ISSN: 2475-6296

Research Article

Journal of Clinical & Experimental Immunology

Surface Engineering of Recombinant RNA Coliphage Qβ to Display gp41 Membrane-Proximal External-Region Epitopes from HIV-1

Alain Bopda Waffo[1, 2*], Loveline N. Ngu[3,4], Rana L. Singleton[1], Timothy Egbo[1], Josué L. Simo[11,13], Carrie A. Sanders[1], Georgia E Ambada[3,7], Nadesh N. Nji[3], Apeh A. Ngoh[8], QianaL. Matthews[1,14], Boakai K. Robertson[1,14], Thibau F. Tchouangueu[3,13], Abel Lissom[3,7], , Swapnil Bawage[1, 2], ColinceTchadji[3,7] Arinze S. Okoli[10], Charles O. Esimone[9], Rebecca Chukwuanukwu[12], Wilfred Mbacham[4,5,6], Lazare Kaptue[11], Godwin W. Nchinda[3*]

[1]*Department of Biological Sciences/College STEM 1627 Hall Street Montgomery, AL 36101*
[2]*Center for NanoBiotechnology Research, 1627 Harris Way Montgomery, AL 36104*
[3]*Laboratory of Vaccinology/Biobanking, CIRCB Cameroon*
[4]*Department of biochemistry, University of Yaoundé I, Cameroon*
[5]*The Biotechnology center, university of Yaoundé I, Cameroon*
[6]*The Department of biochemistry and physiology, Faculty of medicine, university of Yaoundé I, Cameroon*
[7]*Department of animal biology and physiology, University of Yaoundé I*
[8]*Department of biomedical sciences, University of Dschang, Cameroon*
[9]*Department of Pharmaceutical Microbiology & Biotechnology, NnamdiAzikiwe University Awka, Nigeria*
[10]*GenØk - Centre for Biosafety, Tromsø, Norway*
[11]*Université des Montagnes, Bangangté, Cameroon*
[12]*Department of Medical Lab Sciences; NnamdiAzikiwe University, Awka Nigeria*
[13]*Department of biochemistry, University of Dschang, Cameroon*
[14]*Microbiology Program, Department of Biological Sciences, College of Science, Technology, Engineering and Mathematics, AlabamA State University, Montgomery, AL, USA*

[*]Corresponding author
Godwin W. Nchinda, Laboratory of Vaccinology/Biobanking, CIRCB Cameroon, E-Mail: nsehleseh@gmail.com.

Alain BopdaWaffo, Department of Biological Sciences/College STEM 1627 Hall Street Montgomery, AL 36101, E-Mail: abopdawaffo@alasu.edu

Submitted: 16 Nov 2017; **Accepted**: 25 Nov 2017; **Published**: 05 Dec 2017

Abstract

Introduction: *The membrane proximal external region (MPER) of HIV-1 envelope glycoprotein-41 (gp41) is targeted by several broadly neutralizing antibodies whose conserved linear epitopes are promising targets for vaccine design. However, a formidable challenge has remained the difficulty to design and deliver MPER based immunogens for the efficient induction of such broadly neutralizing HIV-1 specific antibodies (bnAb). This is mainly because the linear bnAb MPER epitopes are poorly accessible to the immune system. The overall objective of this study therefore was the development of a novel RNA Qβ phage display system not only for monitoring anti-MPER specific antibody responses but equally as potential immunogens in future HIV-1 vaccine designs.*

Method: *To overcome the challenge of effective presentation of MPER to the immune system we have selectively engineered the surface of the RNA coliphage Qβ to expose all MPER bnAb epitopes. Briefly, DNA representing a 50 amino acids consensus region within the HIV-1 gp41 MPER was fused in frame with the minor coat protein A1of the Qβ phage. Three variant MPER expression cassettes were obtained with the MPER cDNA in frame with the minor coat protein A1 gene, including pQβMPER, pQβMPERHis and pQβMPERN. The expression cassettes were used for the production of QβMPER recombinant phages after transformation of E. coli HB101 strain. Antigencity of the phages was assessed with plasma from long standing anti-retroviral naïve HIV-1 infected people from the CIRCB AFRODEC cohort while immunogenicity studies were done in female Balb/c mice.*

Results: *The initial titers of all recombinant phages including QβMPER, QβMPERHis and QβMPERN were 10^4plaque forming units/ml (pfu/ml). This was significantly lower ($P<0.001$) relative to the 10^8pfu/ml of wild type phage, but was scaled up to 10^{14}pfu/ml. The fusion of MPER and Qβ genes was confirmed by RT-PCR followed by gel electrophoresis and sequencing. Specific recognition of some reported bnAb epitopes within MPER were confirmed in ELISA using the three recombinant QβMPER phages together with an MPER restrictive peptide as antigens and the bnAb 4E10, Z13e1, 2F5 and 10E8 as antibodies. Next the prevalence of MPER-specific antibodies was determined in plasma from long standing antiretroviral naïve HIV-1 infected participants of the CIRCB AFRODEC cohort. The greater majority (84%) of participants' plasma showed MPER peptide specific reactivity with anti-MPER specific* IgG antibody titers ranging from 200 to 409600 comparative to background IgG antibody titer with the Qβ phage alone as antigen or plasma from seronegative participants. In immunogenicity studies in Balb/c mice the recombinant phages QβMPERN and QβMPERHis induced significantly high Anti-MPER-specific IgG antibody responses ($P<0.04$) in at least 60 % of mice following three inoculations of each recombinant phage.

Conclusion: *Thus, these novel recombinantQβMPER phages can be used to monitor MPER-specific immune responses in HIV-1 exposed or infected people. In addition the recombinant QβMPER phages could be used as immunogens either alone as demonstrated here in mice or in combination with other strategies for the induction of MPER specific immunity against HIV-1.*

Keywords: MPER, Gp41, Hiv, Epitopes, BnAb, Qβ, Ni-NTA-Nanogold, 4E10, Z13e1, 2F5 and 10E8

Author Summary

The membrane proximal external region (MPER) of HIV-1 envelope gp41 subunit is a highly conserved region which is vital for both viral infectivity and fusion. In addition it also bears several linear contiguous epitopes targeted by anti-HIV-1 broadly neutralizing antibodies. This makes MPER an attractive candidate for the development of a vaccine against HIV-1 infection. Unfortunately several attempt to exploit MPER as a potential HIV-1 vaccine immunogen has yielded limited success. This is mainly due to poor delivery of MPER derived immunogen to the immune system in their native conformation. Here we have engineered the surface of a 25 nm sized coliphage Qβ to display 12 molecules of MPER per virus like particle. This allows efficient recognition by broadly neutralizing antibodies 2F5, 4E10, Z13e1 and 10E8 which target conserved epitopes within MPER. The recombinant coliphages could also detect MPER-specific antibodies in polyclonal plasma from well 84% of long standing anti-retroviral naïve HIV-1 infected people. In addition the recombinant QβMPER phages were also immunogenic in at least 60% of mice inoculated thrice with the phage particles. Thus, our recombinant QβMPER phages could be used either in monitoring MPER specific antibody responses or as a component of future vaccines against HIV-1 infection.

Introduction

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) consists of trimers of gp120 which are exposed on the surface and a gp41 subunit which anchors the protein into the membrane [1, 2]. This makes HIV-1 Env the only target for anti-HIV neutralizing antibodies because of its exposure on the surface of the virus [3]. Some HIV-1 infected people produce Env targeted monoclonal antibodies with a strong ability to neutralize a broad spectrum of HIV-1variants [4-8]. These broadly neutralizing antibodies (bnAb) target epitopes which are conserved in both gp120 and gp41 across several strains of HIV and as such represent promising targets for an anti-HIV antibody based vaccine development [8, 9]. Unfortunately several attempts to exploit these conserved targets to generate Env based immunogens for the induction of bnAb against HIV has yielded limited success [10-15]. Here, we have applied recent innovations [16, 17] in our group to generate novel immunogens targeting conserved bnAb epitopes in the HIV-1 gp41 membrane proximal external region (MPER). This is mainly because the HIV-1 gp41 MPER is one of the most conserved regions of Env playing vital roles in viral infectivity and fusion.

In addition to being highly conserved the MPER region is also targeted by several bnAb including 2F5, 4E10, Z13e1, m66, m66.6, 10E8 and CAP206-CH12 [10-15, 18, 19]. Some of these bnAb like the 10E8 are amongst the broadest and most potent bnAb reported thus far [9]. These MPER-specific neutralizing antibodies hold promise for vaccine development because they were isolated from HIV-1 infected people and have also been shown to be highly protective in animal challenge experiments [20-22].

However, a number of barriers have hampered the exploitation of MPER as a suitable immunogen for the induction of HIV neutralizing antibodies [20, 21, 23-25]. First, the linear MPER bnAb epitopes are only transiently exposed to the immune system and are located within hydrophobic domains which are strongly influenced by lipid membranes, making it less immunogenic. Secondly, the presence of several immunodominant non-neutralizing regions upstream of MPER within HIV gp41 focuses B cell responses away from MPER [21, 23, 24, 26]. Thirdly, some MPER specific bnAb like 2F5 and 4E10 are autoreactivec [27-31], which poses a challenge for inducing such antibodies to combat HIV infection. We hypothesize based on the linear contiguous epitopes within MPER, that; selective focusing of these conserved epitopes upon the surface of a recombinant coli phage Qβ could allow efficient MPER recognition by anti-MPER specific antibodies and consequent enhanced antigenicity and immunogenicity. Thus, to overcome the challenge of effective presentation of MPER to the immune system, we have selectively engineered the surface of a coliphage Qβ to expose all reported linear MPER based bnAb epitopes. Here, the surface of each recombinant QβMPER phage was engineered to display 12 molecules of MPER per phage particle.

Qβ phages are small positive stranded RNA viruses found throughout the world in bacteria associated with sewage and animal feaces [32, 33]. The infectious Qβ virion is about 25 nm in diameter with just four genes in a genome size of 4220 nucleotides [34]. The genes encodes the subunit II (β) a replicase, a major coat protein, a maturation protein (A2) and the read through minor coat protein (A1) [35-42]. Phage Qβ genome is surrounded by an icosahedral shell of 180 coat protein molecules, termed capsomers, that comprise 132 residues of amino acids each. A capsomer is made up of five-stranded antiparallel β-sheet "core" and a hairpin and 2 contiguous α-helices on the outside [43, 44, 45]. These capsomers are linked together by disulfide bonds in covalent pentamers and hexamers with a stoichiometric ratio of 12:20 and a well-known crystal structure [40]. The minor coat protein A1 protein shares the same initial codon with the coat protein and is produced during the translation when the coat protein stop codon UGA triplet (about 400 nucleotides from the initiation) is suppressed by low level of ribosome mis-incorporation of tryptophan at the coat protein termination signal [46-49]. The life cycle of Qβ phage starts by the adsorption of the phage on the F^+ of bacteria via the A2 protein followed by the injection of the RNA into the cytosol [37]. Once in the host, ribosomes bind to the start region of the coat protein gene, forming a complex which frees the replicase start gene for translation [50]. The replicase produced, would replicate more negative sense viral RNA that would serve as template for viral RNAs [51, 52] progeny production. The viral RNA would then be translated into other viral proteins. Finally, the coat proteins initiate the virus assembly [53].

