

Induction of Antibodies Specific for Gp41 of HIV-1 by Gene Gun DNA Vaccination

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Abstract

All attempts to induce broadly neutralising antibodies such as mAb 2F5 and mAb 4E10 targeting conserved epitopes in the membrane proximal external region (MPER) of the transmembrane envelope (TM) protein gp41 of HIV-1 failed so far. In contrast, in previous studies, immunising with the ectodomain of the TM protein p15E of different gammaretroviruses, we successfully induced neutralising antibodies. These antibodies recognised epitopes located in the fusion peptide proximal region (FPPR) and in the MPER of p15E. The epitope in the MPER of p15E corresponds to that of the mAb 4E10 in gp41 in terms of location within the protein and partial sequence homology. In order to present the MPER of gp41 (containing the 2F5 and 4E10 epitopes) membrane-associated, rats were immunised with DNA constructs corresponding (i) to the entire gp41, (ii) to the C-terminal helix of gp41 and (iii) to hybrid proteins composed of a backbone derived from p15E of a gammaretrovirus with inserted FPPR and MPER from gp41 of HIV-1. After transfection *in vitro* these proteins were found expressed at the cell surface and the accessibility of the 2F5 epitope was demonstrated by flow cytometry. However, DNA vaccination in rats resulted only in low titres of antibodies specific for the MPER of HIV-1, and none of the sera was neutralising.

Keywords: HIV-1; gp41; MPER; FPPR; DNA vaccination; Gene gun

Introduction

Eliciting broadly neutralising antibodies against the human immune deficiency virus 1 (HIV-1) is still considered to be an inevitable goal of HIV vaccine research. Because of the variability of its envelope proteins the conserved domains of these proteins represent a good potential target for broadly neutralising antibodies. One of these conserved domains is the membrane proximal external region (MPER) of the transmembrane envelope (TM) protein gp41 of HIV-1. Two of the most potent broadly neutralising antibodies against HIV-1, mAb 2F5 and mAb 4E10 bind to epitopes located in the MPER of gp41 [1,2]. This domain was considered to be an excellent target region for vaccine development [3]. However, numerous immunisation studies using proteins containing the epitopes of mAb 2F5 and mAb 4E10 failed to induce broadly neutralising antibodies so far [4,5].

In contrast to the situation with HIV-1, neutralising antibodies against different gammaretroviruses such as porcine endogenous retrovirus [6,7], feline leukaemia virus [8-10] and Koala retrovirus [11] have been easily induced by immunisation with the recombinant ectodomains of their TM proteins. The epitopes recognised by the immune sera were located in the N-terminal fusion peptide proximal region (FPPR) and in the C-terminal MPER of p15E. The MPER of PERV, FeLV and KoRV is homologous to that of gp41 of HIV-1 and despite the evolutionary distance between gammaretroviruses and HIV-1 a limited sequence homology of the epitopes was identified [6-11] (Figure 1a).

When studying the mechanisms of neutralisation by mAb 2F5 and mAb 4E10, complex coherences between the structure of the MPER and antibody binding were observed [12-14]. Interaction of both antibodies with lipids, their long hydrophobic CDR3 loop and cross-reactivity with cardiolipin have been discussed controversially as possible reasons for the failure of all previous immunisation attempts [15-17]. Since there is evidence for intramolecular interactions between the MPER and the FPPR during infection and neutralisation [18-22] and a peptide corresponding to the sequence of the FPPR has been shown to increase binding of mAb 2F5 to its epitope [14], it was suggested that the FPPR

