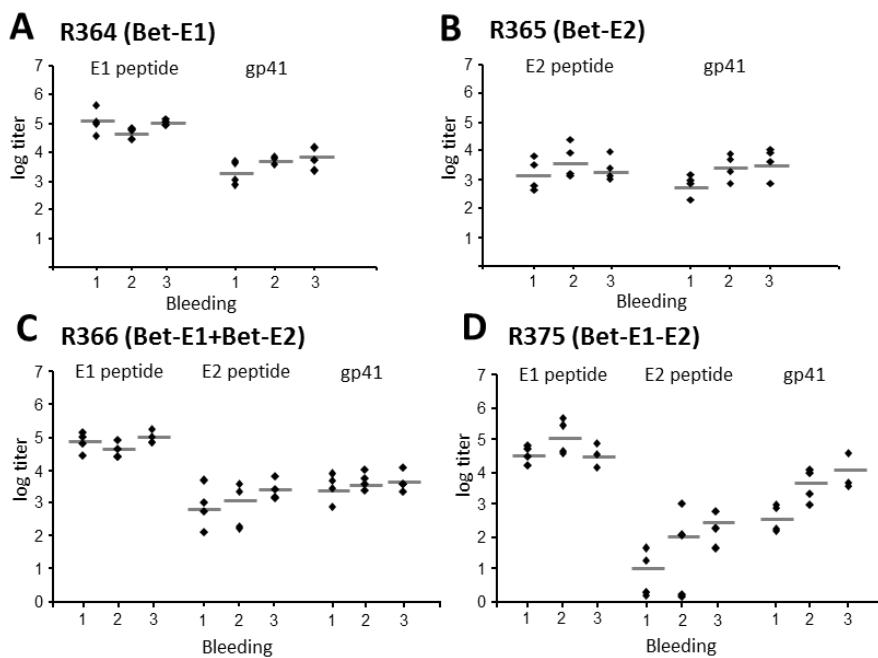


Fig. 34. Reactivity of sera from animals immunised with Bet/HIV-1 proteins against recombinant gp41 and synthetic E1 and E2 peptides as measured in an ELISA. Sera were serially diluted in a range of 10^2 – 10^7 . The endpoint titres of (A) sera from rat group 364, (B) sera from rat group 365 and (C), (D) sera from rat groups 366 and 375, all titrated against the E1 and/or E2 peptides as well as recombinant gp41, are given. The administered antigen is indicated in brackets. Figure reprinted from Mühle *et al.*, 2013a.

To further examine the epitope specificity of the induced HIV-1 antibodies, epitope mappings using microarrays spotted with overlapping peptides of the extended FPPR and MPER domains and two representative sera showing high overall antibody responses were performed (Fig. 35). In group 364 which obtained the Bet-E1 antigen a clear response to the FPPR was observed, with the epitope TLTVQARQL being the only antigenic determinant. The HIV-1 specific antibody response in group 365 (antigen Bet-E2) concentrated to the MPER domain with ELLELDKWA and ELLELDKWASLW

as main epitopes, which overlapped with the residues targeted by 2F5 (underlined). Antisera which were immunised with the Bet-E1 and Bet-E2 mixture (group 366) showed a predominant response to the FPPR epitope TLTQVQARQL and also recognition of MPER residues NEQELLELDKWASLW including the 2F5 epitope. The pattern observed in group 375 (Bet-E1-E2 antigen) was biased. Both sera reacted clearly with the FPPR epitope TLTQVQARQL, but the E2 response was variable, with one serum recognising residues NEQELLELDKWA whereas no MPER response was found in case of the other. The epitope mapping data were consistent with the previous ELISA results and confirmed the overall lower E2 reactivity observed there. In terms of consistency and strength in which 2F5 epitope-specific antibodies were induced, the Bet-E2 antigen appeared to be superior to all other antigens.

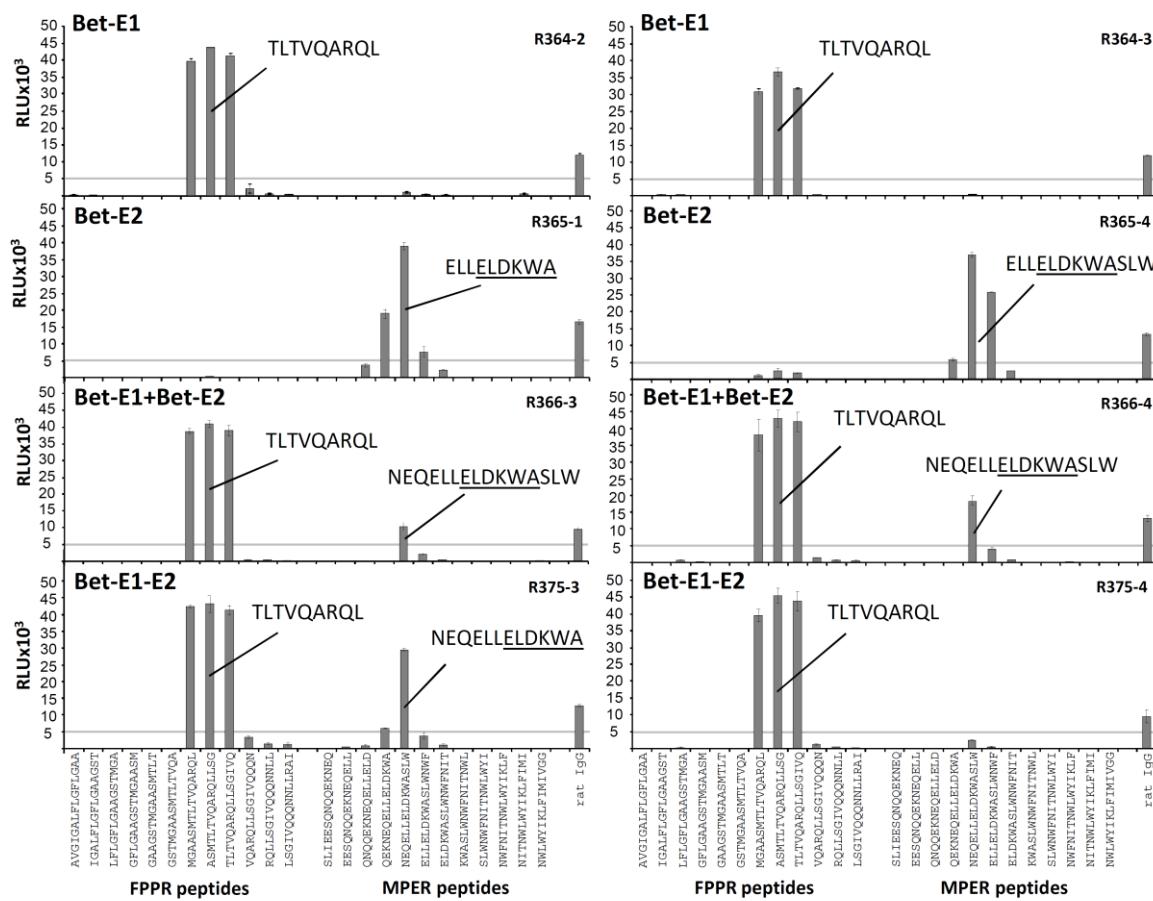


Fig 35. Epitope mapping of the sera from immunised animals. Microarrays spotted with overlapping 15-mer peptides of the prolonged HIV-1 FPPR and MPER domains were incubated with antisera and bound antibodies detected by a fluorescent secondary anti-rat antibody. Results are plotted as relative light units (RLU) against the individual FPPR and MPER peptides. The group number, the administered antigen and the recognised epitopes as defined as central amino acids shared by overlapping peptides exceeding a 5000 RLU intensity threshold (grey line) are indicated. As internal control, anti-rat IgG spotted on the slides were measured in parallel. Figure taken from Mühle *et al.*, 2013a.

The previous analysis of antisera focused on short synthetic peptides of the FPPR and MPER domain and gp41 produced in bacteria. However, binding of the induced antibodies to native Env as presented on the virus surface or on infected cells would be essential to mediate virus neutralisation. To address this question, C8166 cells infected with HIV-1 and non-infected cells were examined by FACS and indirect immunofluorescence for antibody binding. Analysing the same sera as in the epitope mapping experiments together with a rat pre-immune serum as control, different degrees of binding to such cells were observed. Whereas the pre-immune serum did as expected show negligible staining, sera containing FPPR-directed antibodies bound weakly and sera containing MPER antibodies strongly to the infected cells (Fig. 36A). In immunofluorescence analysis, these sera showed signals with varying appearance. The representative serum from rat group 364, containing only FPPR-directed antibodies caused weak and diffuse staining with no clear pattern. Conversely, all antisera that contained MPER-directed antibodies (rats groups 365, 366, 375) gave spot-like signals distributed on the surface of the

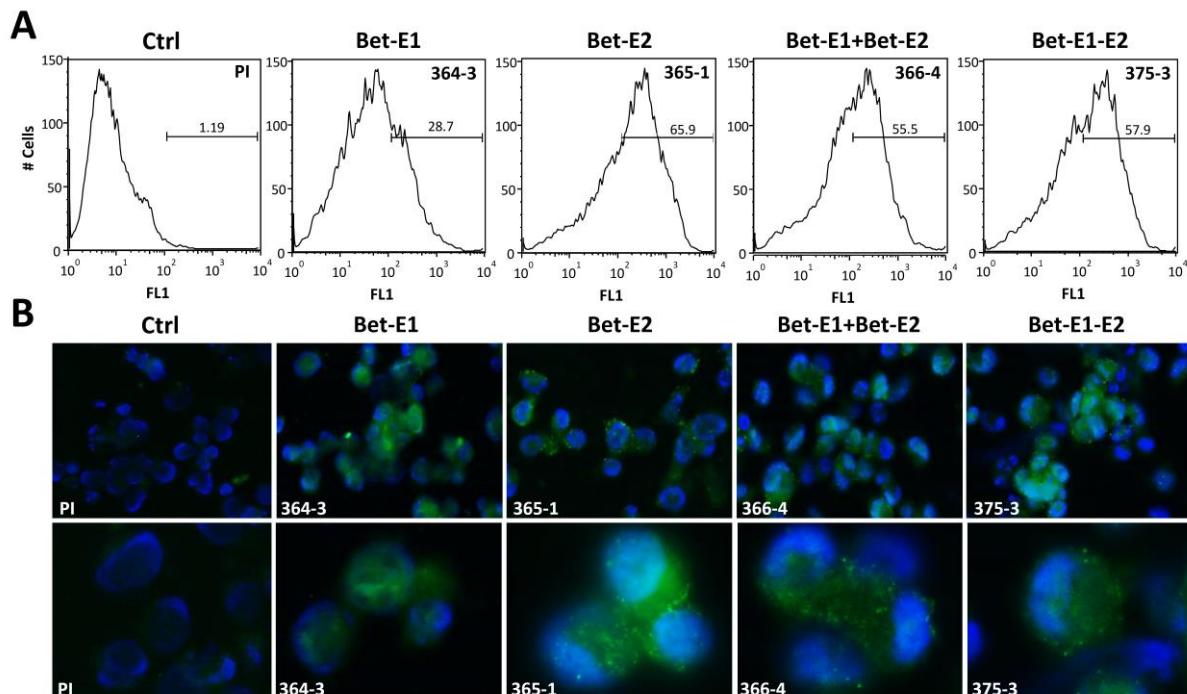


Fig. 36. Binding of induced antisera to HIV-1 pNL-4.3-infected C8166 cells. (A) Analysis of antibody binding by flow cytometry 72 h after infection. Cells were probed with pre-immune rat serum (PI) or antisera from a representative animal from the rat group immunised with the antigen indicated above. (B) Indirect immunofluorescence of C8166 cells 48 h after infection. Fixed cells were transferred to slides where they were incubated with the same sera as in (A). Rat IgGs were detected by an Alexa-Fluor 488 antibody and analysed at a magnification of 20x (upper panel) or 60x (lower panel). No reactivity was observed for the control (Ctrl), but antisera showed either diffuse staining when containing FPPR-targeting antibodies (rats 364) or distinct spots predominantly on the cell surface in antisera where MPER-directed antibodies were present (rats 365, 366 and 375). Figure from Mühle *et al.*, 2013a.

cells (Fig. 36B). As the MPER epitope has been found to be highly accessible in the gp41 fusion intermediate state after attachment of gp120, these spots most probably represented antibodies binding to cell-produced or infecting virus. Despite the beneficial antibody properties found (high titre, binding to residues overlapping with the 2F5 epitope, binding to native Env), none of the sera did prevent HIV-1 infection in a TZM-bl indicator cell line-based neutralisation assay at serum dilutions of 1:50 (222).

