



HIV-1 Membrane-Proximal External Region Fused to Diphtheria Toxin Domain-A Elicits 4E10-Like Antibodies in Mice

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ABSTRACT

The production of broadly neutralizing antibodies (bNAbs) is a major goal in the development of an HIV-1 vaccine. The membrane-proximal external region (MPER) of gp41, which plays a critical role in the virus membrane fusion process, is highly conserved and targeted by bNAbs 2F5, 4E10, and 10E8. As such, MPER could be a promising epitope for vaccine design. In this study, diphtheria toxin domain A (CRM197, amino acids 1–191) was used as a scaffold to display the 2F5 and 4E10 epitopes of MPER, named CRM197-A-2F5 and CRM197-A-4E10. Modest neutralizing activities were detected against HIV-1 clade B and D viruses in the sera from mice immunized with CRM197-A-4E10. Monoclonal antibodies raised from CRM197-A-4E10 could neutralize several HIV-1 strains, and epitope-mapping analysis indicated that some antibodies recognized the same amino acids as 4E10. Collectively, we show that 4E10-like antibodies can be induced by displaying MPER epitopes using an appropriate scaffold. These results provide insights for HIV-1 MPER-based immunogens design.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is still an epidemic around the world despite a significant reduction in rates of infection and death by effective antiretroviral therapy (ART). Developing a protective HIV-1 vaccine is an ideal way to prevent HIV-1 infection and control HIV-1 transmission. The HIV-1 envelope (Env) glycoprotein, a precursor gp160 protein of gp120 and gp41, is the sole antigen exposed on the virus surface and plays an essential role in the virus replication cycle by mediating fusion between the viral and cellular membranes [1]. With the application of novel technologies, such as single B cell sorting, deep sequencing, and microneutralization assays, there are now dozens of broadly neutralizing antibodies (bNAbs) isolated from HIV-1-infected patients that can target the Env protein [2–4]. All of these bNAbs map to six major antigenic sites on Env: the CD4 binding site [5–7], the V1/V2 loop [8,9], the V3 loop with Asn332 glycan patch [10,11] the gp120/gp41 interface [12], the gp41 fusion peptide region [13,14] and the membrane-proximal external region (MPER) [15–17].

Among the vulnerable sites on Env, the MPER epitope is highly

conserved and has a tryptophan-rich, linear epitope that is recognized by bNAbs including 2F5, Z13e1, 4E10, m66 and 10E8 [18]. Structural analysis shows that these bNAbs target the fusion-intermediate conformation, and prevent membrane fusion by interfering with the formation of the six-helix bundle consisting of antiparallel coiled coils formed by the N-terminal and C-terminal heptad repeats of gp41 [19,20]. Although numerous immunization studies have focused on the MPER, bNAbs have not yet to be successfully elicited by MPER immunogens. This is likely due to the poor immunogenicity of MPER and the structural shield when MPER interacting with membrane [21].

Several different approaches have been employed to address the challenges associated with MPER immunogenicity: some authors have used liposomes and scaffolds to present the MPER epitope, whereas others have created chimeras of MPER and virus-like particles for expression [22–26]. The Diphtheria toxin (DT) is a protein vector frequently used for expression studies. In particular, the mutant form, CRM197, which harbors an inactivating Gly52Glu mutation, is often used as an intramolecular adjuvant to enhance the immunogenicity of polysaccharides, haptens, Meningitec and Menveo for meningitis