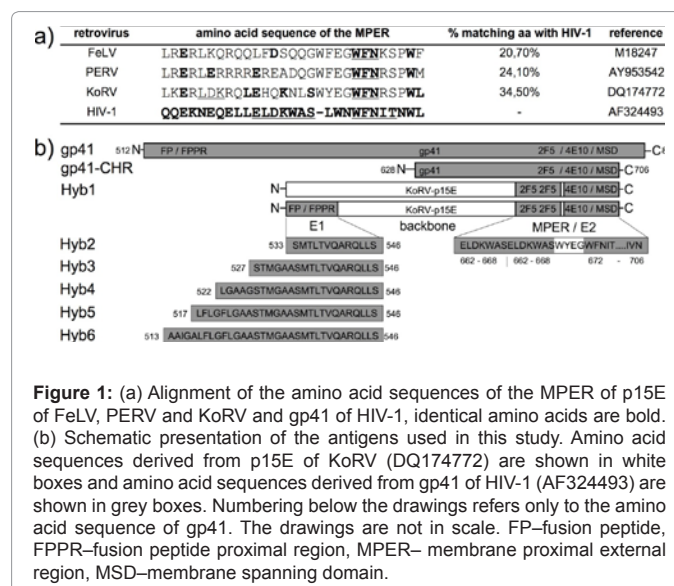


Figure 1: (a) Alignment of the amino acid sequences of the MPER of p15E of FeLV, PERV and KoRV and gp41 of HIV-1, identical amino acids are bold. (b) Schematic presentation of the antigens used in this study. Amino acid sequences derived from p15E of KoRV (DQ174772) are shown in white boxes and amino acid sequences derived from gp41 of HIV-1 (AF324493) are shown in grey boxes. Numbering below the drawings refers only to the amino acid sequence of gp41. The drawings are not in scale. FP–fusion peptide, FPPR–fusion peptide proximal region, MPER– membrane proximal external region, MSD–membrane spanning domain.

may be required to generate a conformation which will be able to induce broadly neutralising antibodies of the 2F5 and 4E10.

Enhanced binding of mAb 2F5 and mAb 4E10 to their epitopes was observed in a lipid environment compared with free MPER peptides [23-26]. These findings raise the question, whether membranes or

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Primers/probes	Primer sequence (5' - 3')
MSD1-rev	tatgaataatttataaccacagccaatttggatgttgaaccatcc
MSD2-rev	tcttaaactaccaagcctcctactatcattatgaataatttat
MSD3-rev	ctgctgcagtcgaatctactatagaagtacagcaaaactattctaaactaccaagc
E1-for	gcgggatccacagcagcatgacactaacagtgcaggcagccaaactactactttgact agcctccagatt
Hyb3-for	cgggatccagcactatgggcccagcgcagtcagtcagctgcagctgacag
Hyb4-for	cgggatccttaggagcagcaggaagcactatgggcccagcgcagc
Hyb5-for	cgggatcctgttctctgggttcttaggagcagcaggaagc
Hyb6-for	cgggatcccagcagcagtaggagctctgcttcttgggtctta
gp41-for	ccagatctgcagtggaataggagctttgttcttggg
gp41-rev	cctgagctgcagataccacagccaatttggatgt
CHR-for	gaagatctatggagtgggacagagaaataac
CHR-rev	cctgagctgcagataccacagccaatttggatgt
hGAPDH-for	ggcagatctgctgcgctgagtagc
hGAPDH-rev	tggtccacaccatgacga
hGAPDH-probe	Hex-ttcaccacatggagaaggctggg-BHQ-1
68i	ggarcagciggaagcaciattgg
69i	cccagacigtgagjitaaca
HIV-1-probe	FAM-tccgctgggcccacctcctggaggctc-BHQ-1
T7-prom	taatacgactcactataggg

Table 1: Primers and probes used for the generation of constructs and for real time PCR.

lipids are required for the induction of such neutralising antibodies. One way to present the MPER of gp41 in a natural lipid environment is DNA vaccination allowing the MPER to be associated with the cell membrane. Although DNA vaccination was mainly used for induction of MHC-I specific cytotoxic cellular immune responses, it can also be used for the induction of humoral immune responses [27-30].

In this study, DNA constructs corresponding to the entire ectodomain of gp41, to the C-helical region of gp41 (gp41-CHR) as well as to hybrid constructs containing a gammaretrovirus backbone and sequences of the epitopes of mAb 2F5 and mAb 4E10 together with the FPPR were used in gene gun immunisation studies. Expression of gp41 and the hybrid antigens on the cell surface was demonstrated *in vitro*, and after immunisation of rats antibodies specific for gp41 were found. These findings may help to develop new strategies for a cell membrane-associated presentation of potential HIV vaccines in order to induce neutralising antibodies against conserved domains in gp41.