3.3 Development of improved loop-stabilised HIV-1 FPPR/MPER antigens

The data presented above demonstrated that during immunisation with the Bet/HIV-1 antigens mainly antibodies with specificity to the 2F5 epitope were induced whereas 4E10-like antibodies were absent in the sera. Since only the antigenicity of 4E10 was substantially improved in presence of the FPPR domain, any potential advantage achieved by the formation of the 4E10 FPPR/MPER conformational epitope did not translate into practice due to the low immunogenicity of the 4E10 epitope in the designed loop antigen. Further attempts were therefore undertaken to design proteins that allow improved 2F5 binding to take advantage of the higher 2F5 epitope immunogenicity. The following sections describe experiments performed in this regard.

3.3.1 FPPR/MPER antigen design and purification

As mentioned earlier, the initial loop antigen used in the Bet-E1-E2 protein was derived from the *ata* peptide modified to contain HIV-1 FPPR and MPER sequences. The lack of enhanced binding of 2F5 to this initial antigen was probably a result of suboptimal spacing or residue contacts, which could arise from a divergence between the theoretical positioning of residues evaluated by bioinformatics and the finally obtained protein structure. In order to find a positioning in which formation of the affinity-enhancing β -turn is possible, an extended set of antigens was designed in which the FPPR and MPER alpha helical flanking regions were stepwise shortened by single amino acid deletions (Fig. 37A). As both domains have mainly alpha helical character, these single amino acid deletions should result in shifting of both helices against each other, with an about 70° turn per deleted amino acid residue (Fig. 37B). Consequently, FPPR and MPER domains could contact at various angles with each other and the

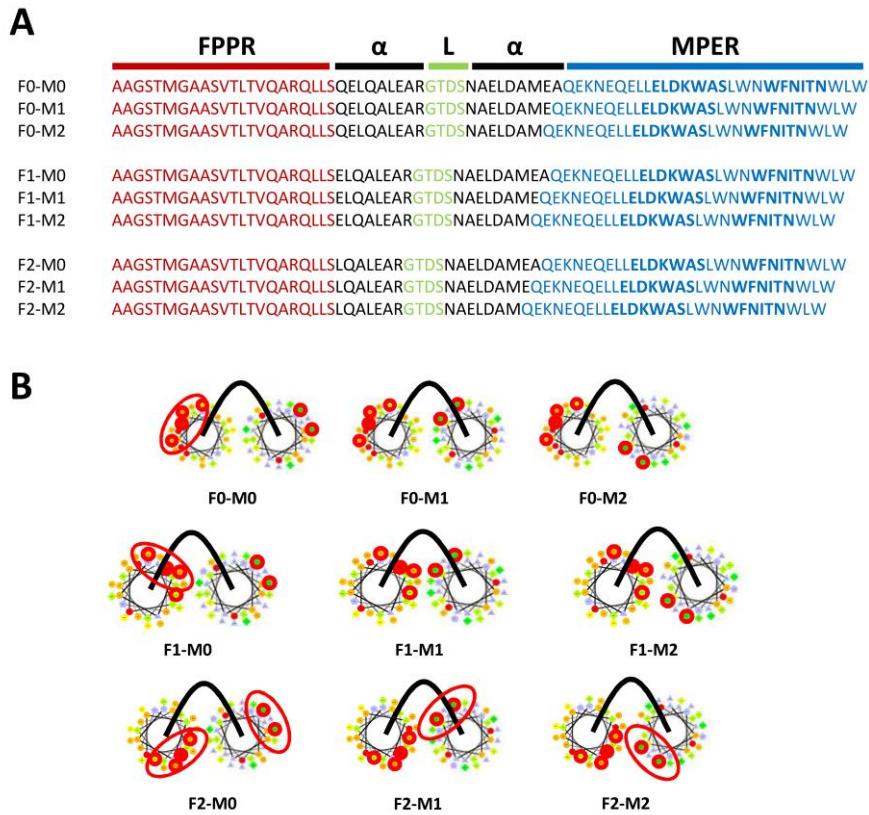


Fig. 37. Recombinant antigen sequences and design. (A) Amino acid sequences of the nine recombinant helix-loop-helix antigens comprising the fusion peptide proximal region (FPPR), the membrane proximal external region (MPER) connected by a loop element (L) and the alpha helical flanking amino acids (α). The epitopes of the broadly neutralising antibodies 2F5 (ELDKWAS) and 4E10 (WFNITN) in the MPER are highlighted in bold. The antigens are named corresponding to the number of amino acid deletions introduced into the flanking regions of the FPPR (F0, F1, F2) or MPER (M0, M1, M2). (B) Alpha helical wheel projections of all antigens. Critical residues for 2F5 binding in the MPER (D and W residues of ELDKWA) and opposite residues in the FPPR are marked as red circles to visualise the influence of single amino acid deletions on the positioning of these selected amino acids. For purification, all antigens also contained a N-terminal His-tag after a Gly-Ala-rich linker sequence (not shown).

2F5 epitope may be presented at different faces of the antigen. Starting with the longest construct synthesised by GeneArt, deletions were achieved via PCR mutagenesis and the corresponding constructs named after the number of deletions introduced into the FPPR and MPER flanking regions (F0, F1, F2 or M0, M1 and M2 respectively). The mutated sequences were then subcloned into pQE-30 Xa expression vector with an N-terminal His-tag for purification and spacer amino acids as in the original Bet-E1-E2 antigen (SGSAGAGAGGG). Expression optimisation was performed as described before, followed by large-scale expression in SCS-1 bacteria and TB medium. For purification, the sarcosyl extraction and refolding strategy established for the FV TM proteins was used, giving yields of about 3–5 mg of protein per litre expression culture. On SDS-PAGE analysis, these proteins were highly pure (> 95%) and migrated at their expected size (Fig. 38A). In line with this, the proteins eluted as a single

peak in gel filtration analysis (Fig. 38C). Notably, all antigens appeared at a retention volume corresponding to an approximate molecular weight of 35 kDa (14 ml, Fig. 38C). This was about threefold the size that was expected for monomeric proteins (12 kDa, ~20 ml retention volume), indicating that the antigens spontaneously trimerised in solution. To further investigate the oligomerisation state, all antigens were subjected to native PAGE analysis. Here, the proteins appeared to varying degrees as monomers, dimers and trimers as apparent by their shifted migration pattern within the gel (Fig. 38B). Among the introduced deletions, those of the FPPR but not of the MPER flanking regions had the strongest impact on trimer stability. Whereas the F0 and F2 constructs were predominantly trimerised, the F1 deletion mutants were almost exclusively dimers (Fig. 38B). In a next step, the reactivity of the produced proteins with the monoclonal antibody 2F5 was tested in a non-denaturing dot blot assay with serially diluted antigens or synthetic HIV-1 FPPR and MPER peptides (Fig. 38D). As in native PAGE experiments, shifting of the FPPR residues had the strongest impact on antibody binding and deletion of two FPPR flanking amino acids (F2 constructs) significantly enhanced the signal intensity for 2F5 but also 4E10 (Fig. 38D). To a lesser extent, rotating the MPER also improved recognition, particularly for the 4E10 antibody where a slight increase in binding with every additional MPER deletion was evident (Fig. 38D). Most importantly, the FPPR/MPER antigens were better recognised as the HIV-1 MPER E2 peptide spotted at equal amounts, again supporting the beneficial role of the FPPR residues for epitope stabilisation or orientation (lane E2, Fig. 38D). These results demonstrated that selective modification of the FPPR/MPER orientation influences the intermolecular interactions of the antigens, leading to an altered pattern of epitope accessibility for the monoclonal antibodies 2F5 and 4E10.