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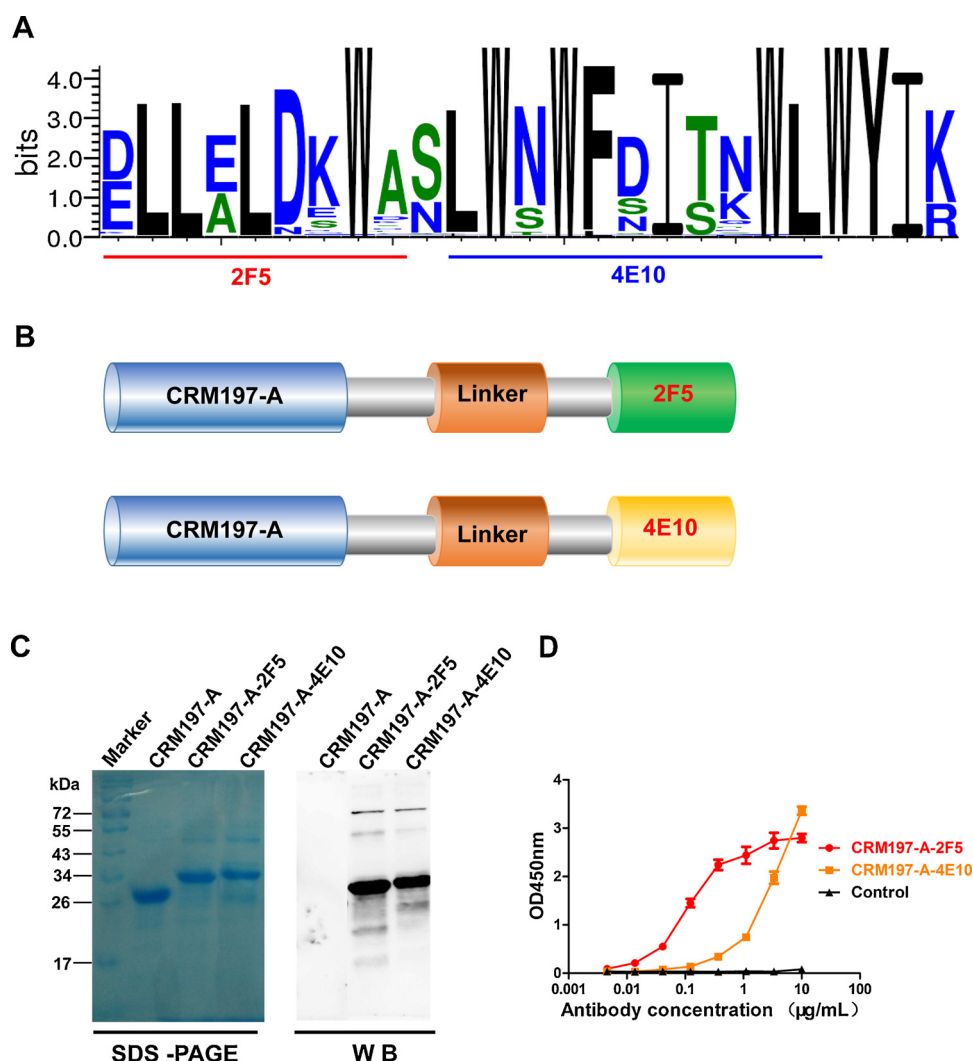


Fig. 1. Construction and characterization of CRM197-A-2F5 and CRM197-A-4E10 fusion proteins. (A) The MPER conservative analysis by WebLogo, in which 1000 Env sequences from HIV sequence database website <http://www.hiv.lanl.gov/> were used. The epitope targeted by 2F5 and 4E10 is indicated. (B) Schematic diagrams of the CRM197-A-2F5 and CRM197-A-4E10 fusion proteins. The epitopes targeted by 2F5 and 4E10 were respectively linked to the CRM197-A C-terminus with a 15-aa long flexible linker (GGGGSGGGSGGGGS). (C) SDS-PAGE and western blotting analysis of the purified CRM197-A-2F5 and CRM197-A-4E10 fusion proteins. The 2F5 and 4E10 mAbs were used as the detecting antibodies. Protein CRM197-A served as the negative control. (D) Antigenicity of the CRM197-A-2F5 and CRM197-A-4E10 fusion proteins by ELISA. The CRM197-A-2F5 and CRM197-A-4E10 fusion proteins were coated into 96-microwell plates and reacted to mAb 2F5 and 4E10, respectively.

[27–31]. DT has three domains: a receptor-binding domain R (aa 385–535), a transmembrane domain T (aa 201–384) and a catalytic domain A (aa 1–191) otherwise referred to as CRM197-A [32,33]. Wang and colleagues reported that higher neutralization titers can be achieved by fusing the human papillomavirus (HPV) major capsid protein L2 peptide with CRM197-A as opposed to CRM197 or CRM389 (aa 1–389) [34]. The other instance is the truncated HEV capsid protein (E2) fused to CRM197-A showed 10-times higher immunogenicity than that of particulate p239 in mice and conferred comparable Hepatitis E protection as Hecolin in non-human primates [35]. Taken together, CRM197 has been corroborated as immunogenicity enhancer through either chemical covalent coupling to polysaccharide in full-length form or fusion expression using its A domain.

