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10E8-like neutralizing antibodies against HIV-1 induced using a precisely designed conformational peptide as a vaccine prime

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Recent studies have demonstrated that the membrane-proximal external region (MPER) of human immunodeficiency virus 1 (HIV-1) glycoprotein 41 contains a series of epitopes for human monoclonal antibodies, including 2F5, Z13e1, 4E10, and 10E8, which were isolated from HIV-1-infected individuals and show broad neutralizing activities. This suggests that MPER is a good target for the development of effective HIV-1 vaccines. However, many studies have shown that it is difficult to induce antibodies with similar broad neutralizing activities using MPER-based peptide antigens. Here, we report that 10E8-like neutralizing antibodies with effective anti-HIV-1 activity were readily induced using a precisely designed conformational immunogenic peptide containing the 10E8-specific epitope. This immunogenic peptide (designated T10HE) contains a 15-mer MPER-derived 10E8-specific epitope fused to T-helper-cell epitopes from tetanus toxin (tt), which showed a significantly stabilized α-helix structure after a series of modifications, including substitution with an (S)-α-(2′-pentenyl) alanine containing an olefin-bearing tether and ruthenium-catalyzed olefin metathesis, compared with the unmodified T10E peptide. The stabilized α-helix structure of T10HE did not affect its capacity to bind the 10E8 antibody, as evaluated with an enzyme linked immunosorbent assay (ELISA) and surface plasmon resonance binding assay (SPR assay). The efficacies of the T10HE and T10E epitope vaccines were evaluated after a standard vaccination procedure in which the experimental mice were primed with either the T10HE or T10E immunogen and boosted with HIV-1 JRFL pseudoviruses. Higher titers of 10E8-like antibodies were induced by T10HE than that by T10E. More importantly, the antibodies induced by T10HE showed enhanced antiviral potency against HIV-1 strains with both X4 and R5 tropism and a greater degree of broad neutralizing activity than the antibodies induced by T10E. These results indicate that a 10E8-epitope-based structure-specific peptide immunogen can elicit neutralizing antibodies when used as a vaccine prime.

MPER, peptide immunogen, vaccine prime, neutralizing antibody

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Until now, no effective vaccine that can induce broad neutralizing antibodies for human immunodeficiency virus (HIV) has been developed because its envelope glycoproteins (Envs) are highly mutable and carry massive glycans [1]. Although most of the antibodies induced in natural HIV-1 infections in humans are often quite strain-specific or even completely nonneutralizing, it is now evident that 10%–25% of infected individuals could eventually induce broad neutralizing antibodies (BNAbs) [2]. In recent years, a few of these BNAbs have been isolated from such individuals using state-of-the-art single-B-cell-based sorting and antibody cDNA cloning technology [3–9]. Four such BNAbs, 2F5, 4E10, Z13e1, and 10E8, bind to continuous

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epitopes on the membrane-proximal external region (MPER) of HIV-1 glycoprotein 41 (gp41) [10–12].

MPER is conserved among all HIV-1 subtypes [13,14]. The cocrystallization of MPER with these antibodies provides valuable information for the design of vaccination strategies. The structure of the 2F5 epitope displays different forms in the presence or absence of 2F5 BNAb, showing a kinked extended structure [15] or a 3_{10} helix, respectively [16,17]. The structure of MPER contains two helices when it is complexed with the antigen-binding fragment (Fab) of 10E8. The important residues of MPER that bind to the 10E8 BNAb are Trp672, Phe673, Trp680, and Lys/Arg683 [12], which show a typical conformational epitope structure. 10E8 is a BNAb newly identified in 2012, which amazingly neutralized ~98% of the 180 viruses tested at an IC_{50}<50 μg mL^{-1}, so it is very likely that the 10E8 epitope on MPER is an excellent candidate epitope for the development of an effective HIV-1 vaccine. However, there has been little research in this area, so in this study, we used a 10E8-epitope-based immunogen as the bait to elicit 10E8-like antibodies. Like anti-HIV drugs that are specific to conformational targets [18], epitope-based vaccine constructs must also be designed precisely according to the epitopes of known BNAbs to elicit BNAbs-like neutralizing antibodies. For example, Joseph et al. [19] modified the original VRC01 epitope on gp120 with computational assistance, and the new format, eOD-GT6, showed improved binding to multiple VRC01-class BNAbs and their germline precursors. The modified immunogen eOD-GT6 can efficiently activate both germline and mature VRC01-class B cells, suggesting that a germline-targeting strategy can potentially be used to design a vaccine prime. In our case, although the MPER of gp41 contains a series of well-characterized BNAbs, including 2F5, 4E10, Z13e1, and 10E8, it is conformationally flexible in the format of a synthesized peptide [20]. Attempts have been made to induce 2F5- and 4E10-like neutralizing antibodies by conjugating the 2F5 or 4E10 epitope to scaffolds [20,21]. However, these have failed, partly because the scaffolds themselves are highly immunogenic, which inevitably causes the immune system to produce numerous nonneutralizing antibodies. In this study, we generated a stabilized helical conformation of the 10E8 epitope by chemically modifying the original sequence of the 10E8 epitope rather than introducing a scaffold, to avoid the production of unwanted antibodies.

MPER-derived peptide antigens, including the 10E8 epitope peptide, are poorly immunogenic [22]. It is very important to conjugate the 10E8 epitope peptide to an appropriate carrier molecule, and ideally, the carrier should stimulate T-helper-cell responses to efficiently induce a 10E8-epitope-specific humoral response [23]. Current research indicates that $CD4^+$ T cells are essential for the induction of effective antiviral immunity in both $CD8^+$ T-cell responses and B-cell responses *in vivo* [24,25]. In this context, the well-characterized immunogenic T-cell epitopes within the tetanus toxin (tt) could increase the activation of $CD4^+$ T-helper cells by our 10E8-epitope-based immunogen, whereas trials with the whole tt protein should be avoided because it has a known immunosuppression function [23].