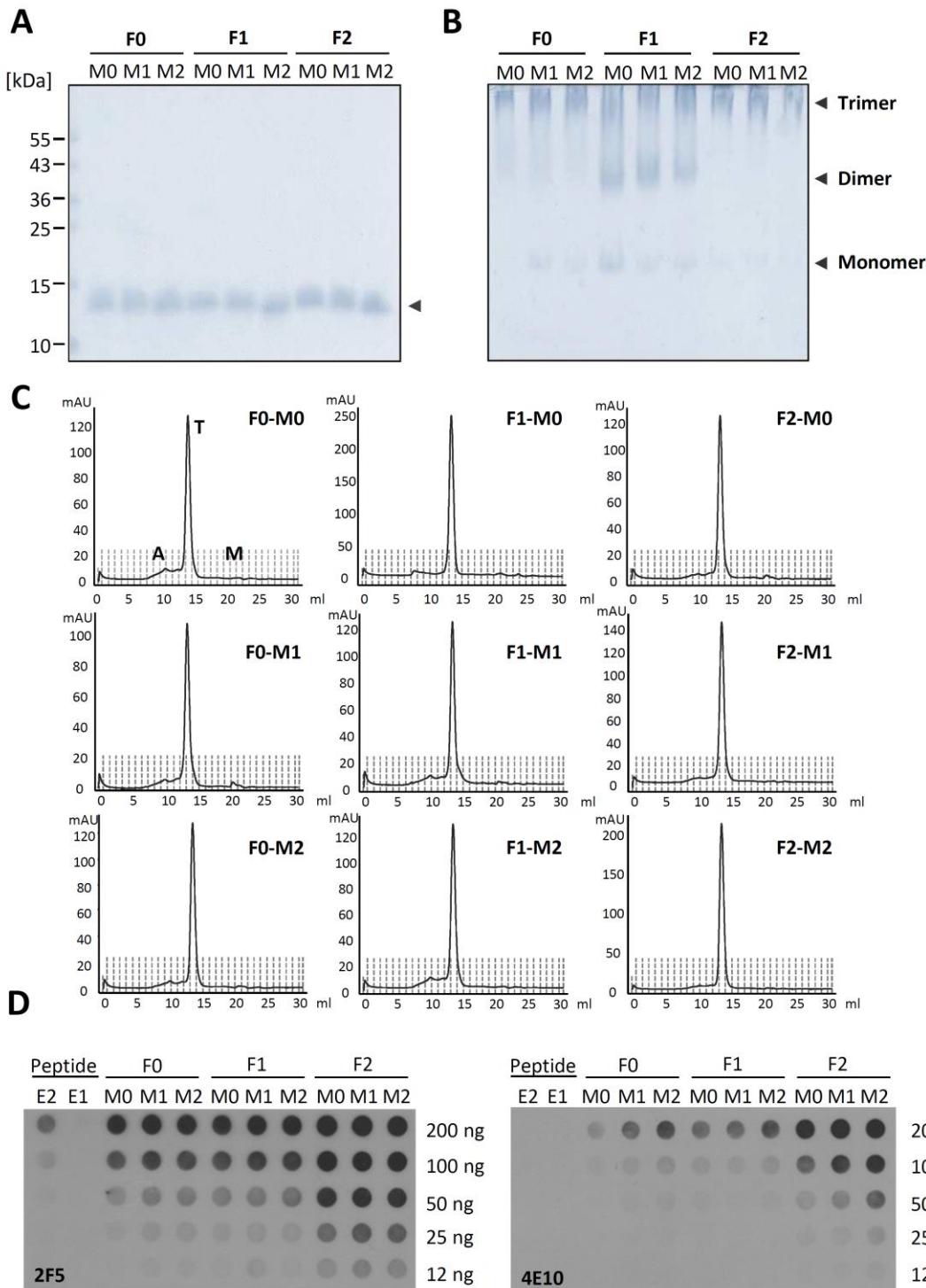


Fig. 38. FPPR/MPER antigen purification and characterisation. (A) SDS-PAGE analysis of the recombinant proteins after Ni-NTA affinity purification. The arrow indicates the proteins at the expected molecular weight of ~12.5 kDa. (B) Native PAGE analysis of purified proteins reveals different tendencies to build monomers, dimers and trimers. Recombinant proteins (3 µg) were mixed with sample buffer without SDS or DTT and separated on native PAGE without boiling of the samples. Truncation of the FPPR flanking regions had the strongest impact on trimerisation, whereas the influence of MPER mutations is negligible. (C) Analytical gel filtration of purified proteins on a Superdex 75 10/300 HR column with PBS, 0.1% sarcosyl at 0.4 ml/ min. Proteins elute as trimers in a main peak at 14 ml (T, ~35 kDa) next to some aggregated material (A, ~8 ml) and the monomeric form (M, ~20 ml). The column was calibrated with ovalbumin and lysozyme (45 kDa and 17 kDa, not shown) (D) Binding of the monoclonal antibodies 2F5 (0.3 µg/µl) and 4E10 (0.5 µg/µl) to serially diluted FPPR/MPER proteins or HIV-1 FPPR and MPER (E1, E2) peptides, each transferred to PVDF membranes by vacuum at indicated concentrations. Deletions in the FPPR flanking region permit a better binding of 2F5 and 4E10.

3.3.2 Immune response to FPPR/MPER antigens in immunised mice

To assess how far the observed differences in antigenicity relate to the immune response induced by these antigens, all FPPR/MPER proteins were used for immunisation experiments. The purified FPPR/MPER antigens were emulsified in incomplete Freund's adjuvant and injected three times into BALB/c mice in a three weeks interval followed by final bleeding after additional two weeks (Fig. 39A). Except for the PBS control group and the pre-immune sera, in all sera from the final bleeding antibodies against gp41 were detected in Western blot analysis (Fig. 39B). Average antibody titres as determined by ELISAs using recombinant gp41 reached levels between 10^3 and 10^5 . Sera from animals immunised with F2-M1 gave the highest response (Fig. 39C). Titres in a similar range were observed when synthetic HIV-1 FPPR and MPER peptides (E1 and E2) were used as antigen. However, dependent on the antigen injected, the strength of the response to either FPPR or the MPER differed (Fig. 39D). Upon rotation of FPPR residues, the E1 response increased steadily, with the most distinct pattern when two MPER flanking region deletions were present (Fig. 39D, upper panel). For E2 immunogenicity, both, rotating the FPPR as well as the MPER helix influenced the induced antibody pattern, with the strongest E2 reactivity achieved with the construct F2-M1 (Fig. 39D, lower panel). Interestingly, only for the constructs able to stably trimerise in native PAGE (F0, F2, Fig. 38B) a strong impact of FPPR or MPER rotation on the antibody response could be detected, whereas the dimeric F1 constructs were basically unaffected (Fig. 39D). Taken together, these data showed that all antigens were immunogenic and that the differences in antigen properties also affected the resulting immune response. A positive correlation was found between the oligomerisation state and gp41 antibody titres. However, the increased antigenicity of the constructs for antibodies 2F5 and 4E10 predicted only in part the strength of the observed MPER immune responses in these experiments.

3.3.3 Epitope specificity of induced antibodies

Antisera of each group were pooled to fine-map the recognised epitopes using HIV-1 FPPR and MPER peptide microarrays (Fig. 40). Independent of the introduced deletions, one epitope, TLTQVQARQL, was recognised in the FPPR by all antisera (Fig. 40, Table 12). Reactivity against the MPER peptides was mainly directed against the 2F5 epitope (ELDKWA) and no antibodies against the 4E10 epitope were

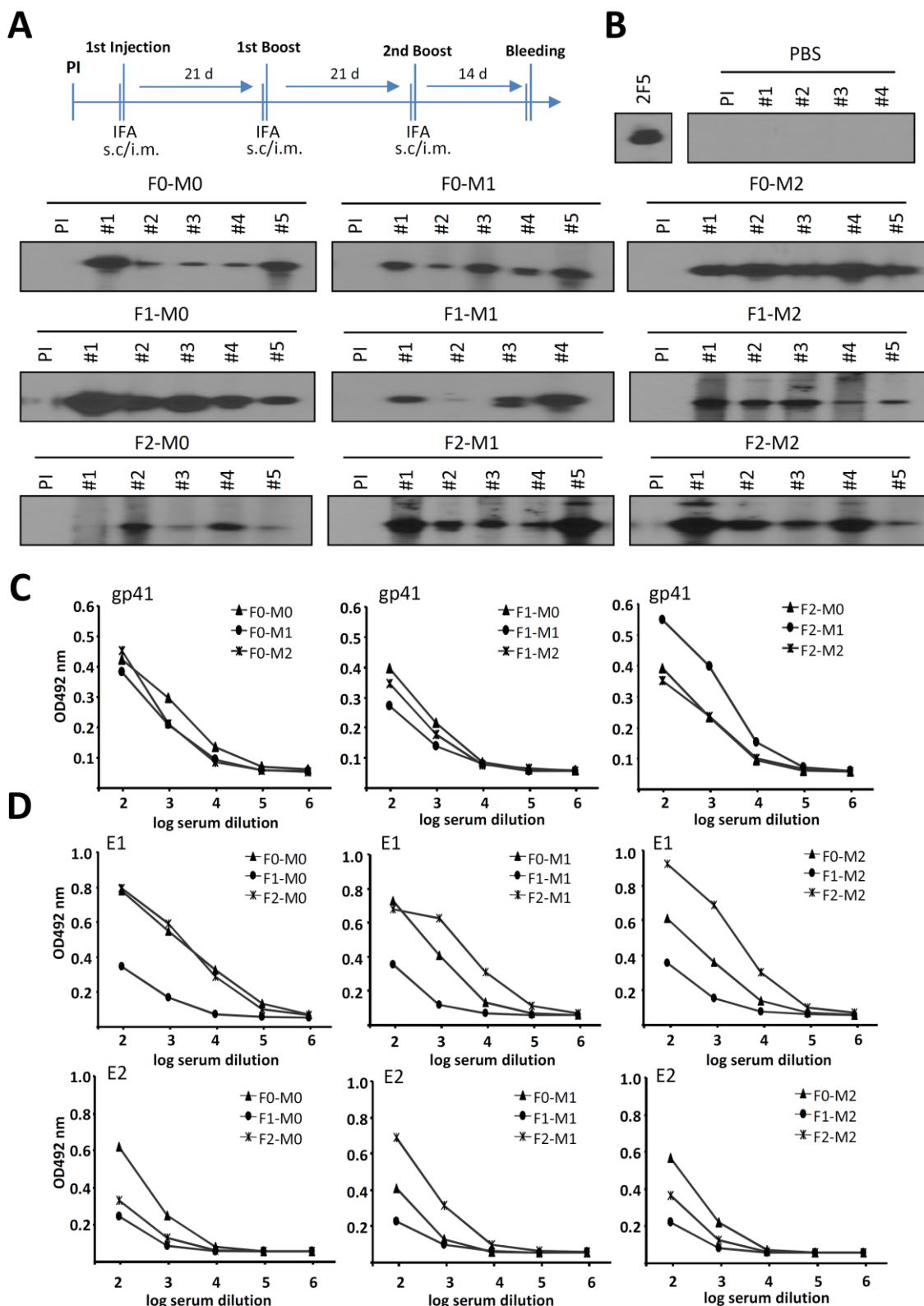


Fig. 39. Immunisation and antibody response to FPPR/MPER proteins in vaccinated mice. (A) Immunisation schedule, adjuvant and route of administration for the conducted immunisation study in mice PI- pre-immune serum, IFA-incomplete Freund's adjuvant, s.c.- subcutaneous injection, i.m.- intramuscular injection. (B) Western blot analysis of antisera from individual mice (#1-5) after the final bleeding (dilution 1:400) using purified gp41 as antigen. PI – pre-immune serum pool of the group indicated. (C,D) Titration of the antisera against recombinant gp41 or (D) HIV-1 FPPR and MPER peptides (E1, E2 respectively). The group mean absorbance ($n=5$) for the indicated serum dilution is given.

evident. Notably, epitopes in the MPER shifted slightly to the N- or C-terminus of the ELDKWA sequence, correlating with the rotation of the MPER domain (Fig. 40, Table 12). From all antisera tested, sera from mice in group F1-M2 recognised an epitope with complete identity to the 2F5 epitope.