In this study, we sought to design an improved method for the production of MPER bNAbs. We fused MPER epitopes (specifically, the 2F5 and 4E10 epitopes) separately to the C-terminus of CRM197-A using a GGGGSGGGSGGGGS linker. The recombinant CRM197-A-2F5 and CRM197-A-4E10 proteins were expressed in *Escherichia coli* and then tested in immunogenicity assays in mice. The results show a broader and more potent neutralizing activity of immune sera from the CRM197-A-4E10 group compared with the CRM197-A-2F5 group. We screened monoclonal antibodies from mice injected with CRM197-A-4E10, and acquired 5 antibodies with 4E10-like properties that recognized the same key amino acids as 4E10 and showed cross-neutralization against HIV-1 strains. Overall, these results provide important insight for MPER-based HIV-1 vaccine design and offer

evidence for the promising role of MPER in the production of HIV-1 immunogenic determinants.

2. Materials and methods

2.1. Construction, expression and purification of epitope fusion proteins

The 2F5 (ELLELDKWA) and 4E10 (LWNWFDITNWL) epitopes were linked to the CRM197-A C-terminus by a GGGGSGGGSGGGGS linker and cloned into the pTO-T7 vector. Plasmids were then transformed into *E. coli* BL21 competent cells and cultured overnight at 37 °C in solid LB medium containing kanamycin. The single bacterial colonies were inoculated in liquid LB medium containing antibiotics, and cultures were grown to an OD₆₀₀ of 0.8. Fusion proteins (respectively designated CRM197-A-2F5 and CRM197-A-4E10) were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25 °C for 12 h. Bacterial cell pellets were harvested by centrifugation at 7000 × g for 10 min in a Beckman Avanti J-26 s centrifuge. Cell pellets were re-suspended with lysis buffer (5 mM Tris-HCl, 1 mM EDTA, 300 mM NaCl), sonicated (SONICS VCX800), and then centrifuged at 25,000 × g for 10 min at 20 °C. The fusion proteins were then purified by washing the inclusion bodies thrice each in buffer with and then without 0.5% Triton X-100. Proteins were solubilized in 20 mM Tris-HCl pH 8.0 with 8 M urea, and insoluble debris was removed by centrifugation for 10 min at 25,000 × g. The supernatant was dialyzed into 20 mM TB8.0 over a gradient containing 6 M, 4 M, 2 M, 1 M, and no urea, and then