The immunization strategy is another crucial factor in vaccine studies. Recently, much attention has been directed toward the heterologous prime-boost strategy, which was shown to be effective in nonhuman primates in 1992 [26]. That pivotal report provided a practical approach to inducing the most effective humoral immune responses to weak immunogens. In this study, we fully appreciated the importance of the prime-boost strategy and incorporated it into the immunization strategy for our 10E8-epitope-derived vaccine construct. However, even the effects of the best prime-boost strategies can vary from patient to patient. Therefore, in this study, we also extensively explored different prime-boost approaches.

In summary, here we report a 10E8-epitope-based peptide vaccine construct, designated the "TT-10E8 helical epitope" (T10HE), consisting of a MPER-derived 10E8-specific 15-mer peptide fused to T-cell epitopes (tt). The hydrocarbon stapling method was used to stabilize the 15-mer 10E8 epitope peptide in a rigid helical conformation. We then tested the hypothesis that a rigid conformation is suitable for using as a vaccine prime with a T10HE prime-pseudovirus boost strategy.

1 Materials and methods

1.1 Ethics statement

All procedures for the use and care of experimental animals were approved by the Institutional Animal Care and Use Committee of Tsinghua University and the Ethics and Welfare of Experimental Animals Committee of Tsinghua University (permit number: 13-CYH5). All experiments were performed according to institutional guidelines.

1.2 Peptides, reagents, cells, and viruses

The peptides, shown in Figure 1, were designed and synthesized. MPER (RRRNEQELLELDKWASLWNWFDITNWLWYIRRRR), TT peptide (FNNFTVSFWLRVPKVSASHLE), T10HE peptide (FNNFTVSFWLRVPKVSASHLE-PEG2-LWNWF-S5-ITN-S5-LWYIR-PEG2-KK), and T10E peptide (FNNFTVSFWLRVPKVSASHLE-PEG2-LWNWFDITNWLWYIR-PEG2-KK) were purchased from CPC Scientific Inc. (Sunnyvale, CA, USA). CpG-ODN 1826 (5′-TCCATGACGTTCCTGACGTT-3′) was synthesized with a backbone of nuclease-resistant phosphorothioate by Sangon Biological Engineering Technology Co. (Shanghai, China). The 10E8 monoclonal antibody used in this study was kindly provided by Dr. Huang JingHe (National Institutes of Health (NIH)). Freund's ad-

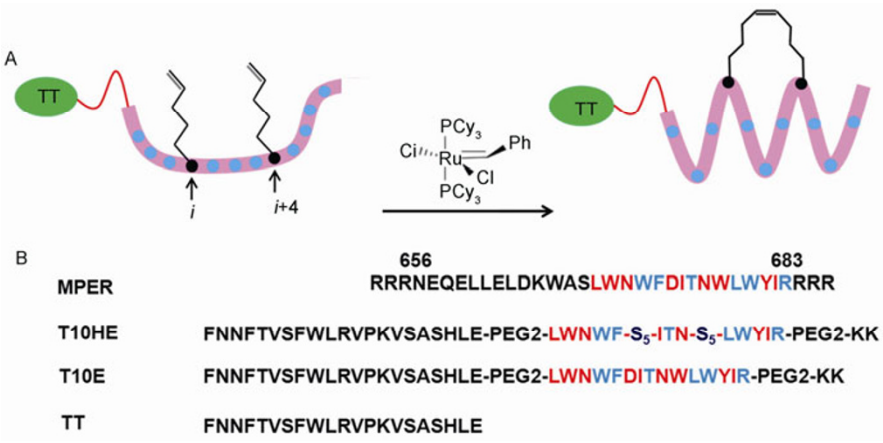


Figure 1 Design and sequences of the synthesized peptide immunogen. A, Schematic representation of the synthesized peptide T10HE with an enhanced α-helix. TT, tetanus toxoid (residues 947–967); *i* and *i*+4, hydrocarbon-stapled sites. B, The amino acid sequences of MPER, T10HE, T10E, and TT. The last 15 residues of MPER are shown in red and blue. The critical residues for 10E8 binding are highlighted in blue. PEG2, flexible linker (–$NHCH_2CH_2OCH_2CH_2OCH_2CO$–); S5, hydrocarbon-stapled site [(S)-α-(2′-pentenyl) alanine].

juvant and incomplete Freund's adjuvant were purchased from Pierce (Rockford, IL, USA). Green fluorescent protein (GFP)-transduced human osteosarcoma (ghost) cells expressing CD4 and coreceptors CCR5 and CXCR4 and plasmids pSF162-Env, pCNE3-Env, pCNE28-Env, and pCNE49-Env were kind gifts from Dr. Zhang LinQi of Tsinghua University. HEK 293T cells and plasmids pNL4-3.Luc.R^-E^-, pJRFL-Env, and pHXB2-Env were obtained from the NIH AIDS Research and Reference Reagent Program. Anti-human IgG was purchased from Sigma (St. Louis, MO, USA). Anti-mouse IgG was purchased from Dako (Glostrup, Denmark).

1.3 Circular dichroism (CD) analysis

The secondary structures of the synthetic peptides were determined with CD spectroscopy, as described previously [27,28]. Briefly, the individual peptides were prepared in phosphate-buffered saline (PBS) containing 50% trifluoroethanol (TFE) at a final concentration of 10 mmol L^{-1}. Data were obtained on an Applied Photophysics Pi-Star 180 spectropolarimeter (Surrey, UK) with predetermined parameters (3 nm bandwidth, 0.1 nm resolution, 0.1 cm path length, 1 nm wavelength interval from 200 to 260 nm) and corrected by the subtraction of the background solvent. The α-helix content was calculated from the CD signal by dividing the mean residue ellipticity at 222 nm.