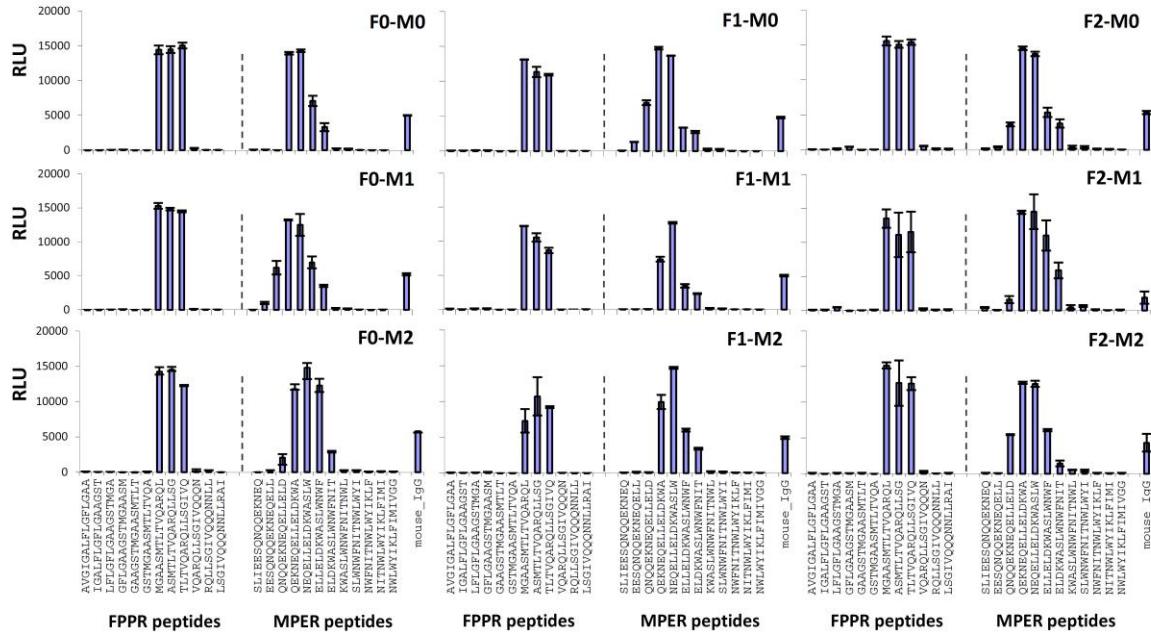


Fig. 40. Epitopes recognised by the immune sera of FPPR/MPER protein immunised mice. Microarrays spotted with 15mer overlapping peptides were probed with serum pools of the indicated mice group at a dilution of 1:100 and bound antibodies were detected by a fluorescent secondary antibody. Epitopes were defined as shared amino acids with signal intensity above 4000 relative fluorescence units (RLU). Dependent on the administered antigen, shifting or broadening of the antibody response to the 2F5 epitope ELDKWA in MPER is observed, whereas FPPR epitopes were basically identical (see also Table 12).

Table 12. Epitopes recognised by group-pooled antisera after immunisation with indicated loop antigens.

Antigen	Epitope	
	E1 domain	E2 domain
F0-M0	TLTVQARQL	ELLELDKWA
F0-M1	TLTVQARQL	NEQELLELD
F0-M2	TLTVQARQL	ELLELDKWA
F1-M0	TLTVQARQL	ELLELD
F1-M1	TLTVQARQL	NEQELLELDKWA
F1-M2	TLTVQARQL	ELDKWA
F2-M0	TLTVQARQL	ELLELDKWA
F2-M1	TLTVQARQL	ELLELDKWA
F2-M2	TLTVQARQL	ELLELD

3.3.4 Analysis of the neutralising capacity of induced antisera

To test for potential HIV-1 specific neutralising antibodies in the produced antisera, neutralisation tests based on TZM-bl indicator cells were performed (Table 13). Although the individual constructs differed by only few amino acids, some of the sera showed titratable neutralising activity at serum dilutions of 1/10-1/80 from which representative data of the 1/40 dilution are summarised in Table 13. Substantial neutralisation was observed only with sera from animals immunised with antigens F1-M1 or F1-M2 and

Table 13. Neutralisation of HIV-1 pNL-4.3 and SIVmac239 by mice antisera at a 1/40 dilution on TZM-bl indicator cells.

Sample	Neutralisation ^a	
	HIV-1	SIVmac239
Non-infected	-	-
Infection	-	-
2F5 (10 µg/ml)	70%	-
PBS-PI	20%	-
PBS-1	29%	-
PBS-2	17%	-
PBS-3	-	-
PBS-4	-	-
F0-M0-PI	-	-
F0-M0-1	56%	-
F0-M0-2	29%	-
F0-M0-3	41%	-
F0-M0-4	1%	-
F0-M0-5	-	-
F0-M1-PI	-	-
F0-M1-1	34%	-
F0-M1-2	25%	-
F0-M1-3	28%	-
F0-M1-4	26%	-
F0-M1-5	-	-
F0-M2-PI	-	-
F0-M2-1	-	-
F0-M2-2	-	-
F0-M2-3	37%	-
F0-M2-4	29%	-
F0-M2-5	25%	-
F1-M0-PI	27%	-
F1-M0-1	30%	-
F1-M0-2	30%	-
F1-M0-3	42%	-
F1-M0-4	24%	-
F1-M0-5	74%	22%
Sample	Neutralisation ^a	
	HIV-1	SIVmac239
F1-M1-PI	13%	-
F1-M1-1	57%	-
F1-M1-2	68%	-
F1-M1-3	57%	-
F1-M1-4	69%	-
F1-M2-PI	26%	-
F1-M2-1	80%	-
F1-M2-2	59%	22%
F1-M2-3	43%	-
F1-M2-4	56%	-
F1-M2-5	58%	-
F2-M0-PI	13%	-
F2-M0-1	25%	-
F2-M0-2	58%	-
F2-M0-3	52%	-
F2-M0-4	62%	-
F2-M0-5	65%	-
F2-M1-PI	5%	-
F2-M1-1	50%	-
F2-M1-2	41%	-
F2-M1-3	55%	-
F2-M1-4	54%	-
F2-M1-5	59%	-
F2-M2-PI	18%	-
F2-M2-1	60%	-
F2-M2-2	72%	-
F2-M2-3	61%	-
F2-M2-4	70%	14%
F2-M2-5	64%	-

^a percent difference to infection control, orange ≤ 50% neutralisation, red ≤ 70% neutralisation.

all constructs having two deletions in the MPER flanking regions (F2-X antigens, Table 13). Importantly, this neutralising activity clearly exceeded the level of background neutralisation observed in some of the pre-immune sera or antisera from the PBS group. Furthermore, weak neutralisation of SIVmac239 was found only in three sera, indicating that neutralisation was indeed HIV-1 specific. Due to the low amounts of antisera available from mice, serum characterisation was limited to the experiments performed above. As the number of immunisations necessary to obtain nAb, the breadth of neutralisation as well as further confirmation of MPER-mediated neutralisation e.g. by using affinity purified IgGs was also of interest or needed, the immunisation study was repeated in hamsters using the antigens that yielded nAb (F1-M1 to F2-M2). Proteins were emulsified in IFA (250 µg/animal/immunisation), administered through the same route (i.m and s.c) and at a time schedule as before (three weeks interval), but boosting was continued for up to five times with the aim to further increase nAb levels. For reasons that are not completely clear, the overall immune responses induced in hamsters was extremely low (gp41 ELISA reactivity ~0.15 AU at serum dilution of 1:100 compared to ~0.3 AU at dilutions of 1:1000 in mice), the induced antibodies were only directed against the FPPR and no neutralisation was observed (data not shown). This suggested that the applied immunisation procedure might have to be altered to obtain robust immune responses in hamsters or that species-specific differences exist that prevent the formation of MPER-directed antibodies here. Repetition of the study should therefore be performed, preferably in rats, guinea pigs or rabbits, from which larger amounts of serum can be obtained.

3.4. Construction of replicating FFV/HIV-1 hybrid vectors

The previous results demonstrated that the Bet/HIV hybrid strategy can be successfully applied to induce HIV-specific antibodies with advantageous characteristics. In combination with the improved loop antigens obtained through systematic optimisation, this approach thus seemed most promising for a transfer into replicating FFV. The following section describes the construction of such hybrid vectors and their subsequent characterisation *in vitro*.

3.4.1 Construction of chimeric FFVs containing HIV-1 epitopes

Chimeric viruses were constructed by two sequential cloning steps (Fig. 41). First, a unique *NheI* restriction site together with a His-tag was fused to the C-terminus of Bet in the FFV molecular clone, giving rise to the plasmid pCF7-Bet-His. In this construct, HIV-1 sequences were afterwards introduced

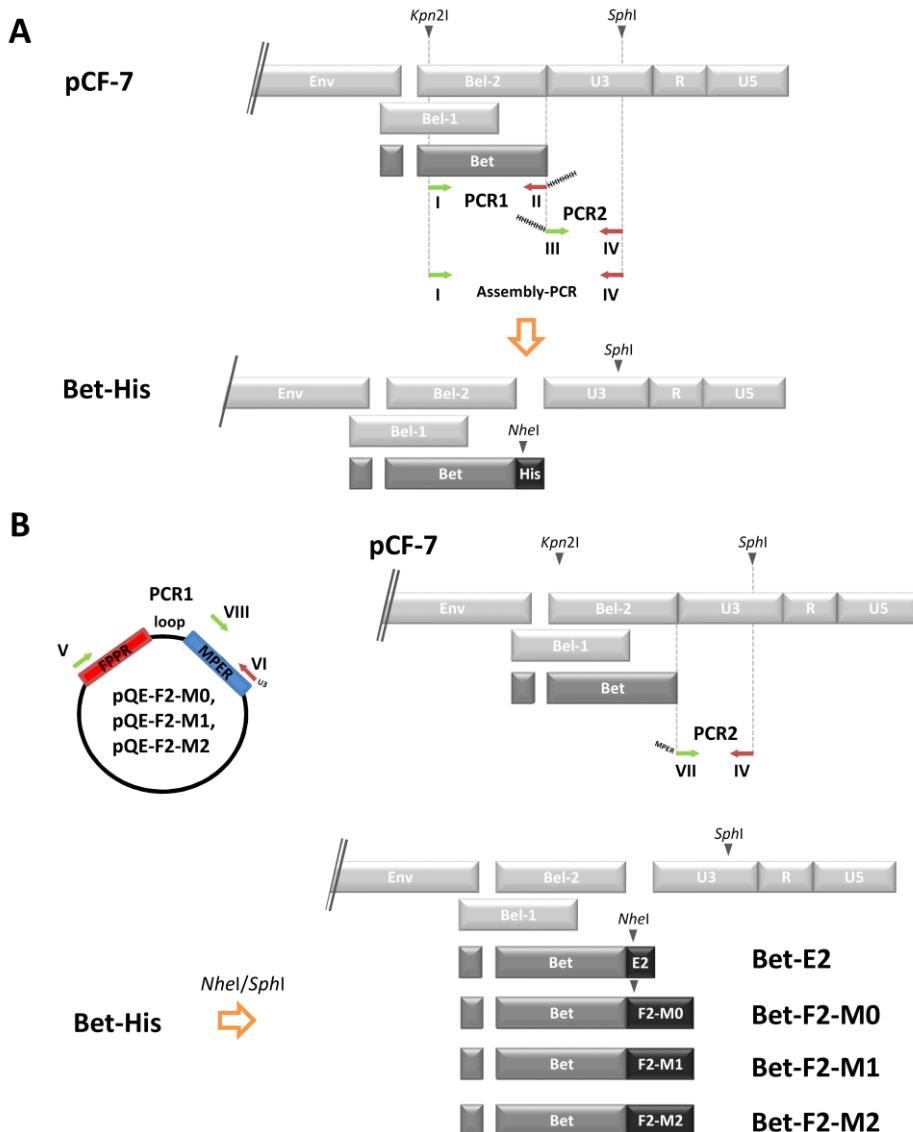


Fig. 41. Construction of FFV vaccine vectors. (A) Nucleotides encoding Asp and Ser residues corresponding to a *NheI* restriction site and a C-terminal His-tag were introduced directly before the Bet stop codon by assembly PCR using primers I and II for PCR 1 and primers III and IV for PCR 2, both having the nucleotide sequence of the His-tag in common. After amplification of the full-length PCR amplicon with primers I and IV and PCR1 and PCR2 DNA as template, digestion with *Agel* (compatible with *KpnI* overhangs) and *SphI* allowed introduction into the *KpnI/SphI*- digested molecular clone pCF-7 yielding the plasmid Bet-His. (B) Plasmids Bet-F2-M0, F2-M1 and F2-M2 were cloned by amplification of HIV-1 sequences from the prokaryotic expression plasmids pQE-F2-M0, F2-M1 and F2-M2 described in the text. In PCR1 primers V containing a *NheI* restriction site and VI encoding nucleotides of the U3 region were used. PCR 2 took the wild type pCF-7 plasmid as template and included parts of the MPER in the 5' primer overhang and amplified the U3 region beginning after the Bet stop-codon down to the *SphI* restriction site with primers VII and primer IV. The full-length amplicon was obtained after an additional PCR using outer primers V and IV. Plasmid Bet-E2 was prepared with primers VIII encoding a *NheI* restriction site and primer VI with plasmid pQE-F2-M0 as template for PCR 1. PCR 2 was identical to cloning of Bet-F2-X constructs described above. The full-length amplicon was constructed after an additional PCR using outer primers VIII and IV. Full-length inserts were digested with *NheI* and *SphI* and cloned into the identically treated Bet-His construct. All plasmids were sequenced in the cloned regions to confirm insert integrity. Green arrows: forward primer; red arrows: reverse primer. Restriction sites are in italics. Figure taken from Mühle *et al.*, 2013b.