Materials and Methods

Generation of the antigen-coding constructs

The sequences of gp41-CHR and gp41 (Figure 1) were amplified by PCR (primers: CHR-for and CHR-rev; gp41-for and gp41-rev) (Table 1) using the HIV-1 molecular clone pNL4-3 (AF324493) as a template. The hybrid sequences were generated by mutagenesis PCR using primers partly homologous to the KoRV-p15E sequence (DQ174772) and providing the HIV-1 gp41 specific sequences derived from pNL4-3. The sequence of hybrid 1 (Hyb1) was generated by three sequentially performed PCRs using (i) primers T7-prom and MSD-1-rev on the template of pCal-n-KoRV-p15E [11], (ii) primers T7-prom and MSD-2-rev on the template generated in step (i), (iii) primers T7-prom and MSD-3-rev on the template generated in step (ii). The sequence of Hyb2 was generated using Hyb1 as template and performing mutagenesis PCR with the primers E1-for and MSD-3-rev. The hybrids Hyb3 to Hyb6 were generated similarly combining the reverse primer MSD-3-rev with the desired forward primers Hyb3-for, Hyb4-for, Hyb5-for and Hyb6-for (Table 1 and Figure 1). All PCRs were performed under the following conditions: 95°C/10 min, 35 cycles: 95°C/45 s; 42°C/120

s; 72°C/45 s, final extension: 72°C/10 min using the AmpliTaq Gold (Applied Biosystems, Foster City, USA). All PCR products were cloned into the eukaryotic surface expression vector pDisplay (Invitrogen, Karlsruhe, Germany) using the restriction sites *Bgl*II and *Pst*I. The correctness of the reading frames were confirmed by sequencing. Plasmids were purified using the Endo-Free-Maxi Prep Kit (Qiagen, Hilden, Germany).

Transfection of Rat-1 cells

Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany) was used according to the manufactures instructions. 2×10^5 /ml Rat-1 cells were seeded into a 6-well cell culture plate and transfected with 5 µg endotoxin-free plasmid when 80% cell confluence was reached. After 48 hours cells were transferred into DMEM containing 600 µg/ml geneticin (Invitrogen, Karlsruhe, Germany) in order to generate stably transfected cell lines.

Flow cytometry

Stably transfected Rat-1 cells were detached from the culture flask bottom using a cell scraper and washed three times with 10 ml of ice cold PBS. The cells were blocked for 30 min in ice cold PBS containing 10% (v/v) fetal bovine serum. mAb 2F5 (10 µg/ml) was added in blocking buffer. After incubation for 30 min on ice the cells were washed three times with ice cold PBS, and FITC-labelled secondary anti-human IgG antibodies (1:1000, Sigma, Munich, Germany) were added. Cells were incubated in the dark for 30 min on ice and then washed five times with PBS. Finally, 2% (v/v) formaldehyde (Roth, Karlsruhe, Germany) in PBS was added to fix the cells. Surface expression was assayed in a FACSCalibur cytometer (BD, Franklin Lakes, USA). Data were analysed using the CellQuest Pro software (BD, Franklin Lakes, USA).

DNA immunisation

Endotoxin-free plasmids were precipitated onto gold particles (particle Ø 0.9 µm, 2.5 µg DNA/1mg gold). The coated particles were inoculated into the shaved abdominal skin of 4 weeks old Wistar rats (Charles River) using the Helios Gene Gun® (Bio Rad, Munich, Germany) with a helium impulse at 400 psi. Each rat received a triple application (1 µg/shot). Boost immunisations using the same procedure were performed on days 14 and 28 post primary immunisation.

ELISA

Peptide ELISAs were performed as described elsewhere [31]. 200 ng/well of the FPPR-derived peptide (aa524-547) (GAAGSTMGAASVTLTVQARLLLS) or the MPER-derived peptide (aa655-679) (NEQELLELDKWASLWNWFDITNWL) (JPT, Berlin, Germany) was coated on Nunc immunoplates (Nunc, Denmark) overnight at 37°C. Sera were diluted 1:350 in blocking buffer. To detect antibodies specific for gp41 or KoRV p15E, purified recombinant gp41 (aa530-683, reference K03455, 400 ng/well) and recombinant KoRV p15E (aa489-597, reference DQ174772, 200 ng/well) [11] were diluted in water and coated overnight at 37°C.