1.4 SPR assay

The binding of synthesized peptides T10HE, T10E, and MPER to 10E8 was assayed with a Biacore T200 instrument, as previously described [29,30]. Briefly, full-length 10E8 antibody (IgG) was captured on a CM5 chip (Uppsala, Sweden), according to standard amine coupling procedures, including the activation of L2 with a fresh mixture of 0.2 mol L^{-1} ethyl (dimethylaminopropyl) carbodiimide and 0.05 mol L^{-1} N-hydroxysuccinimide (NHS) for 7 min and capturing 50 μg mL^{-1} 10E8 in 10 mmol L^{-1} sodium acetate (pH 5.5) to the desired level (3000 RU). Ethanolamine was then used to block the residual reactive esters. L1 was used as a reference surface, without any modification. The synthesized peptides T10H, T10E, and MPER were prepared as 2-fold serial dilutions (from 500 to 31.25 nmol L^{-1} in HBS running buffer: 100 mmol L^{-1} HEPES (pH 7.4), 150 mmol L^{-1} NaCl, 3.4 mmol L^{-1} EDTA, 0.01% Tween 20). Each peptide was injected from low to high concentrations in a single-cycle mode for 100 s at the flow rate of 30 μL min^{-1}, followed by a dissociation time of 180 s. The surfaces were regenerated with the injection of 5 μL of 15 mmol L^{-1} NaOH. The data were analyzed with BiaEvalution 4.1.

1.5 Purification of pseudoviruses

Pseudoviruses were prepared as previously described [31]. In brief, HEK 293T cells were cotransfected with pNL4-3.LucR^-E^- and pJRFL-Env. After 24 h, the culture medium was changed to fresh Dulbecco's modified Eagle's medium. The supernatant containing the pseudoviruses was harvested after another 24 h and the pseudoviruses were purified with Lentivirus purification mini kit (San Diego, CA, USA), according to the manufacturer's instructions. The purified pseudoviruses were dialyzed against PBS and stored as aliquots at −80°C. The titers of the pseudoviruses were determined by ELISA for p24.

1.6 Immunization

Twelve eight-week-old female BALB/c mice were assigned to three groups (four mice per group), designated groups A, B and C. Group A mice were primed with the T10HE pep-

tide and boosted with JRFL pseudoviruses. Group B mice were immunized with the T10E peptide and boosted with JRFL pseudoviruses. Group C mice were immunized with JRFL pseudoviruses. The peptides (20 μg per mouse) were mixed thoroughly with Freund's adjuvant for the first immunization and with incomplete Freund's adjuvant thereafter, and injected intraperitoneally. JRFL pseudoviruses (1 μg per mouse) were mixed thoroughly with CpG-ODN 1826 and injected intraperitoneally. The second immunization was given three weeks after the primary immunization, and two boost immunizations were given at intervals of two weeks thereafter. Preimmunization blood samples were collected before the primary immunization. Blood samples were also harvested a week after each boost immunization and the sera were stored at −20°C.

1.7 ELISAs

The T10HE, T10E, and MPER peptides were coated onto microtiter plates (5 μg mL^{-1} in PBS (pH 7.2), 50 μL/well) at 37°C for 2 h, and blocked with 2% nonfat milk (200 μL/well in PBS) at 4°C overnight. Serially diluted primary 10E8 antibody or mouse serum (50 μL/well) was then added and the samples incubated for 2 h. A peroxidase-conjugated secondary antibody, anti-human IgG or anti-mouse IgG, was added to the sera and incubated for 1 h at room temperature. The freshly prepared ortho-phenylenediamine (OPD) solution (10 mg OPD in 10 mL of 0.1 mol L^{-1} citrate buffer (pH 5.0), 20 μL H_2O_2) was added (50 μL/well). After 10 min, the reaction was stopped with 2 mol L^{-1} H_2SO_4. Data were obtained at an optical density of 490 nm (A_{490}) on a Bio-Rad Model 680 (Hercules, CA, USA).

1.8 HIV-1 neutralization assays

A single-cycle pseudovirus infection assay was performed as previously described [27]. Briefly, pseudoviruses were obtained as described above. The titers of the pseudoviruses were determined with a 4-fold serial dilution (starting from 1∶5) in 1×10^5 ghost cell/well, which were prepared 24 h before infection in a final volume of 200 μL. The samples (50 μL/well) and pseudoviruses (50 μL/well) were incubated at 37°C for 1 h, and then added to the ghost cells (100 μL/well). The cells were cultured at 37°C under 5% CO_2. After 48 h, the cells were lysed and the relative luminescence units (RLU) detected with a luciferase kit (Promega, Madison, WI, USA) and a luminometer (Ultra 386, Tecan, Durham, NC, USA).

Mouse antisera lacking 10E8-like antibodies were prepared using T10HE-conjugated microbeads, after 1 mg of T10HE was coupled to NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare, Sweden), according to the manufacturer's instructions. The antisera were incubated with the microbeads for 45 min, the mixture was centrifuged at 1500×*g* for 5 min, the supernatant was collected and the complement was inactivated at 56°C for 30 min.