The RNA coliphage Qβ display system was recently optimized by our group to facilitate mapping of B cell epitopes and the selection of neutralizing epitopes for immunotherapeutic strategies. Several attractive features of Qβ phage renders it suitable for optimization for an HIV-1vaccine immunogens including: short replication time and high mutation rate 10-4/nucleotide [54-60]. This in effect provides an innovative platform for rapid evolution of immunogens through rapidbiopanning, with their respective bnAb, to achieve efficient epitope display. Various immunogens from our group have previously been successfully cloned into the minor coat protein A1 region of the Qβ cDNA and were expressed to produce hybrid phages without compromising the infectivity of the phage Qβ [17, 43].

In this study, a 50 amino acids consensus HIV-1 gp41 MPER peptide sequence derived from an alignment of two clade C and one clade B MPER sequences, was engineered in frame with the minor coat protein

of Qβ. Three types of QβMPER recombinantphages bearing the MPER peptide were obtained after biopanning with MPER targeted antibodies. This consisted of recombinant QβMPER, QβMPERHis with an additional C-terminal hexa-histidine tag and QβMPERN with a C-terminal not1 site respectively. Surface expression of MPER upon the recombinant phage particle was determinedby bytransmission electron microscopy. Specific recognition of four linear MPER based bnAb epitopes was demonstrated in ELISA assay using the three recombinant QβMPER phages together with an MPER restrictive peptide as antigens and the bnAb 2F5, Z13e1, 4E10 and 10E8. The bnAb Z13e1 and 4E10 showed superior recognition of all three recombinant phages comparative to 2F5 and 8E10 in ELISA assays. This was in contrast to binding to an MPER restrictive peptide where 2F5, Z13e1 and 4E10 showed strong recognition. The 10E8 bnAb on the contrary showed the least reactivity with the MPER peptide in ELISA assay. The recombinant phage QβMPERN in addition to strong reactivity with Z13e1 and 4E10 also showed the strongest reactivity with 10E8 far better than binding to the MPER restrictive peptide and as such was selected for subsequent studies. Next, using plasma from long standing antiretroviral naïve HIV-1 infected people (6-15 years post HIV-1 infection) from the CIRCB AFRODEC cohort we could demonstrate the presence of polyclonal antibodies recognizing MPER on the surface of the recombinant QβMPER phages.

Using the MPER restrictive peptide in ELISA assays together with plasma from long standing antiretroviral naïve HIV-1 infected people we selected three sets of MPER reactive plasma to assess the antigenicity of the recombinant phages. This included two sets with low and high titers of anti-MPER specific IgG antibodies and a set completely devoid of MPER reactive antibodies In contrast to Qβ with no binding all three recombinant QβMPER phages showed specific reactivity in all anti-MPER IgG positive samples. The recombinant phage QβMPERN proved superior in its antigenicity in samples positive for anti-MPER specific IgG antibodies and therefore was selected for subsequent analysis. Thus using plasma from long standing antiretroviral naïve HIV-1 infected people (6-15 years post HIV-1 infection) from the CIRCB AFRODEC cohort we could demonstrate the presence of polyclonal antibodies recognizing MPER on the surface of the recombinant phage QβMPERN in 84 % of the participants. However there was neither correlation with plasmatic viral nor with helper $CD4^+$ T cell count and anti-MPER specific IgG antibody titers. In a comparative analysis of the immunogenicity of both QβMPERN and QβMPERHis in Balb/c mice anti-MPER specific antibody responses were detectable in 60 % (6/10) of the vaccinated mice for both recombinant phages.

Previous nonclinical studies had attempted to present MPER upon the surface of liposomes and viruses to the immune system [23, 61-65]. Our strategy differs from previous approaches in that we deliberately concentrate MPER on the surface of a small RNA phage in its native form rendering it more likely to be recognized by the immune system. Efficient tools for monitoring HIV-1 gp41 MPER specific antibodies should be of great importance especially when such antibodies have been implicated in the prevention of the transmission of HIV-1 especially from mother to child [78-83]. Thus, our recombinant QβMPER phages could be used either alone or in combination with other strategies for the induction and monitoring of HIV-1 gp41 MPER-specific immune responses.

Methods

Reagents

Media used for bacteria culture and phages were purchased from Fisher Scientific (Pittsburgh, PA). Restriction enzymes and T4 ligase were purchased from New England BioLabs (Ipswich, MA). RbCl and CaCl2 including other major reagents were purchased from Sigma-Aldrich (St. Louis, MO). DNA preparation for clones screening and PCR clean up kit was purchased from QIAGEN (Valencia, CA).

Microorganisms

E. coli MC1016 (Invitrogen, Grand Island, NY) was used to grow and maintain the plasmids. *E. coli* HB101 was used to grow and maintain pQβ8Not and pQβ8ΔA1and their recombinant products. *E. coli* ATCC 23725 (K12) was used for phage production and titration. Control plasmids with histidine tag were also maintained and amplified with the same cell: pQβHis, pQβΔA1His and pQβΔAhVAPHis.

Design and 3D structural modelling of the recombinant QβMPER phages

A 50 amino acid consensus sequence was obtained after aligning the conserved membrane proximal external region of three envelope genes including one clade B and two clade C sequences. Sequences were based on the codon optimized plasmids pconCgp160-opt (631) [66, 67], p96ZM651gp160-opt(657) [68] and pConBgp160-opt (646) [66, 67] and representative MPER sequences from data previously published by our group [69] and others [70]. The expression cassettes were analyzed using the sequence analysis software DNA strider while taking into consideration the secondary structure of the RNA of the recombinant phage. The designed MPER region included an SSGGG linker together with the restriction sites *NotI, AflII* and *NsiI* for cloning. The first PCR was done by the annealing and extending with primers ABW1 and ABW2 (150 bp). Using partially complementary primers design the product of the first PCR, was used as a template for the second PCR (150 bp). A forward primer ABW3, was used to amplify the MPER introducing an*AflII* restriction sitetogether with a reverse primer ABW4 bringing in an NsiI restriction site and 6 His-tag (272 bp). Similarly the MPER insert was again amplified with the primers ABW5 (forward) and ABW6 (reverse) introducing a NotI restriction site at both ends (245 bp). All restriction enzyme sites are bold and underlined. These primers were designed to maintain the reading frame and the important secondary structure of hybrid phage RNA.

The sequences for the minor coat protein A1 and engineered proteins were modelled using the template based modelling using RaptorX web server (doi: 10.1038/nprot.2012.085). The models were then transformed to view the protein backbone and highlight the secondary structures using MolGro molecular viewer. The structures were next aligned to understand the differences among the protein models.

Cloning

For restriction digestion, the vector plasmids pQβ8ΔA1 and pQβ8Not were incubated with *AflII/NsiI* and NotI restriction enzymes respectively, at 37°C for 2 hrs. The digested plasmids were run on a low-melting 0.8% agarose gel. The band of ~ 3470bp in size was isolated and purified. The purified linearized vector was dephosphorylated using alkaline phosphatase. The PCR fragments (total three) were separately digested and also purified using the

above protocol and digested with the corresponding restriction enzymes accordingly. The dephosphorylated plasmids pQβ8ΔA1 and pQβ8Not were used to ligate the various MPER fragments accordingly. The various purified MPER inserts (from the second PCR products) flanked with *AflII/NsiI* or NotI restriction sites were incubated with the corresponding vector for ligation for 18 hrs at 16°C (or overnight).

Clones screening/verification

The total product of ligation was used to transform *E.coli* HB101 in each case. For each construction 5 to 10 clones were used to prepare DNA for screening. DNA obtained from clones was selected and screened using *NotI* and *BglII* restriction enzyme. The confirmed plasmid was sequenced to validate fusion and ensure that the insert was in frame at the end of the minor coat protein A1.The positive clones were used to retransform *E. coli* HB 101 for phage productivity and plasmid amplification.

Phage production and purification

Two clones from retransformation were selected separately and inoculated into a nutrient media (2YT), and allowed to grow at 37°C for 4 hrs while shaking (150 rpm). Eachculture was then transferred to one liter of 2YT and allowed to grow at 37°C for 18 hrs while shaking (150 rpm). The phage was precipitated using 29g of NaCl and 80g of polyethyleneglycol (PEG_{8000}). The precipitated phage was amplified using an indicator bacteria cell, *E. coli* (K12). One percent of the fresh overnight culture of K12 on nutrient media (TGY) was amplified at 37°C while shaking (180 rpm) for 3-5 hrs to reach an OD_{600} of 0.6-0.8. The phage was inoculated to the culture at a multiplicity of two and incubated at 37°C for 18 hrs on a racking platform (150 rpm). Following incubation, the phage titer was checked and used to determine the scale of amplification. After amplification, the phage was precipitated, and measurements were done to determine titer and morphology.

Determination of recombinant phage yield and plaque quality

Indicator bacteria K12 was grown to log phase (OD_{600} of 0.6-0.8). A volume of 100µl of the culture, 100µl of phage was added to 3.5 ml of TGY-Top agar (with 0.6% agar) and the mixture was poured on the surface of agar plate. The plates were left standing for few minutes to solidify, then incubated at 37°C for 16 hrs and examined for lysis of the *E. coli*. The plaque count was done according to Pace and Spiegelman. The intensity of the variability of plaque size was observed to predict the nature of Qβ and recombinant as a quasi-species.

Reverse transcription (RT) PCR from purified phages

To extract RNA from the purified phage, 185µl of phage, 5µl of β-Mercaptoethanol and 10µL of SDS (20%), incubated at 37°C for 1 hr and centrifuged at room temperature at 10,000 rpm for 10 mins followed by ethanol precipitation. Overall, 2 µg of RNA with 1µl of reverse primer and 1µl of dNTP (10 mM) in a total volume of 13µl (filled with RNase-free water) were incubated at 65°C for 5 mins. This was followed by incubation on ice to allow annealing of primers to the template. Of 5x, 4µl of RT-buffer, 1µl of DTT (0.01x) and 2µl of enzyme superscript III were added to the mixture. The mixture was incubated at 25°C for 5 mins to activate the superscript III enzyme, 55°C for 45 mins for nucleotide insertion followed by enzyme inactivation at 70°C for 15 mins. Of the RT-PCR product, 1µl was used to amplify the cDNA using 0.5µl phusion (HF) enzyme.

Western blot analysis for MPER region expression by the recombinant QβMPER phages

The purified phage was prepared using 92.5µl phage, 2.5µl β-Mercaptoethanol and 5µl SDS (20%), and incubated for 1 hr at 37°C. Of the Laemmli SDS buffer (2x), 100µl were then added and further incubated for 30 mins at 100°C. The prepared phage sample was loaded in a 15% polyacrylamide gel and allowed to run for 1 hr intervals at 30, 50 and 70 volts. The separated proteins were then transferred unto a nitrocellulose membrane, blocked with 1x roti block and probed with the MPER specific monoclonal antibodies 4E10, Z13e1 and 10E8 respectively. Specific recognition of MPER bound 4E10, Z13e1 and 10E8 antibodies was probed with Horse radish peroxidase-conjugated mouse anti-human IgG1 (Southern Biotech) diluted 1:4000 in 0.1x roti block. Bound conjugate was detected using 1-step ultra TMB blotting solution (Thermoscientific) and the HRP reaction stopped by washing with by molecular grade pure water.