Neutralisation assay

A newly developed neutralisation assay based on the measurement of reduction of provirus integration as a marker for neutralisation was used [14,32]. Briefly: a 20 µl serum dilution and 80 µl HIV-1_{11IB} (TCID₅₀=10³/ml) were incubated for 30 min at 37°C/5% CO₂. C8166 cells (5 × 10⁴ cells in 100 µl) were added and after 65 hours the supernatant was aspirated. Then cells were lysed and provirus integration was determined by an HIV-1 specific duplex real-time PCR

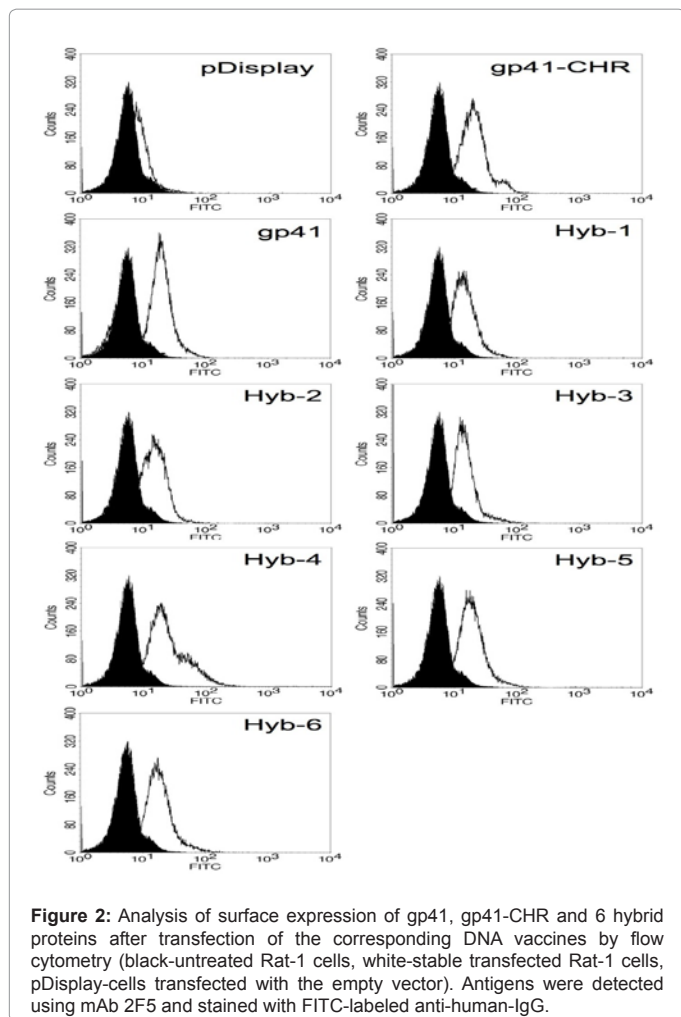


Figure 2: Analysis of surface expression of gp41, gp41-CHR and 6 hybrid proteins after transfection of the corresponding DNA vaccines by flow cytometry (black-untreated Rat-1 cells, white-stable transfected Rat-1 cells, pDisplay-cells transfected with the empty vector). Antigens were detected using mAb 2F5 and stained with FITC-labeled anti-human-IgG.

using the HIV-1 specific primers 68i and 69i [33] as well as an HIV-1-probe (Table 1). To analyse toxic effects of the sera, simultaneously the amount of GAPDH genes (primers: hGAPDH-for and hGAPDH-rev, probe: hGAPDH-probe, Table 1) was analysed. For amplification the real-time cyler MX-4000 (Stratagene, La Jolla, USA) was used with the following cycling conditions: 95°C/8 min+50 cycles: 95°C/45 s– 55°C/60 s–72°C/20 s. Neutralisation (NT) was calculated using the formula: $NT = 100 - 100 / 2^{\Delta\Delta Ct}$. A reduction of 75% provirus integration ($\Delta Ct = 2$) was defined as significant neutralisation. As positive control mAb 2F5 (5 µg/ml) was used.