2 Results

2.1 Design and characterization of the helical immunogen T10HE

A series of BNAbs isolated from HIV-1-infected individuals showed strong binding capacity to MPER, suggesting that MPER is a good candidate epitope for the development of an HIV-1 vaccine. However, MPER is a poor immunogen. To enhance the immunogenicity of the MPER-derived 10E8 epitope peptide, we introduced universally immunogenic T-helper-cell 21-mer epitopes (TT) derived from the tt to the N-terminus of MPER. TT contains three epitopes that can be recognized when associated with different MHC II molecules [32]. We also added a flexible, low-molecular-weight polyethylene glycol linker (PEG2) between the T-cell epitope and MPER to avoid disturbing the structure of MPER. In an effort to form a stabilized α-helical conformation, we substituted aspartic acid and tryptophan (at positions 674 and 678, respectively) with (S)-α-(2′-pentenyl) alanine containing olefin-bearing tethers, followed by ruthenium-catalyzed olefin metathesis (Figure 1A). The substitution positions (*i* and *i*+4) were carefully selected to avoid the disruption of the critical binding interface between MPER and 10E8, as determined from the crystal structure of MPER complexed with the 10E8 Fab [12]. This peptide, with a stabilized helical conformation, was designated the TT-10E8 helical peptide (T10HE for short). We also synthesized a linear analogue, the TT-10E8 peptide, without these modifications (T10E for short) as the control (Figure 1B).

To assess the helicity of T10HE and T10E, a CD analysis was performed at 25°C in 20 mmol L^{-1} PBS (pH 7.2) containing 50% TFE. TFE is a strongly helix-promoting solvent for peptides [33,34]; 50% TFE was used to assay the conformational structure and to increase the solubility of the peptides. As shown in Figure 2, the spectra of T10HE and T10E displayed distinct secondary structures. Two negative bands were observed at approximately 208 and 222 nm, suggesting the presence of α-helix. T10HE showed significant absorption at 208 nm and weak absorption at 222 nm, whereas T10E showed much weaker absorption at 208 nm than T10HE. These results indicate that the introduction of a hydrocarbon bridge increases the helicity of the T10HE immunogen.

2.2 Binding of 10E8 BNAb to T10HE

Next, we tested whether the stabilized helical conformation within T10HE affects its recognition by 10E8 BNAb. We detected the binding of 10E8 to T10HE with ELISA and

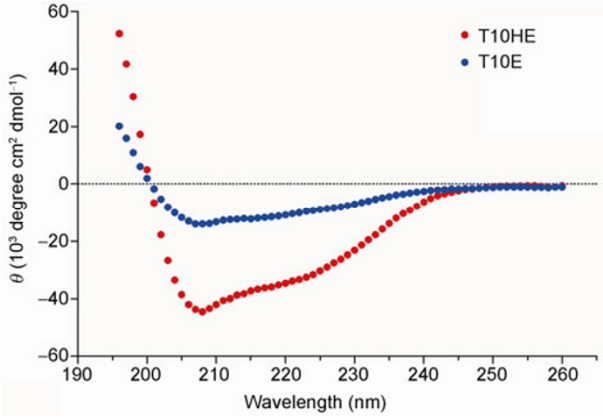


Figure 2 T10HE peptide immunogen has a more-stabilized α-helix structure than T10E. The secondary structures (α-helices) of peptides T10HE and T10E were determined with CD spectroscopy. The preparation of the samples is described in detail in Materials and methods. One representative result from at least two independent measurements is shown.

SPR assay. As expected, 10E8 bound very well to T10HE (Figure 3), and the binding affinities for 10E8 of the three synthesized peptides derived from MPER were in the same order of magnitude (77 nmol L^{-1} for T10E, 61.6 nmol L^{-1} for T10HE, and 33.9 nmol L^{-1} for MPER, respectively) when quantified with the SPR assay (Figure 4). By contrast, the TT peptide, used as the control, was not recognized by 10E8 (Figure 3). These results indicate that the peptide T10HE, with a stabilized helical conformation, maintained the conformational structure of the epitope, which was recognized by 10E8 BNAb.

2.3 Inducing 10E8-like antibody responses with the T10HE prime-JRFL pseudovirus boost strategy

Several studies have demonstrated that it is very difficult to induce antibodies with broad neutralizing activities using MPER-derived peptide antigens. A prime-and-boost-based immunization strategy has been proven to be an effective approach to inducing the best humoral immune responses to poor immunogens. However, the effects of even the best prime-boost strategies can vary from patient to patient. Therefore, we also tried different prime-boost immunization procedures (Figure 5A). Specifically, in group A, we used the T10HE prime-JRFL pseudovirus boost strategy to improve the immunogenicity of the 10E8 epitope (Figure 5A, group A). The critical point in this immunization strategy is that the T10HE peptide was used for the primary immunization (T10HE peptide as the vaccine prime) and the HIV-1 JRFL pseudovirus was used as the boost. Group B (T10E peptide as the vaccine prime) and C (pseudovirus as the vaccine prime) were used as controls.