Tracking the surface localization of the MPER peptide using Ni-NTA-Nanogold®

Ni-NTA-Nanogold® is a gold nanoparticle with a nickel complex with the ability to bind to hexa-histidine tagged proteins. In order to track the exact location of the MPER peptide upon the hybrid phage we developed the unique hybrid phage QβMPERHis where a hexa-histidine tag was engineered in frame with the MPER petide. Here the Ni-NTA is expected to bind specifically to the hexa-histidine tagged protein. Thus the Ni-NTA-Nanogold was employed to track the exact location of the inserted MPER region in the phage particle. Here the 5nm nanogold from Nanoprobes (Nanoprobes,Yaphank, NY, USA) was used for this analysis. The purified phage and Ni-NTA-Nanogold® (1%) was added together before adding to the grid. After a manual mixture, 3µl was added to the grid and 3µl of uranyl acetate was used to stain the grid.

Binding and infectivity

Microtiter plates were coated with recombinant phages at 10^7pfu/well and incubated overnight at 4°C while shaking. The plate was washed 3 times with PBS at different time points. 100µl of the indicator bacteria K12,was added to each well, and allowed to shake for 1hr at 37°C. The culture was then removed and used in plaque assay to determine the amount of plaques formed.

Ethics statement

All human samples in the AFRODEC Cohort were collected with written informed consent under clinical protocols approved by the Republic of Cameroon National Ethics Committee (protocol number 2014/10/499/CE/CNERSH/SP) the CIRCB institutional review board (protocol number 14-11) and the Cameroon government administrative authorization (authorization number 631-1112). The AFRODEC cohort consist of adult HIV-1 infected participants aged 21 to 65 years who were naïve to antiretroviral therapy.

Plasma sample collection and Processing

This was a cross-sectional study, conducted between November 2012 to November 2016, which enrolled anti-retroviral naïve HIV-1 infected people who were 21 years or older. Blood was collected into 4-ml plastic Vacuum blood spray-coated K2EDTA tubes called

Vacutest (Vacutestkirma, Italy). All samples were stored at room temperature and processed within 4 hrs of collection. To obtain plasma, samples were centrifuged at 2,000 rpm for 10 min at 4°C. The plasma fraction was harvested sterile under the hood, aliquoted in small, single-use volumes and stored at -20°C until use. The plasma obtained from anti-retroviral naive HIV-1 infected participants was heat inactivated for 30 minutes at 56°C prior to ELISA assay.

Characterization of the recombinant QβMPER phage byenzyme-linked immunosorbent assay(ELISA)

QβMPER ,QβMPERHis, QβMPERNand Qβ were diluted in PBS and 100 µl containing 10^7 phage particles/well were added to high binding 96-well flat bottom microsorp (Thermo Fisher Scientific) ELISA plates and incubated overnight at 4°C.As a positive control the MPER restrictive peptide (LELDKWASLWNWFDITNWLWYIK, (NIH AIDS Research and Reference Reagent Program, Germantown, USA) was also coated at 100ng/well in PBS and similarly treated like the recombinant phages. The following day, plates were washed 3x with PBST (PBS with 0.05 % Tween-20) and blocked either with 3% BSA or 1x Roti block (Carl ROTH, Karlsruhe, Germany) for one hour at 37°C. Some reported MPER targeted broadly neutralizing HIV-1 antibodies including 2F5, Z13e1, 4E10 and 10E8 (NIH AIDS Research and Reference Reagent Program, Germantown, USA) were diluted in 2% BSA at concentrations of 10, 1, 0.1, 0.01 and 0.001 µg/ml, then 100 µl of the diluted antibodies were added per well of the blocked ELISA plates and incubated for two hours at 37°C. Unbound antibody was removed by washing the plates 5x (198 µl/well) with PBST. The recombinant QβMPER phages bound 2F5, Z13e1, 4E10 and 10E8 antibodies was probed with Horse radish peroxidase-conjugated mouse anti-human IgG1 (Southern Biotech) diluted 1:4000 in 0.1x roti block. Bound conjugate was detected using ABTS substrate and the HRP reaction stopped by adding 50 µl a stop solution according to the manufacturer's protocol (Southern Biotech). The colorimetric signal was measured at 405 nm using a multiscan FC microplate reader (Thermo Fisher Scientific, USA).

Recombinant QβMPER phage Enzyme-linked immunosorbent assay (ELISA)

Similarly ELISA plates (Thermos Fisher Scientific, USA) were coated overnight with 10^7pfu/well of the recombinant phage QβMPERN. Plates were washed 4x with PBST (PBS containing 0.05% Tween 20) and blocked for one hour with 3% BSA in PBST at 37°C. Heat inactivated plasma from anti-retroviral naïve HIV-1 infected participants (members of the CIRCB AFRODEC cohort from 2012 to 2016) was diluted 1:1000 in PBS then added in each well (100 µl) of the antigen coated plates and incubated for two hours at 37°C. The plates were washed 5x (198 µl/well) with PBST after which the bound antibody was probed with a peroxidase-conjugated mouse anti-human IgG (Southern Biotech) diluted 1:2000 in PBS. Bound conjugate was detected using ABTS substrate and stop solution according to the manufacturer's protocol (southern biotech,). The colorimetric signal was measured at 405 nm using a multiscan FC microplate reader (Thermo Fisher Scientific, USA).

Immunogenicity of the recombinant QβMPER phages in balb/c mice

6-8 weeks old female Qβ seronegative Balb/c mice were inoculated each twice subcutaneously in the paws with 10^7 pfu of Qβ, QβMPERHis and QβMPERN at three weeks interval. Three weeks later the mice were anesthetized with Hallothane BP (Piramal Healthcare, Andra Pradesh; india) and boosted intranasally with the same treatment. 14 days post boost blood was collected through ocular vein and sera harvested for subsequent analysis. Starting with a dilution of 1:200 serial two fold dilutions were tested for Qβ specific antibodies after coating ELISA plates with Qβ, QβMPERHis and QβMPERN. To determined anti-MPER specific antibodies high binding ELISA plates were coated with MPER restrictive peptide (as described above. ELISA titer was expressed as arbitrary units (AU) based on five human samples with high anti-MPER specific IgG antibodies.

Results

Design and generation of pQβMPER plasmid vector variants

We previously demonstrated that up to 300 bp of foreign sequences could be inserted into the minor coat protein gene A1 of Qβ phage, without affecting its functionality. In addition, our group has also reported the production of phages with the plasmid harboring/containing A1 deletions. As such, the plasmids pQβ8 and pQβΔA1 were used to generate recombinants containing a 50 amino acid MPER region with and without various linkers (Fig 1A). A consensus 50 amino acid MPER region was obtained after alignment of HIV-1 envelope sequences from three different HIV-1 strains including two clade C and one clade B (Fig1B). The MPER region was amplified with the primers indicated in Table 1 with reference sequences previously published by our group [69] and others [70, 71]. The secondary structure of all recombinant phage RNA was analyzed to ensure that all major features necessary for recombinant phage infection were conserved (Fig 2C to D).

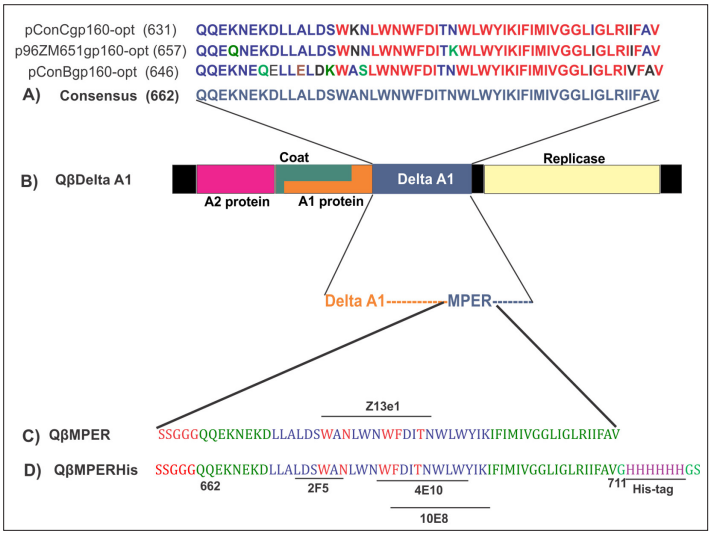


Figure 1: Consensus MPER amino acid sequence, schema of the recombinant QβMPER phages and presentation of the MPER region.

(A) Alignment of three HIV-1 envelope sequences to generate the 50 amino acid MPER region. In (B) schema of Qβ indicating the deletion in the C-terminal end of the minor coat protein A1 where the MPER region was inserted. The consensus sequence of HIV-1 gp41 MPER designed and constructed is fused with the minor coat protein A1 gene end. In (C) amino acid sequence of the recombinant phage QβMPER with an N-terminal SSGGG linker together with the epitope recognized by Z13e1. In (D) amino acid sequences of the recombinant phage QβMPERhis together with the epitope recognized by the antibodies 2F5, 4E10, 10E8, and a C-terminal hexa-histidine tag.

3D modelling to determine the localization of the inserted MPER region within the minor coat protein A1

In order to ascertain the exact location of the MPER region upon the surface of the hybrid phages its amino acid sequence were used for three-dimensional (3D) modeling together with the minor coat protein A1, it derivatives A1Not, ΔA1 and ΔA1MPER (Fig2).

The 3D structural modelling indicated that the MPER region is displayed on the C-terminal end of the minor coat protein A1 as revealed by corresponding changes in its conformation when compared to the wild type minor coat protein A1. This was in contrast to the N terminus of the minor coat protein A1 protein,which apparently was not significantly changed thus conserving itsα-helixes and β-sheets numerically in all models (compare Fig 2A and 2D). Nevertheless, the modification in the C- terminal of the modified minor coat protein A1 seemed to have impacted upon the rotation of its N-terminus (Fig 2D). The effect of amino acids deletion in the minor coat protein A1 was a reduction inβ-sheets of the C-terminus (Fig 2B). Similarly, the addition of an SSGGG linker together with the Not restriction site in place of the deletion in the minor coat protein resulted to a protein with GGR extension that is prominently displayed at the C-terminus (Fig 2C). Finally, conserved MPER consensus region consisting of 50 amino acids sequence was predicted to be inserted into all forms of the minor coat proteins A1 including A1, ΔA1 and ΔA1NotI proteins in C-terminus (Fig 2D). The MPER was confirmed to be exposed at the C-terminus around the β-sheets and between the N-terminal domains. Thus modeling of the C-terminus of the recombinant minor coat protein A1; situated it to the outer surface of the capsid as shown in the ball and stick depiction (Fig 2D).

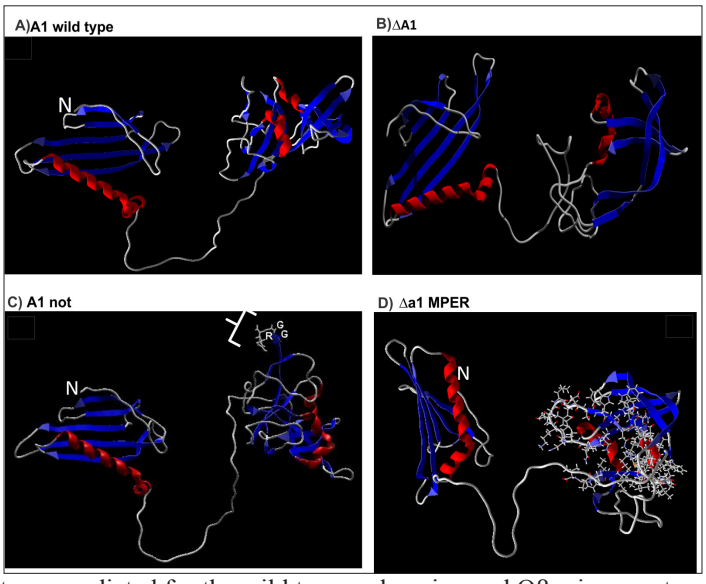


Figure 2: Three dimensional structures predicted for the wild type and engineered Qβ minor coat protein A1.