by using the new *Nhe*I site and the unique *Sph*I site located in the U3 region of the 3' LTR. The required inserts were generated by fusion PCR, joining together the HIV-1 sequences amplified from the loop expression plasmids and the FFV LTR region starting behind Bet and ending with the *Sph*I site. As HIV-1 antigens, the E2 domain and the FPPR/MPER loop antigens F2-M0, F2-M1 and F2-M2 were selected for transfer into FFV. This was based on the previous finding, that including the MPER alone resulted in highest antibody titres with ELDKWA specificity when compared to the initial Bet-E1-E2 antigen. The loop antigens chosen comprised the proteins which contained two deletions in the FPPR flanking region (F2-X) since they were recognised best by 2F5 and resulted, at least in mice, in the induction of neutralising antibodies. As in the recombinant Bet/HIV-1 antigens, spacer amino acids between Bet and the fusion parts were included to assure that HIV-1 epitopes protrude from the Bet carrier and are readily accessible. Figure 42 gives an schematical overview of all constructed FFV clones.

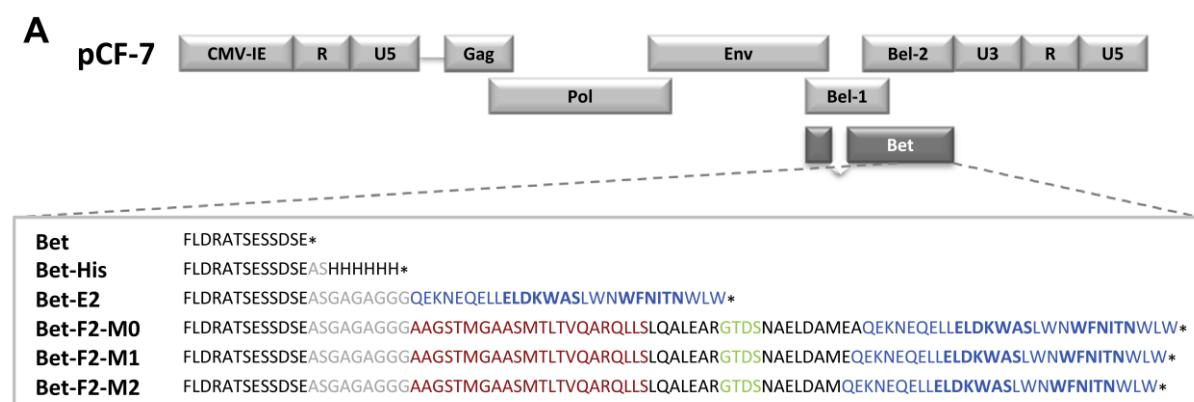


Fig. 42. Construction of chimeric FFV. Schematic illustration of the FFV molecular clone pCF-7 and amino acids introduced in frame to the C-terminus of Bet. The flanking 3' LTR sequences were left unaltered. Between Bet and inserts, spacer amino acids (AS for Bet-His, ASGAGAGGG for the other constructs) were cloned. Labelling of the HIV-1 MPER (E2) or FPPR and MPER domains is corresponding to that in Figure 37. Figure adopted from Mühle *et al.*, 2013b.

3.4.2 Characterisation of chimeric viruses

To test if epitope insertion was compatible with virus production, the constructed plasmids were transfected into 293T cells and supernatants analysed for the presence of infectious particles by titration on FeFAB indicator cells. All vectors gave rise to functional progeny virus, with titres that were comparable to the only slightly modified Bet-His construct and reached $\sim 10^4$ IU/ml (Fig. 43A). Analysing transfected as well as directly transduced cells by immunoblot, all vectors promoted the

expression of the Bet or Bet/HIV-1 fusion proteins at the expected sizes. Furthermore, all constructs modified to contain the MPER epitope also specifically reacted with the 2F5 antibody, confirming that HIV-1 epitope expression is achieved (Fig. 43B). Notably, the enhanced recognition by 2F5 observed for antigens F2-M0 to F2-M2 was compensated by a reduced expression level of these somewhat larger fusion proteins compared to Bet-His and Bet-E2, respectively (Fig. 43B). As the vectors were intended for long-term antigen delivery the stability of replication and transgene expression was an important point to investigate. To address this, supernatants from the individual constructs were passaged every three to four days on permissive feline CRFK cells and virus titres and transgene expression monitored

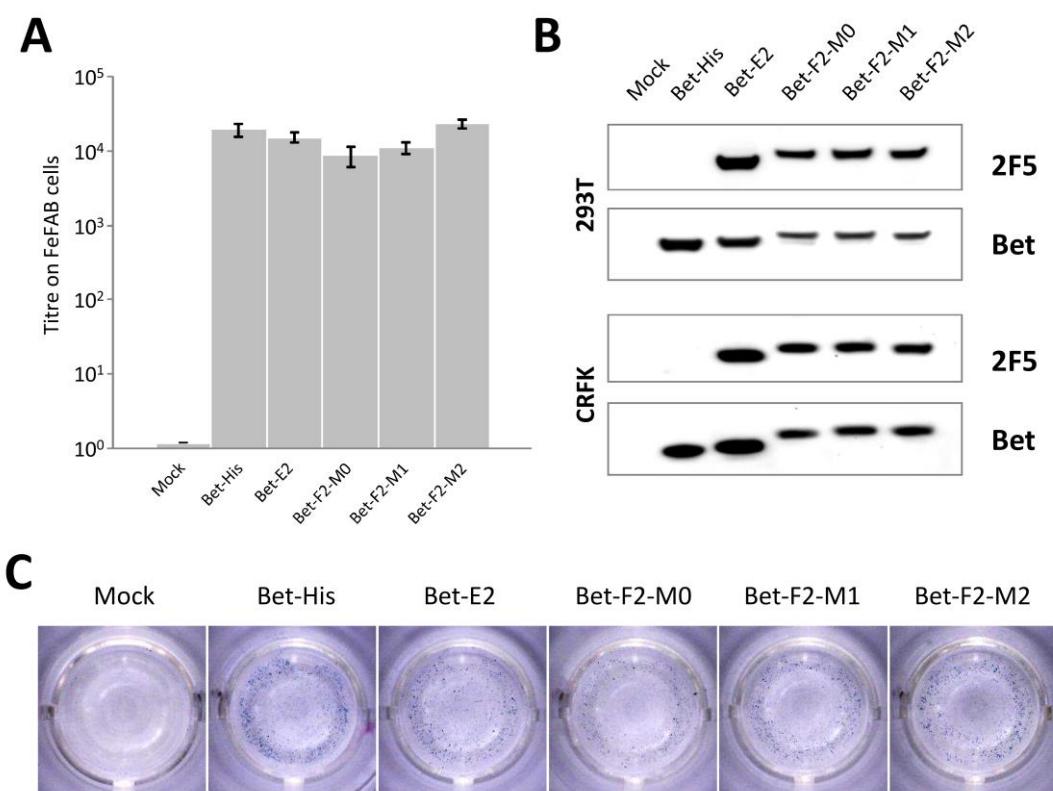


Fig. 43. Characterisation of chimeric FFV. (A) Titration of supernatants from transfected 293T cells on FeFAB indicator cells. Progeny viruses with titres comparable to that of pCF-7 containing only a His-tag were obtained. (B) Transgene expression in transfected 293T and directly transduced CRFK cells using supernatants from transfected 293T cells. Cell lysates were prepared two days post transfection or three days after transduction, separated by SDS-PAGE, immunoblotted and then probed with 2F5 or a Bet specific antiserum. Transgene expression was specifically detected with 2F5 in all chimeric constructs containing the MPER but not Bet-His or a GFP expressing vector (mock control), confirming the integrity of constructed clones. (C) Infectivity of chimeric viruses and/or a GFP expressing vector (mock control) on feline cells. Cleared supernatants from transfected 293T were transferred to FeFAB indicator cells which were stained 3 days later with X-gal solution to verify infection. Figure adopted from Mühlé *et al.*, 2013b.