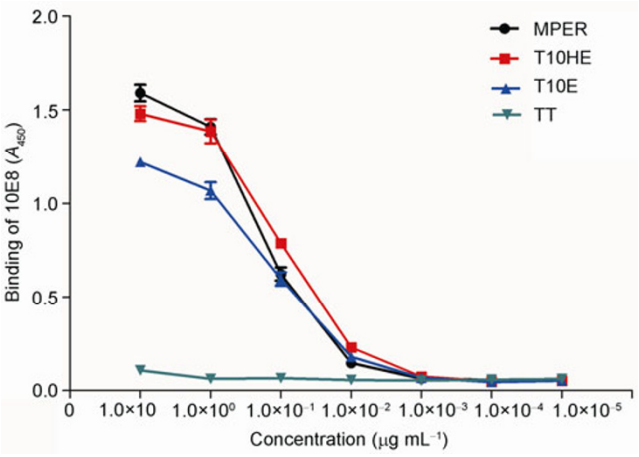
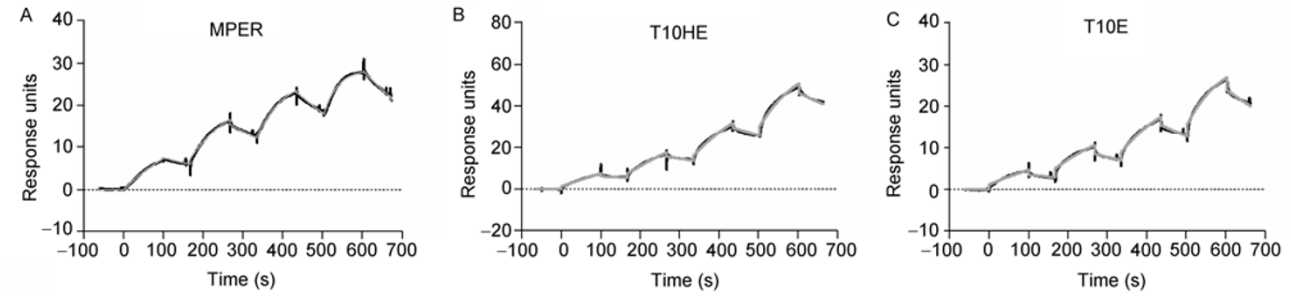


Figure 3 Modified T10HE peptide antigen is recognized by 10E8 BNAb. The binding capacities to 10E8 BNAb of a series of synthesized epitope peptide antigens, T10HE, T10E, and MPER, were assessed with an ELISA. TT was used as the control. Means with error bars of at least two independent experiments are shown.



D

Analyte	*ka* (mol^{-1} L s^{-1})	*kd* (s^{-1})	K_D (mol L^{-1})
MPER	1.11×10^{5}	0.003749	3.39×10^{-8}
T10HE	3.22×10^{4}	0.001987	6.16×10^{-8}
T10E	3.73×10^{4}	0.002869	7.70×10^{-8}

Figure 4 Single-cycle kinetic analysis of the binding of the synthesized peptides to 10E8. A–C, The 10E8 BNAb was immobilized on a CM5 chip and the synthesized peptides T10HE (A), T10E (B), and MPER (C) were passed over it as analytes in 2-fold serial dilutions from 31.25 to 250 nmol L^{-1}. Preparation of the samples is described in detail in Materials and methods. D, Binding constants were calculated by fitting the sensorgrams (A–C, black) to a 1:1 Langmuir model (A–C, gray). *ka*, association rate; *kd*, dissociation rate; K_D, equilibrium dissociation constant. One representative result from at least two independent measurements is shown.

Antisera were collected at the end of the immunization procedure and MPER-, T10HE-, and T10E-specific antibodies were detected with ELISAs. As shown in Figure 5 and Table 1, higher levels of MPER-, T10HE-, and T10E-