Results

DNA immunisation with gp41 and the CHR domain of gp41

DNA constructs, corresponding to the entire gp41 and to the CHR domain of gp41 (gp41-CHR), were generated (Figure 1b). To study expression of the encoded proteins on the cell surface *in vitro*, stable transfected Rat-1 cells were analysed by flow cytometry using mAb 2F5 (Figure 2). Fluorescence signals were detected with all transfected cell lines compared to untreated Rat-1 cells. Since the cells were not permeabilised, the observed staining indicated cell surface expression of all constructs as well as accessibility of the epitope of 2F5 when presented in such a way. Rats were immunised using these DNA constructs as described in Materials and methods. 14 days after the third immunisation, sera were taken and screened for binding antibodies in an ELISA. In nearly all sera (except

serum from rat no. 6), gp41 specific antibodies were detected (Table 2). The highest titre (1:64,000) of gp41 specific binding antibodies was found in the sera from rats 9 to 12, immunised with a DNA construct corresponding to the entire gp41. Sera obtained by DNA immunisation with the gp41-CHR construct showed a titre of gp41-binding antibodies of 1:8,000. When analysed for neutralising activity, none of the gp41 specific sera generated by DNA immunisation was able to neutralise HIV-1_{IIIIB} (Figure 3).

Design of DNA constructs corresponding to TM hybrid proteins

Since DNA immunisation with the entire sequence of gp41 or the CHR of gp41 in a membrane-bound context did not induce neutralising antibodies, a new strategy for antigen design was developed. The sequence, corresponding to the epitopes of mAb 2F5 and mAb 4E10, and the sequence in the FPPR able to increase binding of mAb2F5 and mAb 4E10 to their epitopes [14] were inserted into the TM protein p15E of KoRV. p15E was used as a backbone and from this backbone the regions in the MPER and FPPR shown to contain epitopes recognised by antibodies neutralising KoRV were replaced by the gp41-specific MPER and FPPR domains (Figure 1b). We expected that the helical regions of p15E of KoRV will bring the MPER and FPPR derived from gp41 of HIV-1 in close proximity generating a conformation which allows inducing broadly neutralising antibodies.

DNA immunisation with DNA constructs corresponding to TM hybrids

To demonstrate the expression of the TM hybrid proteins, encoded

Rat no.	Construct	Titre gp41	E1	E2
1	pDisplay	-	0.0	0.0
2		-	0.0	0.0
3		-	0.0	0.0
4		-	0.0	0.0
5	gp41-CHR	1:8000	0.0	0.08
6		-	0.0	0.0
7		1:8000	0.0	0.10
8		1:8000	0.0	0.10
9	gp41	1:64000	0.32	0.42
10		1:64000	0.59	0.51
11		1:64000	0.29	0.37
12		1:64000	0.37	0.46
13	Hyb1	1:4000	0.0	0.08
14		1:4000	0.0	0.08
15		1:4000	0.0	0.09
16		1:4000	0.0	0.06
17	Hyb2	1:4000	0.15	0.12
18		1:4000	0.18	0.06
19		1:4000	0.20	0.09
20		1:4000		0.09
21	Hyb3	1:4000	0.25	0.08
22		1:8000	0.42	0.08
23		1:4000	0.29	0.09
24		1:4000	0.27	0.03
25	Hyb4	1:4000	0.34	0.07
26		1:4000	0.30	0.09
27		1:4000	0.15	0.10
28		1:4000	0.29	0.08
29	Hyb5	1:4000	0.18	0.06
30		1:4000	0.25	0.08
31		1:4000	0.07	0.08
32		1:8000	0.31	0.11
33	Hyb6	1:4000	0.10	0.09
34		1:8000	0.44	0.09
35		1:4000	0.24	0.08
36		1:4000	0.41	0.04

Table 2: ELISA analysis: gp41 specific binding antibodies (OD 492nm/620nm) induced by immunization with different antigens.