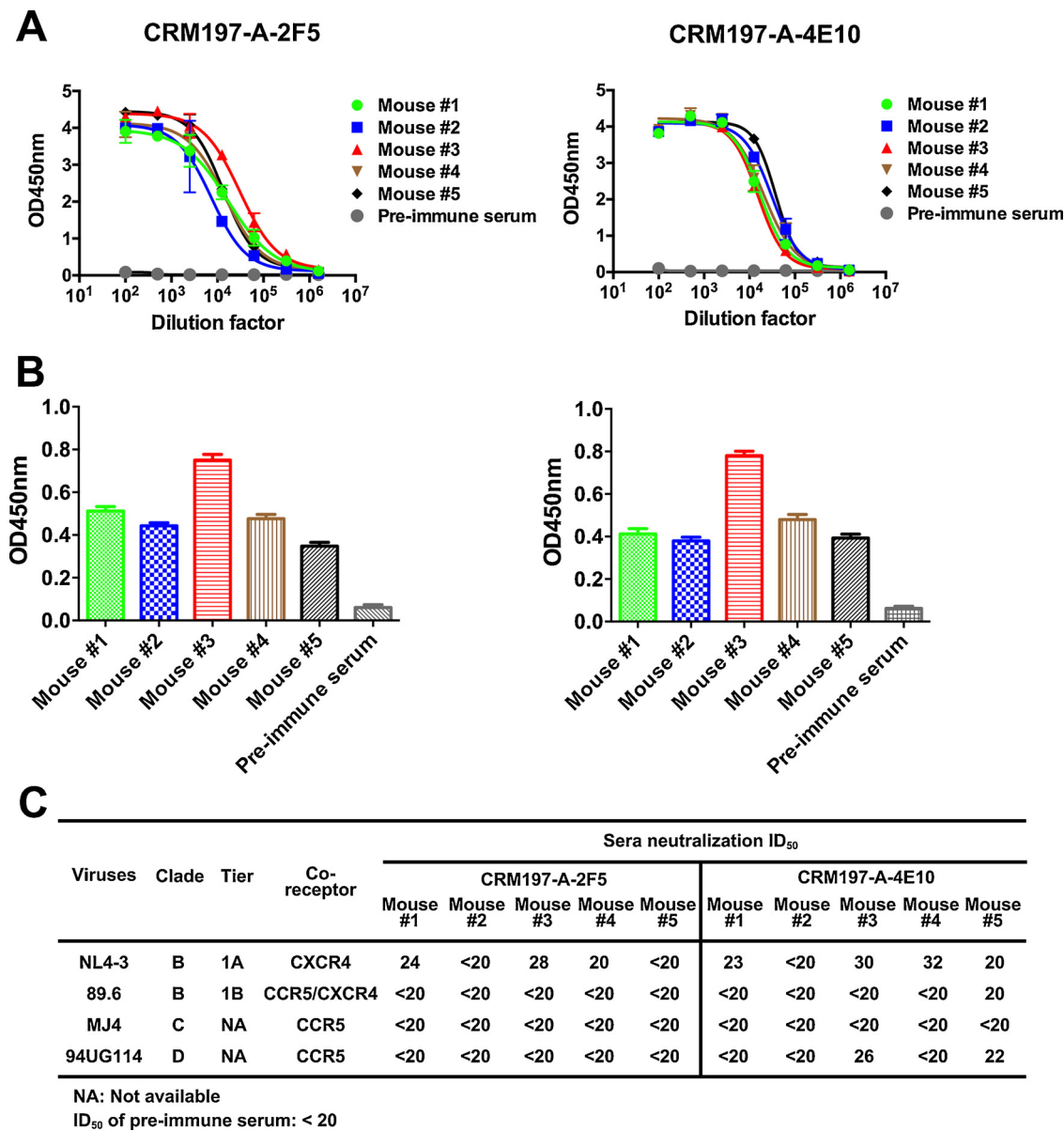


Fig. 2. Immunogenicity assay in mice for CRM197-A-2F5 and CRM197-A-4E10 fusion proteins. Serum taken at 8 weeks after the first immunization were analyzed by ELISA. (A) CRM197-A-2F5 and CRM197-A-4E10 fusion proteins were used as coating antigens. (B) gp41 extracellular domain protein produced by HEK293FT cells was used as coating antigen. Sera were diluted 100-fold. Pre-immune serum were used as a negative control. (C) Neutralizing activity of the immune serum against four HIV-1 viruses: HIV_{NL4-3} (clade B), HIV_{89.6} (clade B), HIV_{MJ4} (clade C) and HIV_{94UG114} (clade D) in the TZM-b1 assay. The results represent three independent experiments.

centrifugated at 25,000 × g for 10 min, as described elsewhere [36]. The supernatant proteins were analyzed by SDS-PAGE and immunoblotting with 2F5 and 4E10 antibodies (obtained from Immune Tech, New York, USA). The purified proteins were stored at −80 °C before further use.

2.2. Enzyme-linked immunosorbent assays (ELISA)

For ELISA assays, proteins were solubilized in carbonate buffer (pH 9.6), and coated into the wells of a 96-well microtiter plate at 37 °C for 2 h. Plates were washed once with PBST and then blocked with buffer (0.5% casein, 2% gelatin, 0.1% preservative (proclin-300) in PBS) overnight at 4 °C. Samples were 3-fold serially diluted with sample buffer (2% BSA in PBS with 0.05% Tween-20) and then added in triplicate into the wells of a 96-well plate for 1 h at 37 °C. The horseradish peroxidase (HRP)-conjugated goat anti-human or anti-mouse secondary

antibody (1:5000 dilution) (Abcam, Cambridge, UK) was used for detection antibody and incubated for 30 min at 37 °C. The OD at 450/630 nm was measured using a microplate reader (Antu Experimental Company, Beijing, China).

2.3. Ethics Statement and Mice Immunization

Animal experiments were approved by the Xiamen University Laboratory Animal Center (Approval number: XMULAC20160051; approval date: 07/03/2016). All procedures were conducted in accordance with animal ethics guidelines and approved protocols. Female, 8-week-old BALB/c mice were purchased from Shanghai Slack Laboratory Animals Enterprise Co., Ltd. Three groups of mice (n = 5 per group) were respectively immunized subcutaneously with PBS (control), CRM197-A-2F5 protein, or CRM197-A-4E10 protein. Mice were primed with 200 µg protein mixed with Freund's complete