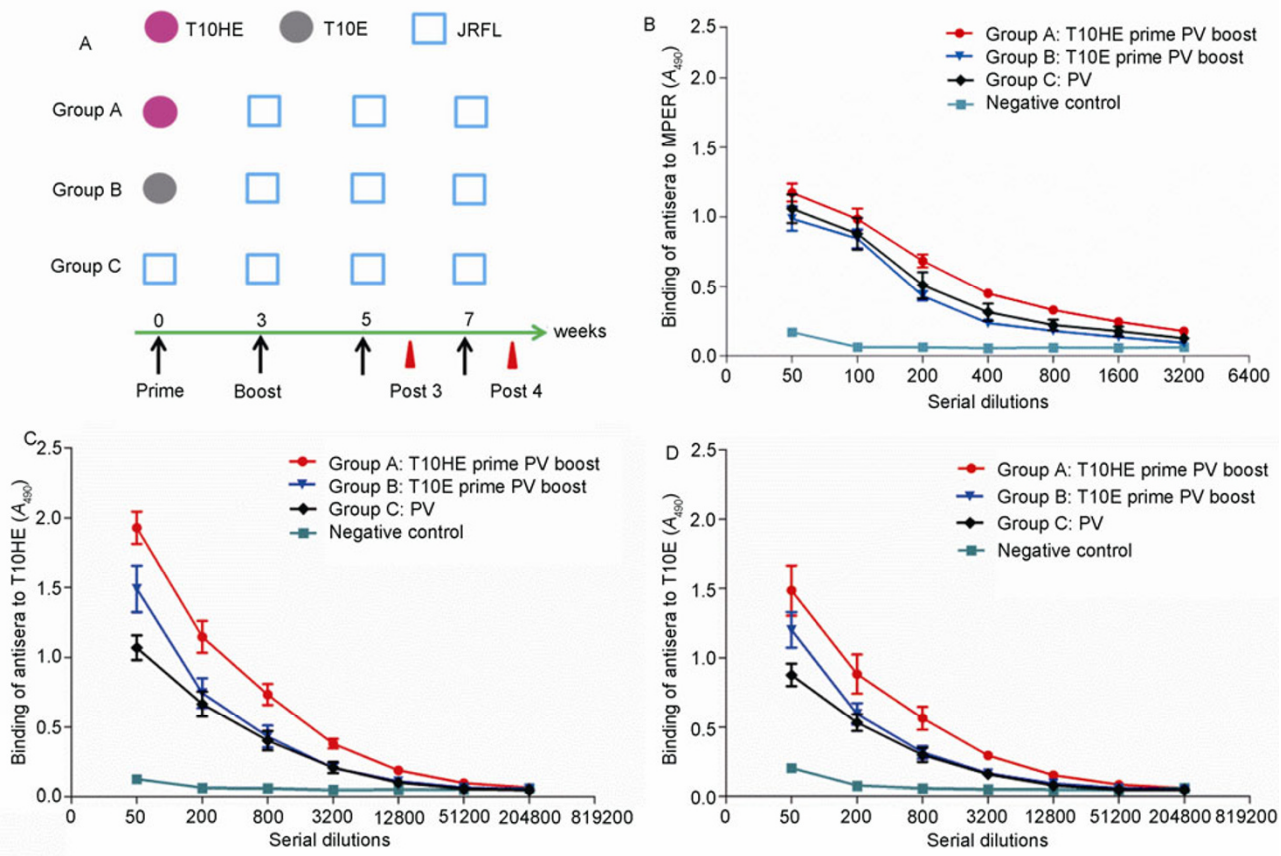


Figure 5 Immunization schedule for BALB/C mice and the detection of antibodies in antisera from groups A, B, and C, induced with three different strategies. A, T10HE, closed purple symbols; T10E, closed gray symbols; JRFL pseudoviruses, open symbols; red arrow, "post 3" and "post 4" sera were collected one week after the third and fourth immunizations, respectively. B–D, Polyclonal antibody responses against MPER (B), T10HE (C), and T10E (D), as assessed by the binding of the post 3 antisera. Sera collected before the immunizations were used as the controls.

Table 1 The titers of specific antibodies of groups A, B and C assessed with the post 3 antisera[a)]

Group	Prime	Boost	Mouse No.	Titers of antibodies specific for			
				MPER	T10HE	T10E	TT
A	T10HE	JRFL	1	1:6400	1:12800	1:12800	1:100
			2	1:6400	1:51200	1:51200	1:100
			3	1:6400	1:51200	1:51200	1:100
			4	1:6400	1:51200	1:51200	1:100
Geometric mean				1:6400	1:36204	1:36203	1:100
B	T10E	JRFL	1	1:800	1:12800	1:12800	1:100
			2	1:800	1:3200	1:3200	1:200
			3	1:1600	1:12800	1:12800	1:200
			4	1:1600	1:12800	1:12800	1:200
Geometric mean				1:1131	1:9050	1:9051	1:168
C	JRFL	JRFL	1	1:6400	1:12800	1:12800	1:50
			2	1:800	1:3200	1:3200	1:50
			3	1:1600	1:12800	1:12800	1:50
			4	1:1600	1:3200	1:3200	1:50
Geometric mean				1:1902	1:6400	1:6400	1:50

a) The experiment was performed in triplicate and repeated once. The mean values from one representative experiment are presented. The immunogenicity of groups A–C was assessed with sera of post 3.

specific antibodies were efficiently induced in group A than in the other two control groups. Anti-HIV antibodies predominated over anti-TT antibodies. The isotypes of the 10E8-like antibodies in these three groups were also determined. As shown in Figure 6, significantly higher levels of the IgG1 subtype were induced in group A (Figure 6A), and moderately higher levels of the IgM and IgG2a subtypes (Figure 6B and C) than in control groups B and C. These results show that the T10HE prime-JRFL pseudovirus boost strategy efficiently induced the 10E8-like antibody response with diverse immunoglobulin subtypes.

2.4 Detection of neutralizing activity of 10E8-like antibodies

Next, we tested the antiviral activity of these 10E8-epitope-specific antibodies. Antisera from the three groups were assayed for their neutralizing activities in a pseudovirus-based assay system using ghost cells expressing CD4, CCR5, and CXCR4 as the target cells. Because JRFL pseudoviruses were used as the immunogen in the boost immunization, we first detected the neutralizing potency of antisera against the JRFL strain (CCR5 tropism). As ex-