(A) A1 wild type (B) ΔA1 (C) A1Not (D) ΔA1MPER (MPER highlighted in ball and stick rendering at the C terminus region of the protein). (N indicates the N terminus of the minor coat protein A1; red and blue colored structures indicate α-helix and β-sheets, respectively).

Genetic construction and characterization of HIV-1 MPER vector

In all designs, the two natural stop codons of the minor coat protein A1 were conserved as well as the intermediate region between the minor coat protein A1 and the replicase gene of Qβ. The oligonucleotides ABW1 to ABW4 were analyzed to eliminate features that could prevent the fusion PCR. Briefly, to obtain the MPER region the oligonucleotides ABW1 (forward) and ABW2 (reverse) shown in Table 1 were annealed and extended by PCR. The resultant PCR product was further extended with the primers ABW3 (forward) and ABW4 (reverse) which introduced a linker and restriction enzyme cloning sites at the ends of MPER region (Table 1). In the design, the ABW3 primer was flanked with *AflII* while ABW4 had *NsiI* and *NotI*. The *Not1* restriction enzyme (bold and underlined) sequence enabled the cloning of the MPER into the variant pQβ8 resulting to the plasmid pQβMPERN (with a c-terminal *Not1* restriction site) while the restriction sites *AflII* and *NsiI* (bold and underlined) were used to obtained the plasmid pQβMPER (S1A). The same gene was obtained with ABW5(forward) and ABW6 (reverse) primers that generate the linker NotI restriction enzyme at both ends (245 bp). These primers were designed to maintain the reading frame and the important secondary structure of the recombinant phage RNA. The pQβMPERHis was obtained by amplifying the previous insert with a reverse primer which incorporates a hexa-histidine tag at the C-terminal of the MPER region (S1B). The resultant expression plasmids were analyzed and all found to contain the MPER region. The positive recombinant plasmids after restriction analysis were further amplified and sequenced. The plasmids containing the inserted MPER region in the right frame was used to transform *E. coli* HB101 for phage production and characterization. Recombinant minor coat protein A1 fused with MPER was expressed after transformation of *E. coli* with recombinant plasmids harboring MPER gene. As a consequence of the surface engineering of the recombinant phages the plaques sizes were predominantly (80%) of small size representing recombinant phages containing the MPER insertion (S2). This difference in plaque size has previously been reported to be a reflection of the quasi species nature of RNA phages [72].

Table 1: Sequences of oligonucleotides designed for MPER PCR construction

ABW1 of 100 bases (5' to 3')
CAGCAGGAAAAAAACGAAAAAGATCTGCTGGCGCTGGATAGCTGGGCGAACCTGTGGAACTGGTTTGAT
ABW2 of 100 bases (5' to 3')
CACCGCAAAAATAATGCGCAGGCCAATCAGGCCGCCCACAATCATAATAAAAATTTTAATATACCACAG
ABW3 of 64 bases (5' to 3')MPER Afl2
TTCGGTAAACATCGAACAATTCATCTAT**CTTAAG**CAGCAGGAAAAAACGAAAAAGATCTGCTGG
ABW4 of 60 bases (5' to 3')Nsi&Not
TTAGAC**ATGCATT**TCATCCTTAGCGGCCGCTTACTACACCGCAAAAATAATGCGCAGGCC
ABW 5 of 33 bases(5' to 3') MPER Not
5'TTAATTCATCTATCTTAAGTCGA**GCGGCCGC**gg
MPER Not His Rev
5' ggTGCTGTCTTAGACATgcatttcatccttagg
ABW6: MPER Not Rev
5' AATAAT**GCGGCCGC**TTACTACACCGCAAAAATAATGCGCAGGCCAATCAGGCC

Recombinant Phage production strategy and genetic characterization

Previously, phages were produced just by transforming *E. coli* HB101 with various expression cassettesresulting to phage titers between 10^8 to 10^9pfu/ml after production was scaled up to 1L of media for 12 hours. However, there was a significant reduction ($P<0.0001$) in the titer of the hybrid QβMPER virus like particles (Table 2 compare titer of Qβ with QβMPER, QβMPERHis) and QβMPERNharboring MPER. This significant reduction in recombinant phage titer necessitated the adoption of novel production strategy to enhanced hybrid phage yield. In this light the recombinantphages obtained after the first round of amplification in one liter, were precipitated prior tosuspension in 10 ml of media followed by 3 more rounds of amplification each with an inoculating multiplicity of infection (MOI) of 3. In order to improve yield *E coli* Q13 the natural host of the phage Qβ was used instead of *E. coli* HB101. This resulted into a significant increase in the yield of the resultant recombinant phages(10^{14}pfu/ml) which were then used for subsequent analysis.

Table 2: Phage viability assay: the affinity, infectivity and viability of phages are shown by an increase in titer after 45 min incubation with $^{E\ coli}$ K12 determined at log phase (OD600~ 0.7)

Phage	Initial Titer (pfu/mL)	Plated (pfu/mL)	Final Titer (pfu/mL)
QβMPERN	10^7	10^5	10^7
QβMPER	10^7	10^5	10^7
QβMPERHis	10^7	10^5	10^7
Qβ	10^8	10^6	10^8

Viability of the recombinant QβMPER phages

The viability of all recombinant QβMPERphages was analyzed in a binding and amplification assay. RecombinantQβMPER phages while bound to the ELISA plates still infected the host *E. coli* Q13 increasing in titer by 2 digits within 30 minutes of incubation. This demonstrates not only viability but equally an ability to infect thehost *E. coli* Q13 and grow while binding to ELISA plates (Table 2). The viable recombinant QβMPERphages were then analyzed by RT-PCR and found to contain recombinant insertions equivalent in length to the 50 amino acid MPER insert. The fusion between MPER RNA and the phage genome was confirmed by sequencing of the amplified product of the RT-PCR. The sequences obtained for QβMPER, QβMPERHis and QβMPERN hybrid phages matched recombinant inserts cloned into the plasmids pQβMPER, pQβMPERHis and pQβMPERN respectively. Thus, the insertion of the MPER region into the minor coat protein A1 of coliphage permits efficient display of the MPER peptide without affecting viability and infectivity.

Western blot anlysis of Recombinant QβMPER phage expression of MPER peptide andLocalization of MPER region upon the recombinant QβMPERHis phage using Nickel gold NTA particles

To characterize thisrecombinantQβMPER phages, protein from partially purified phages were denatured, separated in an SDS-polyacrylamide gel and transferred onto a proteins-absorbing membrane. The membrane was incubated with the MPER targeted broadly neutralizing antibody 4E10. Specific recognition of the MPER region resulted to aclear band in intensity close to the 35kDa A1MPER fusion protein. Various proteins fused to the minor coat protein A1 have previously been used to validate the presence of the insert (Fig 3A). Similar results were obtained when the membrane was incubated with Z13e1 (Fig 3B). In addition recombinant QβMPERN expression as shown in Fig 3C could also be detected with , 8E10 in western blot [15, 71]. Thus, confirming the existence of the MPER region within the recombinantQβMPER phage particles. In order to track the exact location of the MPER region upon the recombinant phage we developed the unique recombinant phage QβMPERHis where a hexa-histidine tag was engineered in frame with the inserted MPER region. Here the Ni-NTA is expected to bind specifically to the hexa-histidine tagged protein. Thus the presence of the MPERHisregion and its surface display was confirmed by transmission electron microscopy usingNickel gold particles (Ni-NTA-Nanogold) that specifically bind to hexa-histidine tag (Fig 3D to G). Therefore, the density of the nanogold particles following specific recognition of the hexa-histidine tag in the recombinant QβMPERHis phages was comparatively very high relative to the wild type Qβ and QβMPERphage without the hexa-histidine tag. In Fig 3D to G, EM images clearly show the association of the nanogold with the virus like particles containing the hexa-histidine tag. This association of nanogold with the tag at the end of the inserted MPER region suggests its expression and exposition on the external surface of the recombinant phage particles. Taking into consideration the

image and the scale, the diameter of the recombinant phage particle was not significantly modified after the MPER peptide insertion.

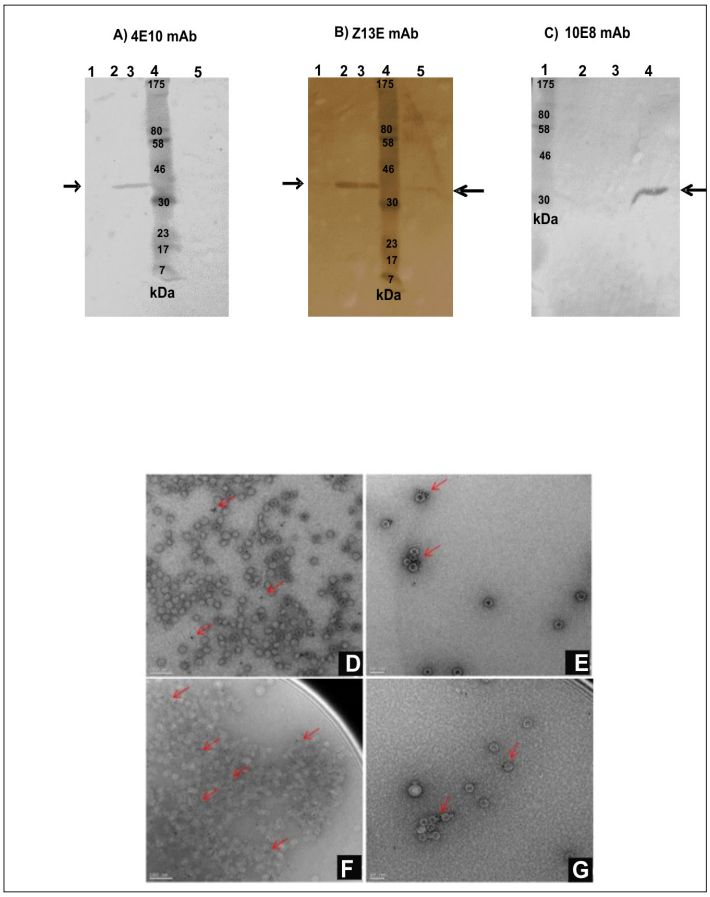


Figure 3: Western blot analysis, antigenicity and electron micrograph of hybrid QβMPER phages

In (A) specific recognition of MPER region by 4E10.Lane 1 isQβ alone, lane 2 is recombinant QβMPERHis, lane 3 is recombinant QβMPERN and lane 4 is the protein marker. In (B) specific recognition of MPER region by Z13e1.Lane 1 is Qβ,lane 2 is recombinant QβMPERHis ,lane 3 is recombinant QβMPERN , lane 4 is the protein marker and lane 5 is recombinant QβMPER .In (C) specific recognition of MPER region by 8E10 in the recombinant QβMPER phage. Electron Microscope (EM) of phage with Nickeled Gold (NTA); in (D) Qβ wild type. (E) Recombinant QβMPERHis with histidine tag; (F) RecombinantQβMPERHis with histidine tag; (G) Recombinant QβMPERHis with histidine tag diluted.