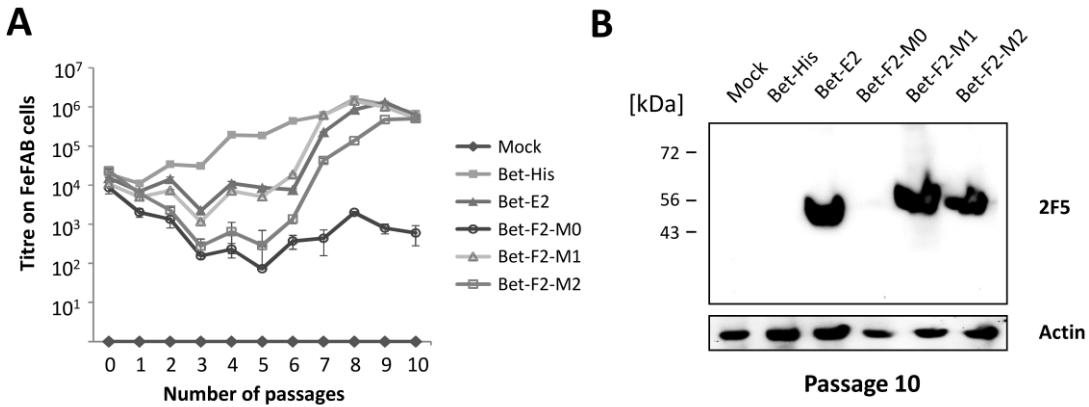


Fig. 44. Viral titres and transgene expression after extended passaging on feline CRFK cells. (A) Titres of chimeric viruses and or a GFP expressing vector (mock control) titrated on FeFAB indicator cells after the number of indicated passages. (B) Transgene expression after passage 10 as detected using the 2F5 antibody. Protein loading was verified using an antibody against actin. Figure taken from Mühle *et al.*, 2013b.

with time (Fig. 44). The viral titre of the Bet-His hybrid steadily increased upon passaging on these cells with final titres approaching 1×10^6 IU/ml. In case of the constructs that contained the MPER or FPPR/MPER inserts, an initial decrease of virus titres was observed. However, starting with passage three and five, FFVs expressing Bet-E2 and Bet-F2-M1 or Bet-F2-M2, respectively, finally also recovered to levels indistinguishable from the Bet-His construct with 1×10^6 IU/ml in passage ten (Fig. 44A). In contrast, the vector expressing the Bet-F2-M0 antigen maintained low level replication ($\sim 5 \times 10^2$ IU/ml) throughout the whole experiment. As this pattern suggested that adoptive arrangements within FFV may occurred to compensate the burden of insert expression, it was essential to examine if transgene expression is maintained also in the final passages. Using cell lysates from passage ten for immunoblotting with 2F5, the presence of the 2F5 epitope could be confirmed for the Bet-E2 as well as the Bet-F2-M1 and Bet-F2-M2 chimeras (Fig 44B). In contrast, the Bet-F2-M0 hybrid did not show any reactivity with 2F5, indicating that it had either lost its transgene or transgene expression could not be detected due to the low levels of replication/number of infected cells. Analysis of cell lysates from passage 1-10 with a Bet-specific antiserum confirmed that the latter was the case and Bet expression vanished after the third round of transfer to fresh cells (data not shown). In summary, this results demonstrated that the constructed FFV hybrid vectors efficiently present the intended HIV-1 epitopes and that replication-competence is maintained in three of four of the constructed hybrid vectors.

4. Discussion

The obstacles in developing a HIV vaccine are manifold. They lie in the nature of HIV infection from which no spontaneous cure in patients has been observed, the high diversity of the virus as well as its rapid development of mutations which hamper universal vaccine development (9). Moreover, quick establishment of HIV reservoirs and viral latency, active immunosuppression and destruction of the biological system responsible for antiviral immunity further complicates the issue (43). The best way to avoid the drawbacks associated with HIV infection and subsequent immune dysfunction would be a vaccine capable to completely block HIV entry and its integration into target cells where it may persist. Here, bnAb represent a promising target to achieve this goal, as such antibodies effectively clear the virus and can provide sterilising immunity (66, 234, 235). Furthermore, they are able to eliminate potential breakthrough infections by mediating killing of infected cells through mechanisms of ADCC or ADCVI and they can block HIV transcytosis at the virological synapse (30, 49-52). Whereas induction of bnAb by immunisation remains a challenge, factors correlating with their emergence during natural HIV infection have been identified and comprise high viral loads during the acute phase, low CD4 T cell counts and the time the individual is infected (35, 36, 236, 237). This suggests a role for high level of Env available during early stages and extended antigenic stimulation to this antigen as a requirement for induction of bnAb and is further supported by the high degree of somatic mutation that can be found in the genes of most of these bnAb (53, 67, 238, 239). Considering this, recent attempts aim to design B cell lineage immunogens to stepwise recapitulate antigenic stimulation over time and to overcome potential depletion of self-reactive bnAb antibodies by immune tolerance mechanisms (67, 77, 240). However, it has to be noted that only 20-30% of HIV positive individuals develop such antibodies even over time (32, 34), that HIV triggered B cell dysfunction might be responsible for the slowed down antibody responses (241-243) and that some studies report the successful induction of bnAb by rather simple immunisation regimens (244-247). Overall, this indicates that in healthy individuals a suitable immunogen presented as B cell prime, to which antibody responses can further improve through affinity maturation might present a way for the effective induction of bnAb in vaccine recipients. The strategy of using replicating vectors for long-term HIV antigen delivery is particularly

suitable in this regard, since affinity maturation to an immunogenic epitope is driven by such a delivery system *per se*. Furthermore, this approach does not require a comprehensive knowledge of antibody maturation pathways, which are complex and difficult to determine (77). The first live attenuated vaccine viruses based on SIVΔnef belong to the most potent inhibitors of a subsequent infection. Unfortunately, their capacity to revert to a more virulent phenotype and issues related to vector integration have excluded their use in humans (248). Nevertheless, the fact that live vectors have an unmatched ability to induce durable and multicomponent immunity against various pathogens has preserved the interest in such vectors high (249, 250). Recently, several reports on the successful application of replicating viruses for preventing disease have reinforced the search and development of safe and efficient vectors as HIV vaccine platforms (120-123, 125, 137, 141, 251, 252). Due to their intrinsic properties, FV meet most of the criteria such a vector should have, including a good safety profile, high immunogenicity, genetic stability as well as a low prevalence in humans. The fact that they replicate in mucosal and lymphoid tissues represents another advantage for their use as HIV vaccine vectors. Despite these obvious benefits, the current study represented the first one aiming to develop FV for the induction of HIV bnAbs and two strategies, based on the FV TM protein and Bet were tested. Although the TM protein based approach did not result in replicating vectors, the data obtained for the FV expressing Bet/HIV fusion proteins are promising and encourage further exploration of this strategy for the purpose of HIV vaccine delivery. In the following sections, the individual parts of the project eventually leading to these replicating vectors will be shortly discussed.

4.1 Characterisation of the humoral immune response to the FFV TM protein

Although Env-specific antibodies can be frequently detected in FV positive antisera, any detailed studies on their epitope specificity were absent in the beginning of this project or based on the SU subunit only (217). That TM protein-directed responses have not been investigated for FV might be explained by the difficulties usually associated with the production of sufficient amounts of them (253, 254). In this study milligram amounts of recombinant PFV and FFV TM proteins with defined structure were produced with the help of a newly developed assay for optimisation of protein expression (220). Thereby, systematic optimisation of protein expression was the key for obtaining about 8-20-fold increased yields

compared to standard expression conditions. Similar significant differences were observed when parameters were optimised for other project-related (FFV Bet, E1-E2 loop antigens) and non-related (PERV gp70, KoRV p52) proteins, demonstrating the importance of matching vector, host and growth conditions to achieve high production rates. The initially described set-up was designed to select for highest overall expression since both TM proteins were highly insoluble. However, including a centrifugation step after lysis also allowed screening for best conditions for soluble proteins and was used to identify ideal parameters for soluble expression of FFV Bet (Fig. 29). This point is an important improvement, because soluble expression warrants biological activity and integrity of proteins including any conformational epitopes. It also avoids establishing a refolding strategy which is usually cumbersome and time-consuming (255-257). The assay was useful for TM proteins known to be hard to express but also easily adoptable to proteins of other origin. Since screening conditions were highly reproducible in large-scale, this assay represents an efficient tool to rapidly overcome expression problems for new protein targets and largely reduces efforts and costs of subsequent downstream-processing steps.

The availability of both TM antigens was a prerequisite to study TM-directed immune responses during FV infection and after immunisation. By analysing about 400 cat sera in a newly established ELISA, it was demonstrated that the FFV TM protein is an important antibody target during natural FFV infection and that TM responses correlated well with FFV Gag and Bet seroreactivity (160). It therefore represented a reliable antigen for serological screening and was used to determine for the first time the prevalence of FFV infection here in Germany, which was found to be 39% (221). This prevalence is consistent with results obtained in other European areas (201, 258-260) but has been found to vary from 4-70% in other geographical regions (261-264). The significant increase of FFV infection observed after one year of birth might be explained with attainment of sexual maturity, where cats start roaming and get in contact to other cats increasing the probability of infection. The positive correlation found for the castration status and FFV infection could be due to similar reasons, because straying of castrated rather than non-desexed animals is tolerated by pet-holders. Alternatively, castration itself might influence the behaviour of the animal. Since purification protocols for the PFV TM protein have also been established,

it would be interesting to analyse seroreactivity also against the PFV TM protein in primate and non-human primate serum samples where current testing also relies mainly on Gag and Bet (161, 265, 266). However, as access to substantial amounts of such sera was not given during the time of this thesis, this issue will be subject of future experiments.

Having identified several cat sera with varying degrees of FFV TM reactivity (serum titres ranging from 1:100-1:20.000), mapping of the epitopes recognised by these specimens was an essential information for the subsequent FFV/HIV-1 epitope exchange. Here, an important result was obtained when sera from naturally infected cats and TM protein-immunised animals were compared. Whereas immunised animals developed antibodies against the FPPR and the central region of FFV TM, naturally FFV infected cat sera contained antibodies exclusively directed to the FPPR and MPER regions but reactivity to the central, glycosylated domain was absent (Fig. 20). This suggested that the immune response to FFV TM is directly influenced by the properties of the antigen presented to the immune system. In case of the recombinant TM proteins obtained from *E. coli*, differences compared to the authentic FFV Env protein from eukaryotic cells comprised the lack of glycosylation, trimerisation, disulphide-linkages, absence of the SU subunit that might sterically protect some epitopes from being accessible (116) as well as the lack of presentation of the TM protein in a lipid context. Notably, such strong deviations in immune responses were also observed for bacterially-expressed gp41 of HIV-1, but not for the TM proteins of FeLV, KoRV and PERV, which are smaller in size and less or non-glycosylated. This highlights the importance of such comparative structural, functional and immunological studies to assess common and unique requirements for production and purification of antigens from various viruses. As a logical consequence of the differences observed between both expression systems, subsequent experiments relied on eukaryotic expression vectors encoding the full-length FFV Env protein.

Next to their epitope specificity, it was of interest to examine if neutralising activity of the antisera correlated with TM reactivity. Two high-throughput FFV neutralisation assays, based on a novel qRT-PCR measuring FFV provirus integration (Fig. 12) and a modified indicator cell assay scaled down to the 96-well plate format and adopted to automated read-out were established. By comparing the levels

of TM protein binding and FFV-FUV-7 neutralising antibodies, no obvious relationship between both parameters was apparent. This is consistent with results from other groups showing that serotype-specific neutralisation of FFV-FUV or F17/951 isolates is primarily mediated by antibodies to the central and C-terminal domains of the SU protein (217, 267). It would be interesting to examine if neutralisation can be detected when antibodies affinity-purified using the TM proteins are used in these assays, as this might provide extended possibilities for an epitope exchange as performed here.