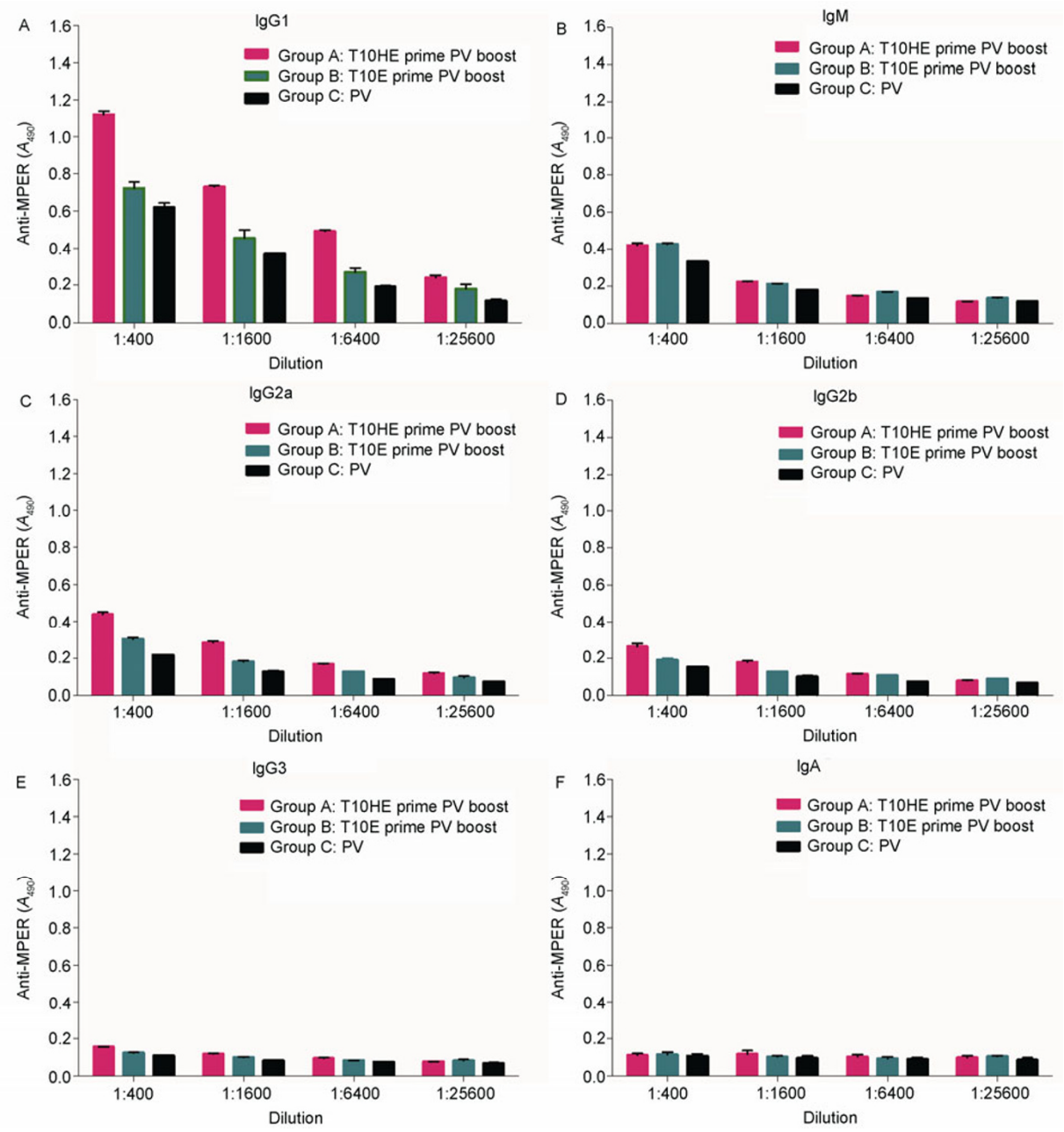


Figure 6 Determination of the 10E8-like antibody subtypes induced with different prime-boost strategies. A–F, Sera collected in the post 4 batch, shown in Figure 5, were assessed. Titers of anti-MPER IgG1 (A), IgM (B), IgG2a (C), IgG2b (D), IgG3 (E), and IgA (F) were measured in triplicate. Data are expressed as means and error bars.

pected, the antisera from group C showed moderate antiviral potency, with IC_{50} of 1∶40 (two mice) and <1∶20 (two mice) (Table 2). Using group C antisera as the baseline, it was striking to see that the antisera of group A displayed significantly enhanced neutralizing potency, with IC_{50} of 1∶160 (three mice) and 1∶80 (one mouse), whereas that of group B could not neutralize 50% of pseudoviruses at a dilution of 1∶20. We then tested another tropic (CXCR4) pseudovirus, HXB2. Antisera from group A efficiently neutralized HXB2 at IC_{50} of 1∶20 (three mice) and 1∶40 (one mouse) compared with the antisera from groups B and C. These results suggest that the antisera of group A exhibited cross-neutralization against pseudoviruses with different tropisms. However, the antisera from all three groups failed to neutralize another laboratory-adapted strain, SF162, at dilutions of 1∶20. The SF162 pseudoviruses were resistant to the antisera from group A, probably because the 10E8 epitope sequence derived from JRFL and T10HE differs from SF162 at position 676, where threonine occurs in T10HE and JRFL, but serine occurs in SF162. We then detected the antisera against pseudoviruses from primary isolates. As shown in Table 2, the antisera from group A neutralized 50% of the CNE3, CNE28, and CNE49 strains at a dilution of >1∶20. Although groups A and B were boosted with the same pseudovirus, different priming resulted in the induction of 10E8-like antibodies with very different neutralizing activities. Group A antisera induced cross-neutralizing antibodies, which showed a certain degree of broad neutralization activity, whereas group B antisera did not. These results suggest that T10HE is a potential vaccine prime for future vaccine designs when combined with a suitable boost strategy.

2.5 Preabsorption of 10E8-like antibodies eliminated the neutralizing capacity of group A antisera

Because the envelope protein of JRFL consists of gp120 and gp41, we tested whether 10E8-like neutralizing antibodies from group A played a major role in an antiviral assay. We preabsorbed 10E8-like antibodies from these antisera to T10HE-conjugated microbeads. As shown in Table 3, after these antibodies were removed, the neutralization capacity of the group A antisera was eliminated. Even at a dilution of 1∶20, they still did not neutralize four of the five pseudoviruses tested in our assay system. When we tested the CNE28 pseudovirus, it was clear that a significant reduction in the neutralization capacity had occurred; the unabsorbed antisera had an IC_{50} of 1∶160 (four mice), which decreased to an IC_{50} of 1∶40 (three mice) or 1∶20 (one mouse) with the absorbed antisera (Tables 2 and 3). These results indicate that the antiviral potency of group A is probably attributable to 10E8-like antibodies.

Table 2 Neutralizing activity of post 4 antisera against infection by HIV-1 pseudoviruses[a)]

Group	Mouse No.	Titers of IC_{50} against HIV-1 pseudoviruses					
		JRFL B, R5	HXB2 B, X4	SF162 B, R5	CNE3 AE, R5	CNE28 AE, R5	CNE49 BC, R5
A	1	1:160	1:40	<1:20	1:40	1:160	1:20
	2	1:160	1:20	<1:20	1:40	1:160	1:40
	3	1:160	1:20	<1:20	1:80	1:160	1:40
	4	1:80	1:20	<1:20	1:80	1:160	1:20
B	1	<1:20	<1:20	<1:20	<1:20	1:20	<1:20
	2	<1:20	<1:20	<1:20	<1:20	<1:20	1:20
	3	<1:20	<1:20	<1:20	<1:20	1:40	<1:20
	4	<1:20	<1:20	<1:20	<1:20	1:40	1:20
C	1	<1:20	<1:20	<1:20	1:20	1:40	<1:20
	2	1:40	<1:20	<1:20	<1:20	1:20	<1:20
	3	1:40	<1:20	<1:20	<1:20	<1:20	<1:20
	4	<1:20	<1:20	<1:20	1:20	1:40	<1:20

a) The experiment was performed in triplicate and repeated at least once.