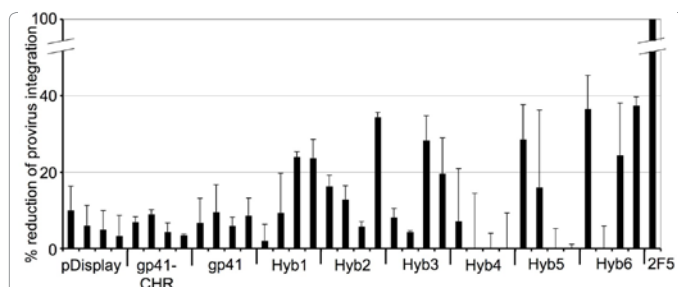


Figure 3: Results of the neutralisation assays using HIV-1IIIIB and antisera induced in rats by DNA immunisation with different constructs as shown in Figure 1. MAb 2F5 was used as positive control. Each value is the result of a triplicate measurement. Significant neutralisation was defined as reduction of provirus integration above 75%.

by the DNA constructs described above, each of the six hybrid proteins (Hyb1-Hyb6) was transfected into Rat-1 cells and stable transfected lines were generated. Flow cytometry analysis of these cell lines using mAb 2F5 showed surface expression and accessibility of the 2F5 epitope in all cases (Figure 2). All six DNA constructs were used for DNA immunisation, as described in Material and methods, and the induced sera were analysed in ELISAs. Only low titres of antibodies specific for synthetic peptides corresponding to the FPPR and MPER of gp41 were found (Table 2). As a rule, the responses against the FPPR-derived peptide were stronger, compared with the responses against the MPER-derived peptide. To study the presence of neutralising antibodies in the immune sera, HIV-1IIIIB was used in a real-time PCR-based neutralisation assay measuring provirus integration. This assay gave similar results when compared with the TZM-bl cell based assay and has numerous advantages [32]. Such neutralisation assays have been used to screen for neutralising antibodies specific for PERV [7], FeLV [8], KoRV [14] and HIV-2 [31]. None of the sera induced by DNA immunisation with the DNA hybrid constructs was able to reduce provirus integration of HIV-1IIIIB (Figure 3).

Determination of antibodies directed against the p15E backbone

Since the antibodies induced by DNA immunisation using the hybrid constructs Hyb1-Hyb6 were not neutralising HIV-1 (Figure 3), the reactivity of the sera was analysed in more detail. To study the presence of antibodies specific for the p15E backbone, an ELISA using recombinant p15E of KoRV was performed. In all sera from rats immunised with DNA hybrid constructs antibodies against the p15E backbone were detected. This was rather astonishing, because after immunisation with p15E of different gammaretroviruses including KoRV, mainly antibodies against the FPPR and the MPER, but not against other parts of p15E were detected [6-10]. Surprisingly, sera from untreated control rats and from rats immunised with gp41 or gp41-CHR DNA constructs also contained antibodies against p15E of KoRV (Figure 4). These data indicate that in contrast to previous experiments antibodies against p15E of a gammaretrovirus were found in naïve Wistar rats not yet used for immunisation. The preformed antibodies against the p15E backbone may have interfered with the immune response against the hybrid protein containing FPPR-derived and MPER-derived from gp41 of HIV-1. This finding may partially explain the low titre of antibodies against the inserted HIV-1 sequences.

Discussion

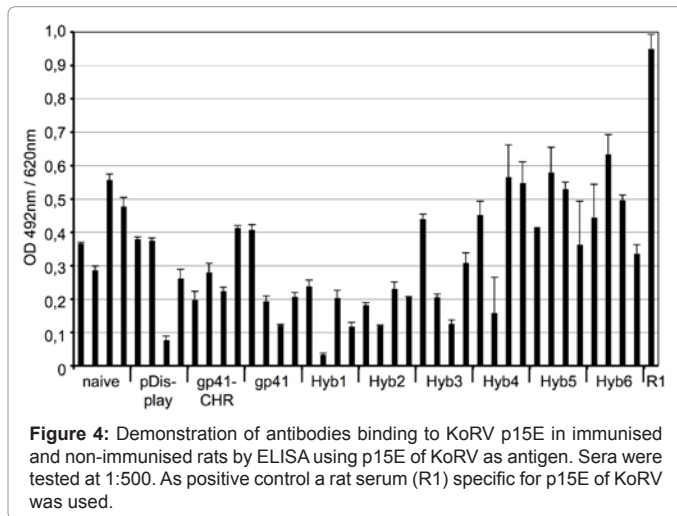
Immunisation of rats with DNA constructs corresponding to gp41,