Antigenicity of MPER restrictiv e peptide, QβMPER, QβMPERHisand QβMPERN with MPER targeting broadly neutralizing antibodies

The recombinant QβMPER, QβMPERHis and QβMPERN phages displaying the MPER peptide and an MPER restrictive peptide were used to assess reactivitytoMPER targeting bnAbsincluding 2F5, Z13e1, 4E10 and 10E8. Briefly ELISA plates were coated with 10^7pfu/well of with QβMPER, QβMPERHis, QβMPERN and MPER restrictive peptides as described in materials and methods then probed with graded doses of the MPER specific antibodies. As expected the MPER targetingmAb 4E10, Z13e1 and 2F5 showed strong reactivity with the MPER restrictive peptide (Fig 4A, B&C). In contrast 8E10 MPER restrictive peptide reactivity was poor almost next to background level following several repeat experiments (Fig 3D). Binding of the recombinant phages QβMPERHis and QβMPERN to 4E10 and Z13e1 were similar to that of the MPER restrictive peptide (compare Fig 4A &B)

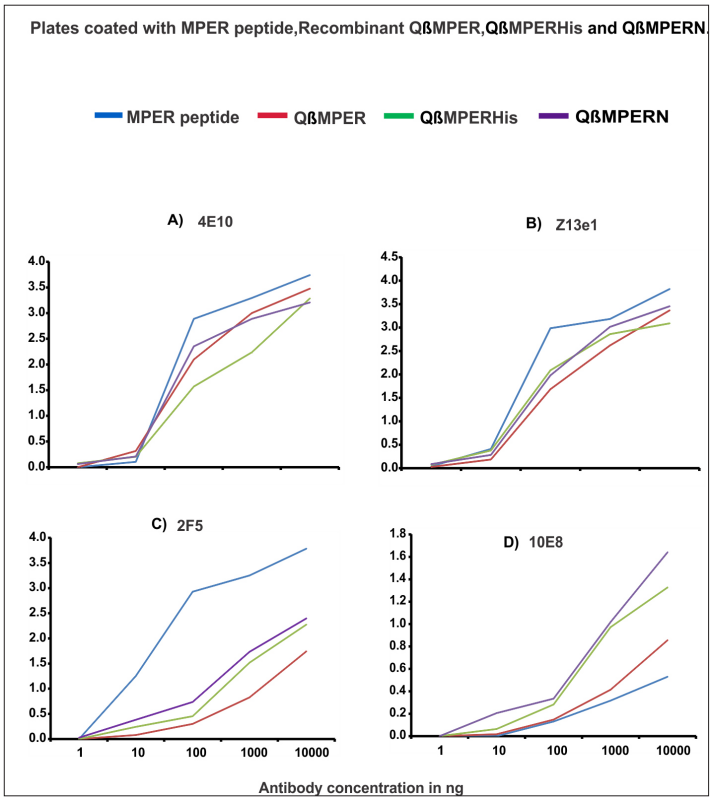


Figure 4: Antigenicity of MPER restrictive peptide, QβMPER, QβMPERHisand QβMPERN with MPER targeting broadly neutralizing antibodies

In (A) (B) , (C) & (D) binding of MPER targeting bnAbs4E10, ZI3e1 ,2F5, and 10E8 to MPER restrictive peptide (blue line), QβMPER (red line), QβMPERHis (green) and QβMPERN (purple line) recombinant phages. ELISA plates are coated with 107pfu/well of QβMPER, QβMPERHis or QβMPERNthen probed with graded doses of the MPER specific antibodies. In (A) reactivity of graded doses of 4E10 with the MPER restrictive peptide as well as the three recombinant phages. In (B), (C) and D same as in (A) but here binding is assessed with graded doses of Z13e1, 2F5 and 10E8 respectively.

Study Population Characteristics

A total of 154 ARV naïve HIV-1 infected participants were included in this study. Most (64%) were females with median age 39 (32 – 47) and the rest males with median age 38.5 (28.75 -46.25). Median CD4 counts were significantly ($p<0.01$)lower in female participants than in their males counterparts:499 cells/mm3 (463-983 – 587.5) Vs. 561 cells/mm3 (404-860) respectively. Whereas the majority of ARV naive HIV^+ participants (57%) showed no significant immune suppression (CD4>500 cells/mm3), 25% had mild immunosuppression (350-499 cells/mm3), 10% had advanced immunosuppression (200-349 cells/mm3) and 8% had severe immunosuppression(1-199 cells/mm3). Well over 92% of participants were in WHO clinical stage 1 and the rest in WHO clinical stage 2. Females showed significantly higher ($p<0.05$) plasmatic viral load than males (Table 3). There were relatively more females participants than males in this study. The female domination of our participants is a reflection of the global

prevalence of HIV-1 infection within our sub region (UNAIDS, 2015).

Table 3: Study population characteristics

Variable	HIV-1-infected participants (n=154)	
Gender	Male	Female
Participants (%)	55 (36)	99 (64)
Median age (IQR)	38.5 (28.75-46.25)	39 (32-47)
Median CD4 count (cells/mm3)	561 (404-860) **	499 (463-983)
CD4>500 cells /mm3 (n/%)	27 (49)	61 (62)
350-499 cells /mm3 (n/%)	17 (31)	21 (21)
200-349 cells /mm3 (n/%)	5 (9)	10 (10)
1-199 cells /mm3 (n/%)	6 (11)	7 (7)
Median viral load (copies/mL)	8579 (489-41237)	19760 (1090- 141839)*
OD Values 0.1 to 0.3 (n/%)	19 (19)	6 (11)
OD values 0.4 to 0.9 (n/%)	71 (72)	41 (75)
0%D values 1 to 2.4 (n/%)	9 (9)	8 (8)

* P<0.05; **P<0.01; IQR= interquartile range, n=number of participants.

Comparative analysis of the antigenicity of the recombinant phages QβMPER, QβMPERHis and QβMPERN for reactivity with polyclonal antibodies in plasma from ARV naïve HIV-1 infected individuals

Since the three recombinant phages QβMPER, QβMPERHis and QβMPERNwere slightly differentin their design, we tested if there will have the same reactivity with polyclonal antibodies in plasma from ARV naïve HIV-1 infected individuals. Here the antigenicity of the three recombinant phages was tested using plasma samples calibrated with the MPER restrictive peptide by ELISA.Using the MPER restrictive peptide in ELISA assay reactive plasma samples were grouped as either low (titers <12800) or high (titers >12800) anti-MPER specific IgG antibodies before being used for the comparative analysis of three recombinant phages.In fig.5A data is shown comparing the antigenicity of QβMPER, QβMPERHis, and QβMPERHis with low tittered anti-MPER specific IgG antibodies. Here the recombinant phages QβMPERHis (p<0.001), and QβMPERN (p<0.001) showed superior reactivity with plasma containing low anti-MPER specific IgG antibodies than QβMPER. In addition the recombinant phage QβMPERN was also superior to QβMPERHis (p<0.0016) in its antigenicity.When the antigenicity of the three recombinant phages (fig. 5B)was compared in plasma samples containing high anti-MPER specific IgG antibodies both QβMPERHis and QβMPERN proved superior to QβMPER (p<0.0011 and p<0.0005 respectively) in their reactivity with the MPER peptide within the recombinant phages. However there was no significant difference (p<0.2427) in the reactivity of both QβMPERHis and QβMPERN with plasma containing high titer anti-MPER specific IgG antibodies.When binding of all three recombinant phages was compared in the 154 plasma samples described abovethe majority of the participants' plasma showed strong reactivity to all three recombinant QβMPER phages, only 20 to 30% showed low reactivity to the recombinant QβMPER phages (S3). These low reactive plasma samples are considered MPER-specific antibodies negative because their OD values were similar to values obtained with plates coated with Qβ.On the other hand, around 10% of participants' plasma demonstrated very strong reactivity to all hybrid phages with OD values ranging from 1 to 2.4. Taken together, this data indicate that the MPER peptide is displayed in efficiently upon all the recombinant QβMPER phages thereby yielding similar antigenicity with polyclonal plasma from ARV naïve HIV infected participants. Thus, a great majority of ARV naïve HIV-1 infected people expressing MPER specific antibodies could be detected by any of our three novel recombinant QβMPER phages.

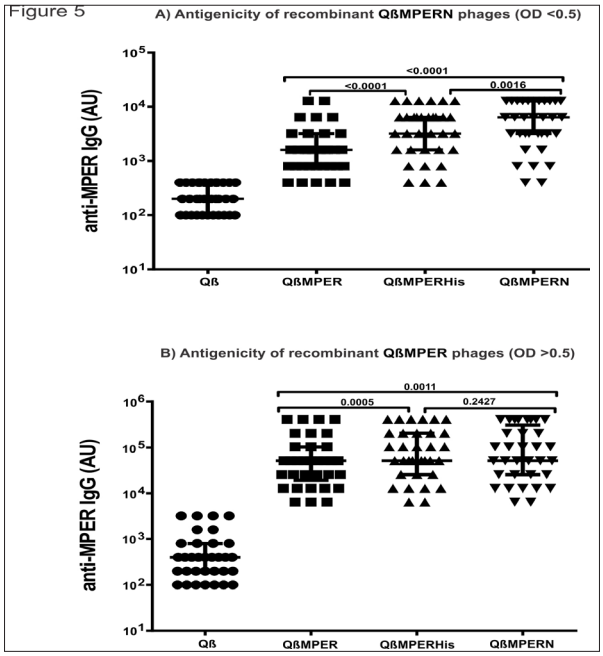


Figure 5: Antigenicity of recombinant phages QβMPER, QβMPERHis and QβMPERN in plasma from ARV naïve HIV-1 infected individuals.

Selected plasma of ARV naïve HIV-1 infected participants was testedfor binding against different recombinant phages including QβMPER, QβMPERHis and QβMPERN. In (A) data is shown for the reactivity ofQβMPER,QβMPERHis and QβMPERN with plasma containing low titer anti-MPER specific IgG antibodies.. Here QβMPERNwas significantly more antigen (P<0.016) than QβMPER, and QβMPERHis. In (B)the three recombinant phages were compared with each for reactivitivity with participants plasma containing high titer anti-MPER specific IgG antibodies.. With plasma containing high anti-MPER specific IgG antibodies QβMPERHIS, and QβMPERN were similar in reactivity but both significantly higher than QβMPER (P<0.0011 and P<0.0005 respectively).The endpoint titers were considered as the highest plasma dilution with a reading above Qβ alone coated wells plus three standard deviations.

Analysis of HIV-1 gp41 MPER specific IgG antibody in plasma from ARV naïve HIV-1 infected people using QβMPERN

Since the recombinant QβMPERN phage displaying the MPER

peptide with a c-terminal Not1 restriction site proved superior to all the recombinant phages in it reactivity with plasma containing low and high titers of MPER specific antibodies we next assess its binding to Plasma collected from 154 ARV naïve HIV infected members of the CIRCB AFRODEC cohort. These plasma samples were collected consecutively from 2012 to 2016. As shown in Fig 6A, 84% (129/154) ofthe participants' plasma showed specific reactivity to the recombinant QβMPERNphage over a wide range of OD values (0.4 to 2.4). In contrast, the highest mean OD value for plates coated with Qβ (no MPER peptide present) was 0.3 and as such all participants with OD values ranging from 0.1 to 0.3 were considered as negative for MPER reactive antibodies (Fig 6A cutoff line indicated).This suggests that a greater majority of our study participants expressed MPER-specific antibodies based upon their reactivity with the QβMPERN recombinant phage.In Fig 6B a comparative analysis is made between anti-MPER specific IgG antibody titers and the different ELISA OD ranges. However, when the different OD ranges were analyzed with respect to their median helper $CD4^+$ T cell count as shown FigB,C&D no apparent correlation was observed between the abundance of MPER-specific antibodies and helper $CD4^+$ T cell count. In this regard, 16% (25/154) of participants were negative for MPER-specific antibodies (Fig 6B). On the other hand, 73% (112/154) of participants showed strong reactivity tothe recombinantQβMPER phages with OD values ranging from 0.4 to 0.9 (Fig 6C). This was in addition to 11% (17/154) of participants showing very strong reactivity (OD values 1 to 2.4) to the recombinant QβMPER phages (Fig6D). Next, when plasmatic viral load was analyzed with respect to the abundance of MPER-specific antibodies there was no correlationwith IgG antibody titers (compare Fig 6E, F&G).Thus the novel optimized recombinant QβMPERNphage detects MPER reactive antibodies in a greater majority of ARV naïve HIV-1 infected individuals.