4.2 The FFV TM protein as HIV-1 epitope scaffold

Compared to classical HIV-1 Env antigens, scaffolding HIV epitopes into immunogenic regions of foreign proteins represents an elegant way to induce antibodies to desired epitopes and allows modification of its structure dependent on the backbone selected (5, 268). In parallel, this strategy prevents responses to unwanted, but otherwise highly immunogenic regions of Env, since only a small part of the full length protein is usually included (5, 87, 89, 269-271). This issue is relevant for the HIV-MPER epitope selected here, since non-neutralising antibodies binding adjacent to the actual MPER epitope may mask critical residues for nAb engagement and thus inhibit virus neutralisation or hide the epitope from being accessible for B cells (272, 273). Introducing HIV epitopes into FV TM proteins was chosen as a strategy as it should principally allow epitope display on the virus surface at a FV typical high density, in a trimeric form and a lipid environment such as in the native HIV-1 context. Next to this, earlier findings showed that MPER-directed antibodies contribute to virus neutralisation during various retroviral infections, suggesting that similar regions might also exist for FV (182, 185-187, 274-276). Despite a direct linkage to virus neutralisation, the experiments clearly showed that the FPPR and MPER domains were immunogenic during FFV infection and rationalised the use of the FFV TM protein as epitope scaffold. After defining immunogenic FPPR and MPER core regions from the epitope mapping data, the final insertion positions for HIV-1 epitopes were selected by combining the immunological data with bioinformatic analysis for secondary structure, amino acid hydrophobicity, surface exposure and spacing to neighbouring functional domains. Whereas this approach did not result in functionally processed FPPR hybrids, HIV MPER insertion into FFV Env was compatible with Env processing and maturation (Fig. 24). Nevertheless, the expressed FFV-Env/MPER hybrids were unable

to promote infection of new cells, excluding their further use for the development of replicating vectors. It might be possible to design chimeric constructs that remain fusion-competent by shortening the introduced HIV-1 sequences, by testing additional insertion positions or by mutation of selected FFV residues to artificially construct a 2F5 or 4E10 epitope. This task is yet quite challenging, as essential FV MPER residues critical for Env function and virus infectivity have not been identified as in case of HIV-1 (78, 277). Since they were released as SVPs, therewith placing the HIV-1 MPER in a lipid environment shown to influence MPER epitope accessibility and structure (188, 278-280), testing immunogenicity of such SVPs was considered worth exploring. The fact that only weak antibody responses to the TM protein and the HIV MPER domain were induced after DNA prime and the SVP boost was most likely a result of the small amounts of SVPs used here (100, 45 and 110 ng of EnvG2, MPER1 or MPER SVPs per rat compared to 250 µg of antigen when recombinant proteins were used), which were probably too low to mount substantial B-cell responses. Furthermore, no adjuvant was used in these experiments to avoid a loss of SVP-integrity during antigen/adjuvant mixing or dissociation of particles through lipid adjuvant components. Enhanced responses might be triggered by including genetic adjuvants during the DNA prime and the use of substantial amounts of SVPs (e.g. 100 µg per animal). However, the production of such large amounts by transient transfection is technically challenging and would have required fermentation of cells, what simply exceeded the capacity of both labs. In the meantime, wildtype and MPER-containing FFV Env sequences have been subcloned into retroviral vectors and SVP expressing stable cell lines were established. Systematic selection of the expression cell lines, growth conditions and media has increased yields several-fold and now allows continuous harvesting from cells, providing access to much higher amounts of antigen. An immunisation study with these SVPs is currently ongoing and will help to evaluate their potential as HIV vaccine candidates (Mühle *et al*, in preparation).

4.3 FFV Bet/HIV-1 fusion proteins induce high titred HIV-1 specific antibodies *in vivo*

In a second approach, HIV-1 epitopes were appended to the FFV Bet accessory protein and recombinant proteins were produced to allow characterisation of such antigens. It was essential that the final products were soluble in physiologic buffers, since a conformational epitope in the Bet-E1-E2 protein would be formed under such conditions only. Despite all efforts, insufficient yields of soluble protein were obtained even after systematic optimisation (Fig. 29). After purification under denaturing conditions, successful renaturation was observed in presence of L-Arginine, which is an established additive that prevents aggregation of insoluble folding intermediates and therefore increases refolding efficiency (281, 282). Interestingly, interaction of renatured Bet with its natural ligand APOBEC (207, 283, 284) was dependent on disulphide bonds in the refolded Bet protein, since only in presence of a redox-system significant binding was observed (Fig. 30). Together with the fact that no distinct APOBEC interaction domain could be identified by using Bet deletion mutants (283), this indicates that a discontinuous surface area stabilised by disulphide bridges might be responsible for creation of the contact interface. Structural characterisation of the Bet protein produced in this study in complex with feline APOBEC would be an interesting project giving insights into the mechanisms FVs have evolved to counteract cellular immunity and which are quite distinct from that described for HIV-1 Vif (207). When the protocols established for Bet were used for Bet/HIV-1 fusion proteins, they were soluble in phosphate buffers and had a defined structure. However, protein solubility was concentration-dependent and solutions exceeding 1 mg/ml had a high tendency to aggregate, preventing determination of affinity constants for some antigens in SPR experiments (Table 11). This observation can either be explained by the presence of trace amounts of misfolded protein in the sample, triggering aggregation of folded proteins or, more likely, by the odd number of 13 cysteines present within FFV Bet. Even in case that most cysteines are involved in protein stabilisation and thus oxidised, a single cysteine residue remains unpaired in an active, reduced state favouring intermolecular interactions or induction of disulphide scrambling, particularly at high protein concentrations where molecule interactions are increased. Supporting this idea, 1-3 free cysteine in preparations of oxidised Bet could be frequently measured using Ellman's reagent (data not shown). Including low amounts of reducing reagent might prevent aggregation at higher protein concentrations, but was not performed here as the effect on other

disulphides and thus Bet structure remained hard to predict. Besides these technical issues, the affinity for mAb 2F5 to the generated fusion proteins was excellent, with comparable or even better binding to the MPER domain when compared to constrained peptides or other MPER epitope scaffolds (5, 88, 89, 271). Moreover, although overall affinity of 4E10 to the antigens was weak, including FPPR residues in one antigen had a beneficial effect on 4E10 binding, demonstrating that this domain indeed positively influences MPER epitope accessibility. The improved binding could result from an increased alpha helical content in C-terminal ELDKWA residues, which has been observed in presence of hydrophobic FPPR residues (95) and found to enhance affinity for 4E10 (86, 90, 91). Independent of the antigen selected, fusing epitopes to the C-terminus of Bet together with some spacer residues was overall an efficient mean to present them in an antibody-accessible form and high levels of antigen-binding and HIV-1 epitope-specific antibodies were induced when these proteins were injected into rats. For all antigens that included the HIV MPER domain, the elicited antibodies predominantly targeted the 2F5 epitope and those were able to bind to native Env presented on infected cells. The dominance of ELDKWA directed responses, whereas 4E10 epitope-binding antibodies were virtually absent, might be an result of better presentation of the 2F5 epitope in the Bet/HIV proteins, since high affinity binding to an epitope is the driving force triggering proliferation and differentiation of B cells to plasma cells (285, 286). It is noteworthy, that in presence of FPPR residues the immune response to that domain frequently exceeded that to the MPER by 1-2 logs. This could present an issue for the use of FPPR-constrained MPER antigens, because high immunogenicity of the FPPR might bias the strength of MPER responses although this domain is actually included for stabilisation only. It would be advisable to introduce modifications of the FPPR that immunologically silences FPPR responses by maintaining its confining properties as has been demonstrated for some therapeutic proteins (287, 288). As in previous studies, the antibodies induced by the Bet/HIV-1 fusion proteins were not able to inhibit HIV-1 infection in TZM-bl cell-based neutralisation assays, despite their high abundance, recognition of the 2F5 epitope and binding to native Env. This suggests that either the antigens did not provide the structural features required for their induction, what is supported by the results obtained later with modified FPPR/MPER antigens, or that their presence in the raised antisera was too low to be detected.

It has to be noted, that in later experiments some of the antisera from rat groups injected with MPER-containing antigens showed indirect antiviral activity in ADCC assays using MPER peptide-loaded CEM cells and human PBMCS (Steve Norley, RKI, data not shown). Considering that enhanced lysis (10-30%) compared to non-pulsed cells or pre-immune controls was observed in this system is remarkable, since binding of human NK effector cells to rat IgGs is mainly limited to the IgG2a subclasses (289), whereas IgG1 and IgG3 are usually found to have highest ADCC activity (30, 290, 291). The actual capacity of the sera to induce ADCC is thus probably higher, but to fully explore antiviral functions of these sera, a system based on rat effector and target cells would be desirable which is not available to our knowledge. In general, mechanisms such as ADCC and ADCVI play an important role for protection from HIV infection and particularly MPER directed antibodies have been found to be involved in blocking viral transcytosis in intestinal epithelial cells or prevent cell-to-cell transmission through the viral synapse (49, 51, 292). They were also considered responsible for the moderate protection observed in the recent RV144 trial (293-295). Exploring such alternative antibody functions is principally indispensable to draw complete pictures of the antibody response induced by novel antigens. Still, as assays are usually established for human and simian antibodies, addressing these questions is challenging for experiments in rodents as performed here. In summary, the described experiments showed that Bet fusion proteins can be successfully employed to induce epitope-specific antibody responses *in vivo*, what prompted further examination of this strategy.

4.4 Improved FPPR/MPER antigens induce HIV-1 neutralising antibodies in mice

Whereas the foregoing results rationalised the use of Bet as carrier protein, the performance of the loop stabilised FPPR/MPER antigen used in these experiments was rather moderate. Although higher affinity for 4E10 was achieved by including FPPR residues, 4E10 epitope specific-antibodies were not induced during immunisation (Fig. 35). Furthermore, in case of 2F5, the loop antigen showed reduced affinity to its epitope compared to the Bet-E2 antigen which encoded the MPER domain alone (Fig 33, Table 11). This supported the view that linker length or the spacing of FPPR and MPER residues in this initial construct was suboptimal for the formation of a 2F5 conformational epitope. Importantly, this situation is divergent to that in earlier experiments using individual FPPR and MPER peptides, which were not

constrained by a linker and therefore can spontaneously assemble into an energetically favoured complex (92, 96, 189). In order to make use of the 2F5 epitope-dominated response observed after injection into rats, it was sought to modify antigens to achieve a FPPR/MPER positioning in which binding for 2F5, rather than 4E10 is enhanced. A set of antigens was cloned in which the FPPR/MPER flanking residues were systematically shortened through single amino acid deletions. In line with the theory that such mutations should alter surface exposure of distinct residues in the predominantly helical domains (81, 296), significant differences in bnAb binding characteristics as well as the propensity of the antigens to form trimers were observed (Fig. 38). The latter finding is consistent with previous results showing that trimer formation is influenced by FPPR residues (93) and extends these data as it suggests that the influence is not only residue but also position dependent. Furthermore, such stepwise truncations also resulted in changes of the strength and quality of MPER-directed immune responses in mice, including the induction of higher titres of binding antibodies and a shift of the epitope recognition pattern in dependence to the antigen administered (Fig. 40, Table 12). Most importantly, only a subset of these antigens was capable to prevent HIV-1, but not SIVmac239 infection in TZM-bl cells when compared to pre-immune control sera. These data strongly suggested that the MPER structure required for induction of nAb is formed only in a distinct positioning of FPPR and MPER residues and it would be worthwhile to decipher the structural differences of these antigens to those that induced binding antibodies only. Notably, these results are consistent with a recent publication by Dawood *et al.*, which reports the induction of bnAbs in mice by use of a cell line expressing a linked NHR/CHR antigen as immunogen (245). For reasons that are not completely clear, the results obtained in mice could not be reproduced when the study was repeated in hamster and in contrast to the immunisations performed in BALB/c mice, the overall immune response observed was weak and neither MPER-directed nor nAb were induced (data not shown). This might be linked to the differences in the genetic background of both species resulting in varied B cell receptor reservoirs or ability to process and present certain peptides on MHC class II receptors. In line with this, distinct HLA alleles associated with HIV/SIV protection or delayed disease progression have been identified in man and primates (297-300). Deviation of experimental results dependent on the animal system and the resultant limitations for predicting efficiency in humans are a well-known issue in HIV vaccine research as well (301). To draw final

conclusions on the ability of the loop antigens to induce protective responses, the study should be performed in humanised mice followed by subsequent HIV-1 challenge, which represents one of the most stringent models to evaluate novel vaccine candidates.