Table 3 Neutralization titers of antisera after MPER-specific antibodies exclusion[a)]

Mouse No.	Titers of IC_{50} against HIV-1 pseudoviruses				
Group A	HXB2	JRFL	CNE3	CNE28	CNE49
1	<1:20	<1:20	<1:20	1:40	<1:20
2	<1:20	<1:20	<1:20	1:40	<1:20
3	<1:20	<1:20	<1:20	1:20	<1:20
4	<1:20	<1:20	<1:20	1:40	<1:20

a) The experiment was performed in triplicate and repeated at least once.

3 Discussion

Because MPER is immunorecessive [31,35] and shows a variety of transient conformations in its native microenvironment [11,15,36,37], the induction of MPER-specific neutralizing antibodies has been very challenging until now. Considerable efforts have been made to induce BNAbs by designing MPER-containing immunogens in the context of various scaffolds, including hepatitis B surface antigen [38], computationally selected scaffolds [20], and gp120 [21]. However, none of these scaffold-based immunogens successfully elicited BNAbs that target MPER-like 2F5, 4E10, or Z13e1. In previous studies, we reported using appropriate scaffolds to support MPER-containing peptides, creating a stabilized format for the 2F5-binding conformation or an α-helix 4E10-binding conformation [31]. Although the anti-MPER antibody titers were improved when these newly designed MPER-containing scaffolds were used as immunogens, most of the induced antibodies were directed against the scaffolds, suggesting that the high immunogenicity of the scaffolds inevitably caused the immune system to produce numerous nonneutralizing and thus useless antibodies. However, short neutralizing epitope-based peptides are rarely used as immunogens because their immunogenicity is poor, and indeed, most of these short peptides must be conjugated to large carriers or scaffold proteins, such as bovine serum albumin or keyhole limpet hemocyanin, to act as vaccines [23]. In this study, we tried to resolve this dilemma by generating a stabilized helix conformation within a MPER-derived peptide by chemically modifying the original sequence instead of introducing a scaffold to avoid unwanted nonneutralizing antibodies. Using the epitope on MPER that is recognized by the recently identified BNAb 10E8, we synthesized the peptide T10HE, which contains an α-helix structure and clearly exposes the predefined binding surface for BNAb10E8. Our CD analysis indicated that this strategy worked well and that the introduction of a hydrocarbon bridge increased the helicity of the T10HE immunogen. These results show that the T10HE peptide, with a rigid helical conformation, possesses the structural basis or potential to induce 10E8-like antibodies. When T10HE was used as the prime immunogen and JRFL pseudoviruses as the boost immunogen, the immunized mice produced more neutralizing antibodies than those immunized with pseudoviruses alone and those primed with peptide T10E, which has a nonrigid conformation. Although the sequence of MPER is conserved among the viruses tested, especially the critical binding sites (W672, F673, T/S676, L679, W680, and R/K683), there are slight variations in the sequence. We found that the last 15-mer sequence of MPER in the CNE3 and CNE28 strains, which are identical, differed in their sensitivity to the serum from group A. CNE49 and HXB2 differ at two residues but showed similar sensitivity. However, SF162, which differs in only one residue from JRFL, was resistant to the antisera. Therefore, we speculate that viral sensitivity to neutralization by the 10E8-like antibody may only be partly related to sequence variations in MPER. We determined the neutralizing capacity of the sera only after the fourth immunization, which suggests that this capacity is dose and time dependent. The increased neutralization with increasing time was partly attributable to the increased antibody titers [31,39]. We collected the mouse sera after the third and fourth immunizations, and the titers after the fourth immunization were higher than those after the third immunization. Neutralization was partly attributable to the antibody maturation and enhanced binding affinity for the immunogens [12,19]. In this study, we also determined the avidity index [40] of the three groups, and found that the 10E8-like antibodies from the sera of group A showed significantly enhanced avidity (P<0.05) over those of groups B and C (Figure S1 in Supporting Information). The isotypes of the 10E8-like antibodies of these three groups were also determined. The enhanced levels of IgG1, IgM, and IgG2a in group A and their increased avidities could also have contributed to the broad neutralizing activities of the 10E8-like antibodies induced. One possible explanation of this increased antiviral activity is that T10HE mimics the native conformation of the 10E8 epitope and may bind to naïve B cells, resulting in the enrichment of these cells in the blood circulation. The native immunogens expressed on the pseudoviruses or viral particles may stimulate these B cells to differentiate rapidly, becoming high-affinity antibody-secreting cells and producing potent and broadly neutralizing antibodies. When all these data are considered together, they indicate that T10HE is an effective vaccine prime, at least in this research context. Whether the use of immunizing peptides as vaccine primes could be a universal strategy requires future confirmation.

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Supporting Information

Figure S1 Avidity index of MPER-specific serum IgG (post 4) measured by chaotropic ELISA using NH_4SCN. * indicates the significant difference in IC_{50} values between groups A, B and C (two tailed *T* test; $P<0.05$; $n=4$). The avidity index was detected by diluting sera to a concentration that corresponds to $A_{490}=1.0$ in an endpoint ELISA, and reacting with antigen which coated to plates for 2 h at 37°C. After washing, serial dilutions of NH_4SCN (4–63 mmol L^{-1}) were added. The plates were washed after 20 min, and the remaining antibodies were detected. The avidity index is the concentration of NH_4SCN required to reduce the optical density (*A*) by 50% compared to that in wells with no NH_4SCN.

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