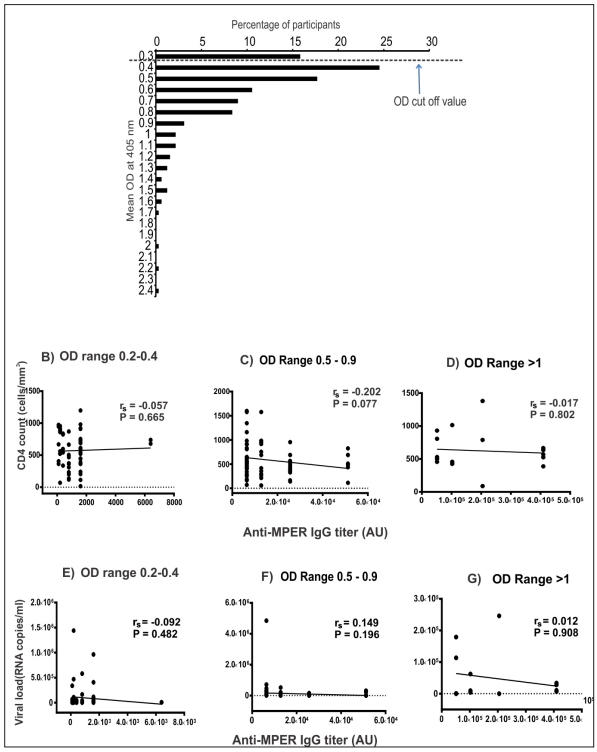


Figure 6: HIV-1 gp41 MPER specific IgG antibody in plasma from ARV naïve HIV infected people.

As shown in fig.6A the majority of plasma from ARV naïve HIV-1 infected people have MPER specific IgG antibodies. In (A) ELISA plates coated with 10^7pfu/well of either QβMPER or Qβ are probed with plasma from ARV naïve HIV-1 infected individuals. Dotted lines indicate maximal OD values for Qβ alone (no MPER region=background). There are a broad range of MPER-specific antibody levels in plasma from ARV naïve HIV-1 infected people. In (B), (C) and (D)spearman´s correlation analysis for the relationship between the abundance of MPER specific antibodies and helper $CD4^+$ T cell count. In (D), € and (F) data is shown for spearman's correlation analysis forthe relationship between plasmatic viral load and ELISA IgG antibody titers. Correlation coefficient (r) and p values (p) are shown in the figures.

Immunogenicity of the recombinant QβMPER phages in balb/c mice

To determine the immunogenicity of the parental phage Qβ, ELISA plates were coated with Qβ as described above. In Fig 7A data is shown for the induction of Qβ specific antibody responses in the vaccine treated group. Qβ specific IgG antibodies were detectable in well over 70% of all the mice that were treated with the phage. This clearly suggests a strong immunogenicity of phage itself. To measure anti-MPER specific IgG antibodies high binding plates were coated with 100 ng/well of MPER restrictive peptide to eliminate cross reactivity with Qβ specific antibodies.A strong recombinant QβMPER specific IgG antibody response was detected in serum of both groups of Balb/c mice vaccinated either with QβMPERHis or QβMPERN (Fig 7A). This IgG antibody response was considered MPER specific because serum from mice receiving Qβ alone showed little or no reactivity with the MPER restrictive peptide. Following three inoculations of the recombinant phages 60% of mice vaccinated with either QβMPERHisor QβMPERN showed detectable anti-MPER specific IgG antibodies. The anti-MPER specific IgG responses following QβMPERHisvaccinationwere similar toresponses following inoculation with the recombinant QβMPERN phage.Thus these preliminary results demonstrate the immunogenicity of the recombinant QβMPER phages in Balb/c mice.

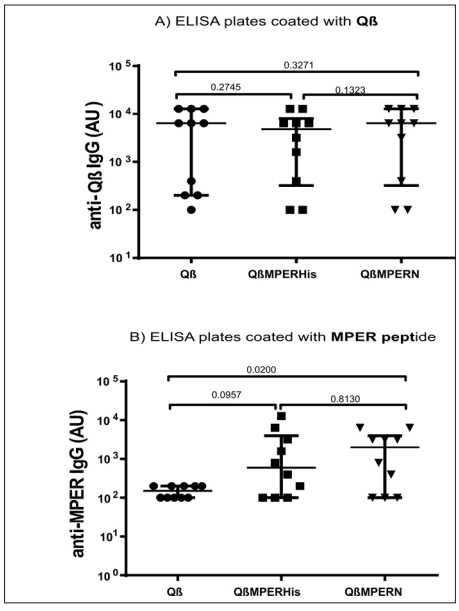


Figure 7: Immunogenicity of recombinant QβMPERHis and QβMPERN in mice

In (A) immunogenicityof Qβ in balb/C mice. Both recombinant QβMPER phages showed similar immunogenicity with the wild type Qβ vector.

In (B) when plates are coated with the MPER restrictive peptide only the recombinant phages could induce MPER specific antibodies. There was no different between the recombinant QβMPER phages in their immunogenicity in mice. Never the less only QβMPERN induced significantly higher ($p<0.020$) MPER specific antibodies than Qβ.

Discussion

The highly conserved gp41 MPER, in addition to being rich in bnAb epitopes, also plays key roles not only in viral infectivity, but also in membrane fusion and as such is a promising target for an anti-HIV antibody vaccine development [21, 71]. However, MPER is weakly immunogenic due to the fact that it's inaccessible to the immune system being masked in the unmodified HIV-1 envelope glycoprotein [73]. A number of strategies aimed at improving the accessibility of MPER and the consequent induction of neutralizing antibodies targeting the MPER bnAb epitopes have met with limited success. In this study, we have engineered the surface of the RNA coliphage Qβ to display all reported contiguous linear MPER epitopes corresponding to the bnAb 2F5, 4E10, Z13e1 and 10E8 through linking to the minor coat protein A1 of Qβ. This resulted to the surface display of 12 molecules of MPER per 25 nm hybrid phage particle thereby concentrating and improving the availability of the MPER based broadly neutralizing determinants for antibody recognition. Thus the recombinant QβMPER phages reacted specifically with their respective bnAbs in ELISA assay and surface-localization of the MPER insert was also confirmed by three dimensional structural modelling and transmission electron microscopy. Similarly, ELISA assays with plasma from long standing ARV naïve HIV infected people revealed selective recognition of the recombinant QβMPER phages by well over 84% of the participants. In addition, three variants of Qβ surface displayed MPER chimeric phages including QβMPER, QβMPERHis, and QβMPERN (S3 and Fig 5A&B) showed strong reactivity with MPER specific polyclonal antibodies in plasma from a greater majority of long standing ARV naïve HIV-1 infected participants. This probably indicates that the conserved bnAb epitopes within MPER are exposed efficiently upon the recombinant phage particles. This would imply that through surface engineering of the RNA coliphage Qβ we have optimized an MPER phage display platform that allows antibody recognition to be focused to its conserved epitopes.

When we analyzed the responses against MPER in plasma from long standing ARV naïve HIV-1 infected people using all therecombinant QβMPER phages, well over 75 % showed strong reactivity. This is in contrast to previous reports which showed MPER-specific responses in all HIV infected participants [70]. Unlike the previous study which included only 35 participants our work involved 154 ARV naïve HIV infected participants. Here plasma from close to 30% of ARV naïve HIV-1 infected individuals did not show any specific reactivity with the recombinant QβMPER phages. This suggests that the induction of MPER-specific antibodies in ARV naïve HIV-1 infected individuals might not be universal. One widely recognized hindrance for the induction of bnAb targeting MPER based broadly neutralizing determinants is masking by the gp120 subunit [74], rev. in [21, 71, 75] which makes them poorly assessable to the immune system. Secondly, adjacent immunodominant epitopes upstream of MPER can also focus B cell responses away from its conserved bnAb epitopes [21, 23, 24, 26, 76]. Thirdly, the MPER is highly hydrophobic and partially embedded in viral and cell membrane making it less available to the immune system. To address this challenge and minimize diversity, we have constructed an MPER consensus sequence [67, 77] by choosing the most common amino acids in an alignment of three envelope glycoproteins consisting of two clade C sequences and one clade B sequence [66–68]. Here the engineered sequences contained all the reported MPER based bnAb epitopes with additional gp41 derived conserved sequences upstream and downstream of the MPER yielding a 50 amino acid peptide insert. This strategy, while eliminating the gp41 based non-neutralizing immunodominant epitopes, ensures that all MPER bnAb epitopes are efficiently exposed upon the surface of the recombinant QβMPER phage particles. Additional amino acids upstream of the 2F5 epitopes have been suggested to be necessary for efficient recognition of the epitope after display upon the cell surface [70]. Transmission electron microscopic analysis of the recombinant QβMPERH is phage stained with Ni-NTA-Nanogold demonstrated the MPERHispeptide to be presented on the surface of QβMPERHis virus like particle. This surface exposure of MPER is expected to focus B cell responses to the conserved bnAb epitopes within MPER.

In summary, results from this study show that all the reported epitopes within MPER were easily recognized by their respective bnAbs and the hybrid phages could be used to assess the antigenicity of MPER specific antibodies in plasma of HIV-1 infected people.

The QβMPERNrecombinant phage was superior in antigenicity than the other two recombinant phages reported in this study. However in preliminary immunogenicity studies in Balb/c mice both QβMPERHis and QβMPERN induced detectable ant-MPER specific antibodies in 60% of the immunized clearly suggesting the potency of the recombinant phages in inducing anti-HIV-1 antibodies. Work is ongoing in rabbits to assess the neutralizing efficacy of such antibodies in Tier II HIV-1 strains.

Conclusion

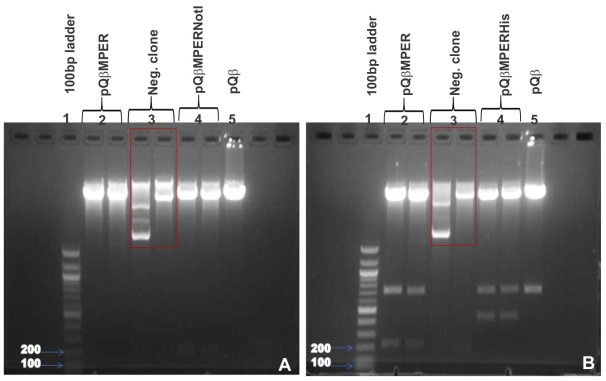
The recombinant QβMPER phages presented here were antigenic for 4E10, 10E8, Z13el, 2F5 and polyclonal antibody from long standing HIV-1 infected people. However, not all HIV-1 infected persons showed reactivity with the recombinant phages clearly indicating that the induction of MPER reactive antibodies was not universal in all HIV infected people. However, there was no correlation between the abundance of MPER specific antibody responses and participants' viral load or helper CD4 T cell counts. Nonetheless, the success of this QβMPER display paves the way for future immunogenicity studies using the recombinant phages either alone as demonstrated here or in combination with other vaccine strategy for the induction of efficient humoral immunity against HIV-1. Additional studies are necessary to assess the ability of the recombinant QβMPER phages in the induction of bnAb and to optimize strategies to use the novel phages for monitoring or predicting HIV-specific immunity.