to the C-terminal helix of gp41 (gp41-CHR) or to hybrid proteins composed of a backbone derived from p15E of the gammaretrovirus KoRV and the FPPR and MPER of gp41 of HIV-1 failed to induce neutralising antibodies. This new vaccination strategy was developed based on some recent findings: First, since immunising with p15E of different retroviruses was successful [6-11], hybrids were used because we expected that the helical regions of p15E bring the MPER and the FPPR of HIV-1, which substituted the FPPR and MPER of PERV, into close proximity, and the antigen may be able to induce neutralising antibodies. Second, a DNA immunisation approach was used to gain a membrane-associated localisation of the MPER expecting that it will be able to induce neutralising antibodies such as mAb 2F5. However, this approach failed despite the cell surface localisation of the expressed p41 or gp41-CHR was shown.

We recently identified a domain in the FPPR of gp41 that was able to enhance binding of mAb 2F5 and mAb 4E10 to their epitopes in the MPER [14]. Other authors had shown an interaction between MPER and FPPR during infection [18-22]. These findings suggested that the interaction between the MPER and the FPPR during infection may generate a conformation able to induce 2F5/4E10-like neutralising antibodies. However, all DNA constructs failed to induce antibodies significantly neutralising HIV-1 (Figure 3).

In other studies, the epitope sequence of 2F5 was added to the C-terminal part of p15E of PERV displayed on vesicular stomatitis virus particles in a membrane bound context [34] or as a rhinovirus hybrid [35] or in a pre-fusion state expressed on the surface of immature virus-like particles from Sf9 cells [36]. Only low, if any, titres of neutralising antibodies were observed. Wang et al. [37] reported the induction of neutralising antibodies after immunisation with a protein containing the CHR, NHR and the MPER, which recognised an epitope in the MPER. Immunising guinea pigs with DNA or VLP based on a HA/gp41 protein with a mutated Cys in the HA subunit, Ye et al. [38] reported a neutralising activity which could be blocked with a MPER-specific peptide, thus demonstrating induction of MPER-specific neutralising antibodies. Using a phage library, an HIV-1 elite controller was identified who developed antibodies against the MPER and neutralising antibodies were induced when immunising with the epitope phage [39]. Neutralising antibodies were also induced when using the epitope and synthetic sequential carriers or a palmitoyl group. On the other hand, immunising with VLP expressing the MPER Kandum Toukam et al. [40] were able to induce MPER-specific antibodies binding the 2F5 epitope, but not neutralising. A conformation-specific display of 4E10 and 2F5 epitopes on self-assembling protein nanoparticles also failed to induce neutralising antibodies [41].

There may be several reasons for the failure of many of the attempts to induce 2F5/4E10-like neutralising antibodies including our new approach: First, although it has been shown, that peptides derived from the FPPR increase the binding of mAb 2F5 to its epitope in the MPER/E2 domain [14] and there is an interaction between MPER and FPPR [18-22], it is still unknown whether this interaction is also required for the induction of 2F5/4E10-like broadly neutralising antibodies. Second, it remains unclear, whether the DNA immunisation generated the correct membrane associated context. HIV particles are budding from special regions of the cell membrane, so called rafts, that are characterised by specific lipid content [42] and it is unclear whether the DNA vaccines were also expressed in such a compartment. Third, conformational restrictions in the antigens expressed *in vivo* may have prevented the induction of neutralising antibodies. Fourth, pre-existing antibodies directed against the p15E backbone (Figure 4) may have



interfered with the antibody response against the gp41-derived FPPR and MPER when hybrids were used.

The finding that pre-formed antibodies against p15E exist in newly delivered rats is an interesting finding during these studies. It is likely that the antibodies reacting with p15E of the KoRV represent cross-reacting antibodies specific for p15E of another related retrovirus probably an endogenous rat retrovirus. There are numerous data showing that endogenous retroviruses can be activated during mitogen stimulation of lymphocytes *in vitro* [43-45] and in immune responses *in vivo* [45,46]. Thus, further studies would be needed to investigate the origin of these pre-existing antibodies.

The present study using a new immunisation approach based on MPER-containing antigens underlines, that a much deeper understanding of the structure of gp41 and the interaction of FPPR and MPER is required to overcome the recent restrictions in the induction of 2F5/4E10-like antibodies.

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