4.5 Chimeric FFV vectors expressing Bet/HIV-1 fusion proteins

Based on the results obtained above, three of the five loop antigens as well as the MPER domain alone were chosen for subcloning into the FFV molecular clone. This selection was basically driven by the observations that these antigens showed highest affinity to 2F5, induced significant levels of ELDKWAS specific antibodies after immunisation and showed the ability to induce nAb in mice. As before, the HIV-1 inserts were fused to Bet with identical spacing/linker residues to assure an antigen structure most closely resembling that of the previously characterised recombinant antigens. Moreover, separating the HIV epitopes from Bet was considered important since they might sterically interfere with the function of Bet, which would be essential to allow effective replication in APOBEC-positive cells (207, 283, 284). In a previous study, this was achieved by including the FFV protease cleavage site between Bet and the transgene (118, 209). However, as secretion of the intact Bet/HIV-1 fusion proteins from infected cells was desirable to maintain epitope conformation and to stimulate surveying immune cells, this was not included here. It is important to note that fusion to Bet in that case most probably prevents the trimerisation of the FPPR/MPER antigens observed when the proteins were analysed alone. It is not clear how this affects their immunogenicity, but internal promoter driven, Bet independent expression of FPPR/MPER antigens with a secretion signal might be considered as an alternative way of delivery of these antigens. The applied cloning strategy fused the HIV-1 inserts to Bet by leaving the subsequent 3' LTR unaltered instead of truncating the corresponding sequences (Fig. 41, Fig. 42). Although such vectors have been found to be genetically less stable compared to those with a truncated 3' LTR, chimeric vectors with an intact LTR were superior in inducing antibodies to the desired epitope (118). Furthermore, the instability observed in these studies was inversely correlated to the insert size. The single MPER domain and the longest FPPR/MPER inserts introduced here were of small or intermediate size (28 and 70 residues) compared to the previous 39, 105 and 150 residues of feline calicivirus stably expressed for up to 150 days, suggesting that they potentially will be maintained in a

similar fashion (118). Consistent with these thoughts, the resulting chimeric vectors gave raise to infectious virus similar to the wildtype virus, expressed the Bet/HIV-1 fusion proteins at the expected sizes and were able to replicate in APOBEC-expressing feline CRFK cells (Fig. 43, Fig.44). Dependent on the cloned insert, virus replication was somewhat compromised in the initial passages, suggesting that adoptive mutations were probably necessary to compensate for expression of the highly hydrophobic HIV epitopes (Fig. 44). In case of the FFV Bet-F2-M0 vector, this resulted in low level replication vanishing after the third passage (data not shown). It is possible that the HIV-1 fusion part in this construct might be misfolded, impairing the ability of Bet to act as APOBEC decoy protein as mentioned above. However, in the remaining constructs integrity of HIV-1 epitopes was obviously not affected as no change in insert size or recognition by 2F5 was evident. This analysis was further extended by sequencing proviral DNA from infected cells of the last CRFK passage (data not shown). There were no or up to four independent mutations within Bet for the Bet-His and the Bet-E2 vector, respectively, but MPER epitope integrity was not affected. The other two Bet-F2-MX vectors were sequenced after cloning. Seven (Bet-F2-M1) and nine (Bet-F2-M2) of 11 clones analysed encoded the entire HIV MPER epitope, whereas the remaining clones expressed truncated forms of Bet due to premature stop codons or insert deletion and fusion to LTR sequences (data not shown). Therefore, some degree of genetic rearrangements does occur in these vectors and it will be important to analyse their behaviour in an *in vivo* situation. Alternatively, a set of additional vectors incorporating the suggestions mentioned above might be constructed and again analysed *in vitro* before proceeding to the animal model. Overall, the initial goal to construct replicating FV vectors that express HIV epitopes has thus successfully been accomplished.

5. Summary

The induction of neutralising antibodies by immunisation represents one of the best strategies to prevent subsequent virus infections. However, whereas such antibodies form the basis of several vaccines available today, attempts to induce protective immune responses against the human immunodeficiency virus (HIV) failed till now. This work aimed to establish and evaluate a novel vaccine approach based on apathogenic, persistently infecting foamy virus (FV) to exploit their continuous antigen delivery and immune stimulation. To allow testing of such novel vectors *in vivo*, the feline FV (FFV) was used as model for later application in cats. In a first approach, the transmembrane envelope (TM) protein of FFV was investigated as potential epitope scaffold. With the help of recombinantly produced antigen it was demonstrated that the TM protein is a strong immunogen during natural FFV infection and used to serologically determine for the first time the FFV prevalence in Germany. After immunogenic regions within FFV TM were mapped, replacing these domains through the membrane proximal external region (MPER) of the HIV TM protein, an epitope recognised by several HIV-1 broadly neutralising antibodies (bnAb), was accomplished. The generated chimeric FFV/HIV-1 Env were released from transfected cells as subviral particles (SVPs), presenting the HIV MPER epitope in a FV typical high density on the particle surface, in a trimeric context and a lipid environment and were thus tested in subsequent immunisation experiments. However, since they gave rise to fusion-defective FFV, they were impractical for application in a replicating system. In a second strategy, fusion proteins of the accessory FFV Bet protein and HIV-1 epitopes were therefore evaluated. Characterisation of these antigens showed that they were efficiently recognised by the preferred HIV-1 bnAb and elicited binding antibodies with identical epitope specificity in immunisation studies. Using further improved HIV-1 inserts this approach could be successfully transferred into the FFV and resulted in the production of infectious, chimeric FFV viruses. Importantly, HIV-1 epitope expression remained stable after extended passaging on feline cells, suggesting that these vectors are suitable for studying the effects of affinity maturation on the emergence of bnAb responses. Taken together, in this work two new HIV-1 vaccine platforms based on SVPs and replicating FFV were developed, which are characterised by a high safety profile. In particular the replicating FFV/HIV-1 chimeric viruses thereby represent attractive vectors for further analysis *in vivo*.

6. Zusammenfassung

Die Induktion neutralisierender Antikörper durch Vakzinierung ist eine der attraktivsten Strategien, um eine nachfolgende Infektion durch Viren zu verhindern. Während jedoch solche Antikörper die Basis für viele bestehende Impfstoffe darstellen, blieben Versuche, vergleichbare Ansätze für das Humane Immundefizienz-Virus (HIV) umzusetzen, bisher erfolglos. Diese Arbeit diente der Etablierung und Evaluierung eines neuen Impfkonzepts basierend auf foamyviralen (FV) Trägerviren, welches sich die apathogene, aber persistierende Infektion und dadurch verlängerte Antigenexpression der FV zu Nutzen machen sollte. Im Fokus stand dabei insbesondere das feline FV (FFV), um generierte Viren später im Tiermodell testen zu können. Zunächst wurde dafür das transmembrane (TM) Hüllprotein des FFV als potentielles Trägerprotein näher untersucht. Mittels rekombinant hergestelltem TM Protein wurde gezeigt, dass dieses ein starkes Immunogen während der natürlichen FFV Infektion darstellt und erstmals zur Bestimmung der FFV Prävalenz in Deutschland eingesetzt. Für den HIV-Impfstoff wurde nach Kartierung immunogener Bereiche im FFV TM Protein ein Austausch dieser Domänen gegen die membran-proximale externe Region (MPER) des TM Proteins von HIV als Epitop mehrerer breitneutralisierender Antikörper (bnAk) durchgeführt. Die generierten FFV/HIV Env Hybride wurden als subvirale Partikel (SVPs) freigesetzt, was Immunisierungsversuche mit diesen Antigenen, die die HIV MPER an der Partikeloberfläche in einer FV-typischer Dichte, in einem trimerisiertem Zustand und einer Lipidumgebung präsentieren, ermöglichte. Mit diesen FFV/HIV Envs pseudotypisierte Viren blieben jedoch replikations-defizient. Als zweite Strategie wurden daher Fusionsproteine bestehend aus dem akzessorischen Bet Protein des FFV und den HIV-1 TM Epitopen hergestellt. Die Charakterisierung dieser Antigene zeigte eine hochaffine Bindung von bnAk gegen HIV-1 an diese Antigene sowie die Induktion bindender Antikörper mit identischer Erkennungssequenz in Immunisierungsstudien. Die Strategie wurde mit weiter optimierten Antigenen erfolgreich auf das FFV übertragen. Die Expression der HIV-1 Epitope blieb dabei auch nach mehrmaligen Passagieren der chimären Viren in felinen Zellen stabil und ist daher für Untersuchungen zur Notwendigkeit der Affinitätsreifung für die Entstehung bnAk besonders geeignet. Zusammengefasst wurden in dieser Arbeit zwei neuartige HIV-Impfstoffsysteme basierend auf hybriden SVPs und replizierenden FFV entwickelt, die sich durch ihre hohe biologische Sicherheit auszeichnen. Insbesondere die replizierenden FFV/HIV-1 Hybride stellen dabei attraktive Vektoren für weitere *in vivo* Versuche dar.

7. References

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8. Appendix

8.1 Publications

Parts of this work have been published in the following manuscripts

1. **Mühle M, Bleiholder A, Kolb S, Hübner J, Löchelt M, Denner J** (2011) Immunological properties of the transmembrane envelope protein of the feline foamy virus and its use for serological screening. *Virology* 412:333-340
2. **Mühle M, Löchelt M, Denner J** (2012) Optimisation of expression and purification of the feline and primate foamy virus transmembrane envelope proteins using a 96 deep well screen. *Protein Expr Purif* 81:96-105
3. **Mühle M, Hoffmann K, Löchelt M, Denner J** (2013) Immunisation with foamy virus Bet fusion proteins as novel strategy for HIV-1 epitope delivery. *Immunol Res* 56:61-72
4. **Mühle M, Hoffmann K, Löchelt M, Denner J** (2013) Construction and characterisation of replicating foamy viral vectors expressing HIV-1 epitopes recognised by broadly neutralising antibodies. *Antiviral Research* 100:314-320
5. **Bleiholder A, Mühle M, Hechler T, Bevins S, Vandewoude S, Denner J, Löchelt M** (2011) Pattern of seroreactivity against feline foamy virus proteins in domestic cats from Germany. *Vet Immunol Immunopathol* 143:292-300
6. **Slavkovic D, Hotz-Wagenblatt A, Lei J, Räthe AM, Mühle M, Denner J, Münk C, Löchelt M** (2013) Identification of the feline foamy virus Bet domain essential for APOBEC3 counteraction. *Retrovirology*, 10:76-96
7. **Mühle M, Bleiholder A, Löchelt M, Denner J** (2014) Establishment and evaluation of a feline foamy virus subviral particle based vaccine strategy for induction of neutralising antibodies against HIV-1, *in preparation*.

8.2 Curriculum Vitae

For reasons of data protection, this curriculum vitae is not included in the online version

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