Acknowledgement

The authors are grateful to Professor Weber for pBRT7Qβ, and to Professor Alexander Chetverin for pQβ7 and pQβ8 plasmids. We thank all participants who consented to participate in this study. We would like to thank the personnel of unites techniques of CIRCB for their help in collecting the blood samples. This project was funded by grants from CIRCB, EDCTP(grant #TA.2010.40200.016) TWAS(#12059RG/bio/af/ac_G), Canada grand challenge to Godwin W Nchinda (#0121-01); from the Center for NanoBiotechnology Research (CNBR) of ASU for grant # NSF-CREST (HRD-1241701) and grant # NSF-AGEP (1432991 BKR) of National Science Foundation to Alain BopdaWaffo.The following reagent

was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pConCgp160-opt(Cat#11407), pConBgp160-opt(Cat#11402) from Dr. Beatrice Hahn and p96ZM651gp160-opt from Drs. Yingying Li, Feng Gao, and Beatrice H. Hahn, HIV-1 anti-gp41 mAb (10E8), from Dr. Mark Connors, Anti-HIV-1 gp41 Monoclonal (4E10), Anti-HIV-1 gp41 Monoclonal (IgG1 Z13e1) from Dr. Michael Zwickand Anti-HIV-1 gp41 Monoclonal (2F5) from Polymun Scientific,HIV-1 MPER (662-684) peptide, 23-mer.

S1: Genetic construction and characterization of HIV-1 MPER vector and phages

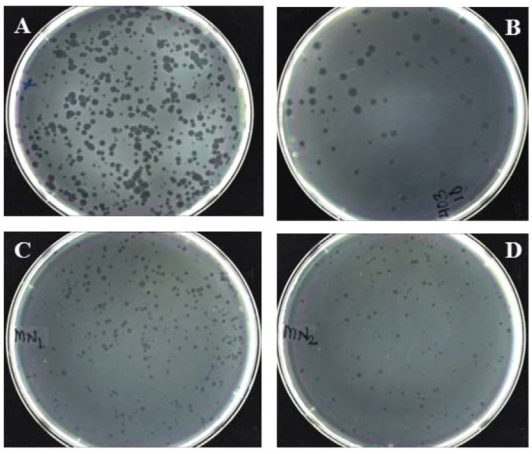


In (A & B) TAE 1.2% agarose gel; Restriction analysis of recombinant plasmid pQβMPER and variants; (A) Product of the recombinant pQβMPER restriction digestion with *NotI* (lane 2). (B) Product of the retransformed recombinant pQ βMPER restricted with *BglI*I (lane 2). Plasmids and gene fragments have the correct size (Lane 2). Negative clones of pQ βMPERN/pQβMPER do not show one of its band down or up (lane 3).

S1 Genetic construction and characterization of HIV-1 MPER vector and phages

In (A & B) TAE 1.2% agarose gel; Restriction analysis of recombinant plasmid pQβMPER and variants; (A) Product of the recombinant pQβMPER restriction digestion with NotI (lane 2). (B) Product of the retransformed recombinant pQβMPER restricted with BglII (lane 2). Plasmids and gene fragments have the correct size (Lane 2).Negative clones of pQβMPERN/pQβMPER do not show one of its band down or up (lane 3).

S2: The morphology of plaques formed on lawn of E coli K12.

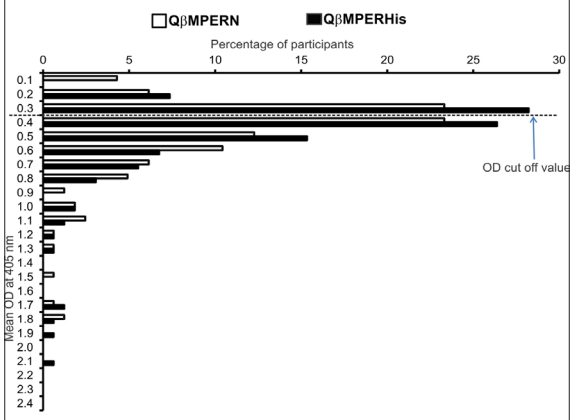


In (A): Qß; (B): QßMPER; (C): QßMPERN; (D): QßMPERHis

S2 Themorphology of plaques formed on lawn of *E coli* K12.

In (C): Qβ; (D): QβMPER; (E): QβMPERN; (F): QβMPERHis.

S3: Antigenicity of the recombinant phages QβMPERHis and QβMPERN.



S3 shows data for the reactivity of the recombinant phages QβMPERHis and QβMPERN with polyclonal antibodies in plasma from ARV naïve HIV-1 infected participants. Dotted lines indicate cutoff as determined with maximal OD values of Qβ alone. Both recombinant phages were reactive with a greater majority of participants' plasma over a wide range of OD.

S3 Antigenicity of recombinant phages QβMPERHis and QβMPERN in plasma from ARV naïve HIV-1 infected individuals

S3 shows data for the reactivity of the recombinant phages QβMPERHis and QβMPERN with polyclonal antibodies in plasma from ARV naïve HIV-1 infected participants. Dotted lines indicate cutoff as determined with maximal OD values of Qβ alone. Both recombinant phages were reactive with a greater majority of participants' plasma over a wide range of OD.

Author Contributions

Conceived and designed the experiments: G.W.N , A.B.W, and L.K
Performed the experiments: L.N.N., J.S., R.C., N.N.N, G. A., A.A. N, A.S.O., T. E., R. S., C. S., A.L., F.T.T.,A.S.O, and J.C.T
Technical assistance: N.N.L, N.N.N, W.M., S.M, N.E.C, C.O.E, L.K, R.L.S, T,E,C.A.S
Analyzed the data: G.W.N, A.B.W, N. N. L
Wrote the paper: G.W.N, A.B.W, Q.L.M

References

1. Liu J, Bartesaghi A, Borgnia MJ, Sapiro G, Subramaniam S (2008) Molecular architecture of native HIV-1 gp120 trimers. Nature 455: 109-113.
2. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC (1997) Atomic structure of the ectodomain from HIV-1 gp41. Nature 387: 426-430.
3. Wyatt R, Sodroski J (1998) The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280: 1884-1888.
4. Landais E, Huang X, Havenar-Daughton C, Murrell B, Price MA, Wickramasinghe L, et al. (2016) Broadly Neutralizing Antibody Responses in a Large Longitudinal Sub-Saharan HIV Primary Infection Cohort. PLoS pathogens 12: e1005369.
5. Mascola JR, Haynes BF (2013) HIV-1 neutralizing antibodies: understanding nature's pathways. Immunological reviews 254: 225-244.

6. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, et al. (2004) Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. The Journal of experimental medicine 200: 761-770.
7. Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, et al. (2009) Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. Nature 458: 636-640.
8. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, et al. (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 326: 285-289.
9. Huang J, Kang BH, Pancera M, Lee JH, Tong T, Feng Y, et al. (2014) Broad and potent HIV-1 neutralization by a human antibody that binds the gp41-gp120 interface. Nature 515: 138-142.
10. Buchacher A, Predl R, Strutzenberger K, Steinfellner W, Trkola A, Purtscher M, et al. (1994) Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS research and human retroviruses 10: 359-369.
11. Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, et al. (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. Journal of virology 67: 6642-6647.
12. Nelson JD, Brunel FM, Jensen R, Crooks ET, Cardoso RM, Wang M, et al. (2007) An affinity-enhanced neutralizing antibody against the membrane-proximal external region of human immunodeficiency virus type 1 gp41 recognizes an epitope between those of 2F5 and 4E10. Journal of virology 81: 4033-4043.
13. Ofek G, Zirkle B, Yang Y, Zhu Z, McKee K, Zhang B, et al. (2014) Structural basis for HIV-1 neutralization by 2F5-like antibodies m66 and m66.6. Journal of virology 88: 2426-2441.
14. Zhu Z, Qin HR, Chen W, Zhao Q, Shen X, Schutte R, et al. (2011) Cross-reactive HIV-1-neutralizing human monoclonal antibodies identified from a patient with 2F5-like antibodies. Journal of virology 85: 11401-11408.
15. Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, et al. (2001) Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. Journal of virology 75: 10892-10905.
16. Singleton J, Osborn JL, Lillis L, Hawkins K, Guelig D, Price W, et al. (2014) Electricity-free amplification and detection for molecular point-of-care diagnosis of HIV-1. PloS one 9: e113693.
17. Skamel C, Aller SG, Bopda Waffo A (2014) In vitro evolution and affinity-maturation with Coliphage qbeta display. PloS one 9: e113069.
18. Huang J, Ofek G, Laub L, Louder MK, Doria-Rose NA, Longo NS, et al. (2012) Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. Nature 491: 406-412.
19. Morris L, Chen X, Alam M, Tomaras G, Zhang R, Marshall DJ, et al. (2011) Isolation of a human anti-HIV gp41 membrane proximal region neutralizing antibody by antigen-specific single B cell sorting. PloS one 6: e23532.
20. Frey G, Peng H, Rits-Volloch S, Morelli M, Cheng Y, Chen B, et al. (2008) A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. Proceedings of the National Academy of Sciences of the United States of America 105: 3739-3744.
21. Montero M, van Houten NE, Wang X, Scott JK (2008) The membrane-proximal external region of the human immunodeficiency virus type 1 envelope: dominant site of antibody neutralization and target for vaccine design. Microbiology and molecular biology reviews : MMBR 72: 54-84, table of contents.
22. Stiegler G, Kunert R, Purtscher M, Wolbank S, Voglauer R, Steindl F, et al. (2001) A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. AIDS research and human retroviruses 17: 1757-1765.
23. Kim M, Song L, Moon J, Sun ZY, Bershteyn A, Hanson M, et al. (2013) Immunogenicity of membrane-bound HIV-1 gp41 membrane-proximal external region (MPER) segments is dominated by residue accessibility and modulated by stereochemistry. The Journal of biological chemistry 288: 31888-31901.
24. Montero M, Gulzar N, Klaric KA, Donald JE, Lepik C, Wu S, et al. (2012) Neutralizing epitopes in the membrane-proximal external region of HIV-1 gp41 are influenced by the transmembrane domain and the plasma membrane. Journal of virology 86: 2930-2941.
25. Ofek G, Guenaga FJ, Schief WR, Skinner J, Baker D, Wyatt R, et al. (2010) Elicitation of structure-specific antibodies by epitope scaffolds. Proceedings of the National Academy of Sciences of the United States of America 107: 17880-17887.
26. Behrendt R, Fiebig U, Kurth R, Denner J (2012) Induction of antibodies binding to the membrane proximal external region of gp36 of HIV-2. Intervirology 55: 252-256.
27. Alam SM, McAdams M, Boren D, Rak M, Scearce RM, Gao F, et al. (2007) The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. Journal of immunology 178: 4424-4435.
28. Finton KA, Larimore K, Larman HB, Friend D, Correnti C, Rupert PB, et al. (2013) Autoreactivity and exceptional CDR plasticity (but not unusual polyspecificity) hinder elicitation of the anti-HIV antibody 4E10. PLoS pathogens 9: e1003639.
29. Haynes BF, Fleming J, St Clair EW, Katinger H, Stiegler G, Kunert R, et al. (2005) Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science 308: 1906-1908.
30. Shen X, Parks RJ, Montefiori DC, Kirchherr JL, Keele BF, Decker JM, et al. (2009) In vivo gp41 antibodies targeting the 2F5 monoclonal antibody epitope mediate human immunodeficiency virus type 1 neutralization breadth. Journal of virology 83: 3617-3625.
31. Verkoczy L, Diaz M (2014) Autoreactivity in HIV-1 broadly neutralizing antibodies: implications for their function and induction by vaccination. Current opinion in HIV and AIDS 9: 224-234.
32. Olsthoorn R, Duin J (2011) Bacteriophages with ssRNA. eLS.
33. Kitti T, Thummeepak R, Thanwisai A, Boonyodying K, Kunthalert D, Ritvirool P, et al. (2014) Characterization and Detection of Endolysin Gene from Three Acinetobacter baumannii Bacteriophages Isolated from Sewage Water. Indian journal of microbiology 54: 383-388.

34. Taniguchi T, Palmieri M, Weissmann C (1978) QB DNA-containing hybrid plasmids giving rise to QB phage formation in the bacterial host. Nature 274: 223-228.
35. Biebricher CK, Luce R (1992) In vitro recombination and terminal elongation of RNA by Q beta replicase. The EMBO journal 11: 5129-5135.
36. Inokuchi Y, Hirashima A (1987) Interference with viral infection by defective RNA replicase. Journal of virology 61: 3946-3949.
37. Karnik S, Billeter M (1983) The lysis function of RNA bacteriophage Qbeta is mediated by the maturation (A2) protein. The EMBO journal 2: 1521-1526.
38. Rumnieks J, Tars K (2011) Crystal structure of the read-through domain from bacteriophage Qbeta A1 protein. Protein science : a publication of the Protein Society 20: 1707-1712.
39. Rumnieks J, Tars K (2017) Crystal Structure of the Maturation Protein from Bacteriophage Qbeta. Journal of molecular biology 429: 688-696.
40. Schroeder R, Barta A, Semrad K (2004) Strategies for RNA folding and assembly. Nature reviews Molecular cell biology 5: 908-919.
41. Witherell GW, Uhlenbeck OC (1989) Specific RNA binding by Q beta coat protein. Biochemistry 28: 71-76.
42. Yates JL, Gette WR, Furth ME, Nomura M (1977) Effects of ribosomal mutations on the read-through of a chain termination signal: studies on the synthesis of bacteriophage lambda O gene protein in vitro. Proceedings of the National Academy of Sciences of the United States of America 74: 689-693.
43. Waffo AB (2004) Evolutionary RNA-coliphage Qβ [Q-beta] Display: Expression Biopathway in E. Coli. Cuvillier Verlag.
44. Machida K, Imataka H (2015) Production methods for viral particles. Biotechnology letters 37: 753-760.
45. Takamatsu H, Iso K (1982) Chemical evidence for the capsomeric structure of phage q beta. Nature 298: 819-824.
46. Firth AE, Wills NM, Gesteland RF, Atkins JF (2011) Stimulation of stop codon readthrough: frequent presence of an extended 3' RNA structural element. Nucleic acids research 39: 6679-6691.
47. Harrell L, Melcher U, Atkins JF (2002) Predominance of six different hexanucleotide recoding signals 3' of read-through stop codons. Nucleic acids research 30: 2011-2017.
48. Hofstetter H, Monstein HJ, Weissmann C (1974) The readthrough protein A1 is essential for the formation of viable Q beta particles. Biochimica et biophysica acta 374: 238-251.
49. Valegard K, Fridborg K, Liljas L (1994) Crystallization and preliminary X-ray diffraction studies of the bacteriophage Qbeta. Acta crystallographica Section D, Biological crystallography 50: 105-109.
50. Klovins J, Berzins V, van Duin J (1998) A long-range interaction in Qbeta RNA that bridges the thousand nucleotides between the M-site and the 3' end is required for replication. Rna 4: 948-957.
51. Blumenthal T, Carmichael GG (1979) RNA replication: function and structure of Qbeta-replicase. Annual review of biochemistry 48: 525-548.
52. Kaminski PA, Desnoues N, Elmerich C (1994) The expression of nifA in Azorhizobium caulinodans requires a gene product homologous to Escherichia coli HF-I, an RNA-binding protein involved in the replication of phage Q beta RNA. Proceedings of the National Academy of Sciences of the United States of America 91: 4663-4667.
53. Muffler A, Fischer D, Hengge-Aronis R (1996) The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for rpoS translation in Escherichia coli. Genes & development 10: 1143-1151.
54. Mekler P (1981) Determination of Nucleotide Sequences of the Bacteriophage Q [beta] Genome: Organization and Evolution of an RNA Virus. Universität Zürich.
55. Chao L (1990) Fitness of RNA virus decreased by Muller's ratchet. Nature 348: 454-455.
56. Domingo E, Holland JJ (1997) RNA virus mutations and fitness for survival. Annual review of microbiology 51: 151-178.
57. Drake JW, Holland JJ (1999) Mutation rates among RNA viruses. Proceedings of the National Academy of Sciences of the United States of America 96: 13910-13913.
58. Elena SF, Sanjuan R (2005) Adaptive value of high mutation rates of RNA viruses: separating causes from consequences. Journal of virology 79: 11555-11558.
59. Jenkins GM, Rambaut A, Pybus OG, Holmes EC (2002) Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. Journal of molecular evolution 54: 156-165.
60. Palasingam K, Shaklee PN (1992) Reversion of Q beta RNA phage mutants by homologous RNA recombination. Journal of virology 66: 2435-2442.
61. Dennison SM, Sutherland LL, Jaeger FH, Anasti KM, Parks R, Stewart S, et al. (2011) Induction of antibodies in rhesus macaques that recognize a fusion-intermediate conformation of HIV-1 gp41. PloS one 6: e27824.
62. Gu L, Farrow AL, Krendelchtchikov A, Matthews QL (2015) Utilizing the antigen capsid-incorporation strategy for the development of adenovirus serotype 5-vectored vaccine approaches. Journal of visualized experiments : JoVE 99: e52655.
63. Hanson MC, Abraham W, Crespo MP, Chen SH, Liu H, Szeto GL, et al. (2015) Liposomal vaccines incorporating molecular adjuvants and intrastructural T-cell help promote the immunogenicity of HIV membrane-proximal external region peptides. Vaccine 33: 861-868.
64. Matyas GR, Wieczorek L, Beck Z, Ochsenbauer-Jambor C, Kappes JC, Michael NL, et al. (2009) Neutralizing antibodies induced by liposomal HIV-1 glycoprotein 41 peptide simultaneously bind to both the 2F5 or 4E10 epitope and lipid epitopes. Aids 23: 2069-2077.
65. Sun ZY, Oh KJ, Kim M, Yu J, Brusic V, Song L, et al. (2008) HIV-1 broadly neutralizing antibody extracts its epitope from a kinked gp41 ectodomain region on the viral membrane. Immunity 28: 52-63.
66. Andre S, Seed B, Eberle J, Schraut W, Bultmann A, Haas J, et al. (1998) Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. Journal of virology 72: 1497-1503.
67. Kothe DL, Li Y, Decker JM, Bibollet-Ruche F, Zammit KP, Salazar MG, et al. (20060 Ancestral and consensus envelope immunogens for HIV-1 subtype C. Virology 352:438-449.
68. Gao F, Li Y, Decker JM, Peyerl FW, Bibollet-Ruche F, Rodenburg CM, et al. (2003) Codon usage optimization of HIV type 1 subtype C gag, pol, env, and nef genes: in vitro expression and immune responses in DNA-vaccinated mice. AIDS research and human retroviruses 19: 817-823.
69. Kamdem Toukam D, Tenbusch M, Stang A, Temchura V, Storcksdieck Genannt Bonsmann M, Grewe B, et al. (2012) Targeting antibody responses to the membrane proximal external region of the envelope glycoprotein of human immunodeficiency virus. PloS one 7: e38068.
70. Molinos-Albert LM, Carrillo J, Curriu M, Rodriguez de

la Concepcion ML, Marfil S, Garcia E, et al. (2014) Anti-MPER antibodies with heterogeneous neutralization capacity are detectable in most untreated HIV-1 infected individuals. Retrovirology 11: 44.
71. Zwick MB, Jensen R, Church S, Wang M, Stiegler G, Kunert R, et al. (2005) Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. Journal of virology 79: 1252-1261.
72. Zamora H, Luce R, Biebricher CK (1995) Design of artificial short-chained RNA species that are replicated by Q beta replicase. Biochemistry 34: 1261-1266.
73. Vassell R, He Y, Vennakalanti P, Dey AK, Zhuang M, Wang W, et al. (2015) Immunogens Modeling a Fusion-Intermediate Conformation of gp41 Elicit Antibodies to the Membrane Proximal External Region of the HIV Envelope Glycoprotein. PloS one 10: e0128562.
74. Karlsson Hedestam GB, Fouchier RA, Phogat S, Burton DR, Sodroski J, Wyatt RT, et al. (2008) The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. Nature reviews Microbiology 6: 143-55.
75. Walker LM, Simek MD, Priddy F, Gach JS, Wagner D, Zwick MB, et al. (2010) A limited number of antibody specificities mediate broad and potent serum neutralization in selected HIV-1 infected individuals. PLoS pathogens 6: e1001028.
76. Enshell-Seijffers D, Smelyanski L, Vardinon N, Yust I, Gershoni JM (2001) Dissection of the humoral immune response toward an immunodominant epitope of HIV: a model for the analysis of antibody diversity in HIV+ individuals. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 15: 2112-2120.
77. Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, et al. (2005) Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. Journal of virology 79: 10108-10125.
78. Robertson CA, Mok JYQ, Froebel KS, Simmonds P, Burns SM, Marsden HS, et al. (1992) Maternal antibodies to gp120 V3 sequence do not correlate with protection against vertical transmission of human immunodeficiency virus. Journal Infectious Diseases 166: 704-709.
79. Ugen KE, Goedert JJ, Boyer J, Refaeli Y, Frank I, Williams WV, et al. (1992) Vertical transmission of human immunodeficiency virus (HIV) infection. Reactivity of maternal sera with glycoprotein 120 and 41 peptides from HIV type 1. Journal Clinical Investigation 89:1923-1930.
80. Alfsen A, Iniguez P, Bouguyon E, Bomsel M (2001) Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1. J Immunol 166: 6257-6265.
81. Conley AJ, Kessler JA II, Boots LJ, McKenna PM, Schleif WA, Emini EA, et al. (1996) The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. Journal of virology.
82. Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, et al. (2010) Broadly neutralizing monoclonal antibodies 2F5 and 4E10, directed against the human immunodeficiency virus type 1 gp41 membrane proximal external region protect against mucosal challenge by simianhuman immunodeficiency virus SHIVBa-L. Journal of Virology 84: 1302-1313.
83. Moog C, Dereuddre-Bosquet N, Teillaud J, Biedma ME, Holl V, Van Ham G, et al. (2014) Protective effect of vaginal application of neutralizing and nonneutralizing inhibitory antibodies against vaginal SHIV challenge in macaques. Mucosal Immunology 7: 46-56.