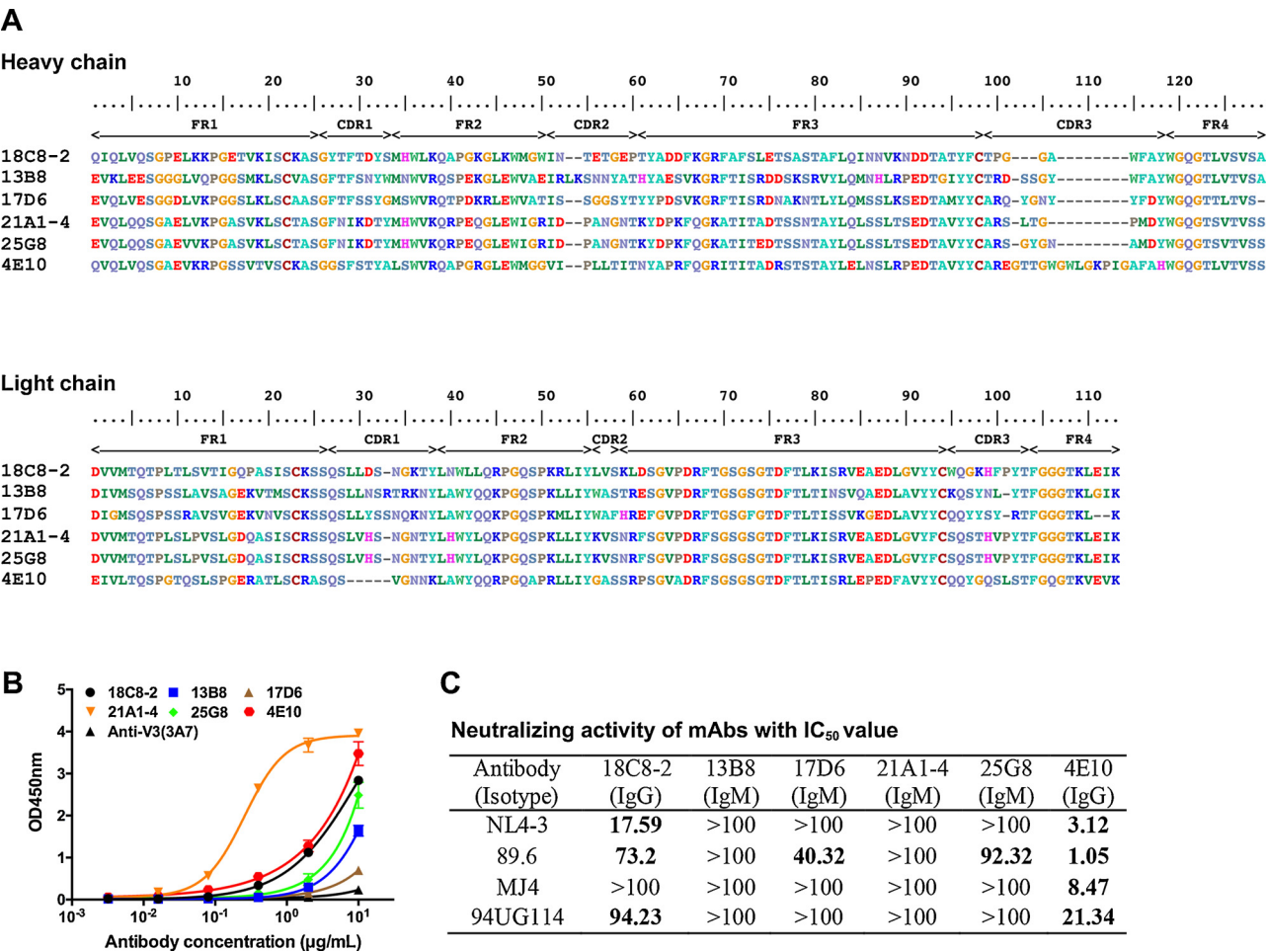


Fig. 3. Characterization of five CRMA197-A-4E10 elicited monoclonal antibodies. (A) Sequence alignment. (B) ELISA analyses of reacting with the gp41 extracellular domain protein produced by HEK293FT cells. gp41 proteins were coated into the wells of a 96-well plate (100 ng/well) and monoclonal antibodies were 5-fold serially diluted from 10 μg/mL. 4E10 was used as a positive control and a V3-specific antibody (3A7, screened in-house) as a negative control. (C) Neutralizing activity. The values shown are the IC₅₀. IC₅₀ < 100 μg/mL are in bold. IC₅₀ > 100 μg/mL indicate that the neutralization was lower than 50% at an antibody concentration of 100 μg/mL.

adjuvant, and then subsequently immunized with 100 μg protein with Freund's incomplete adjuvant. The immunization schedule comprised four doses at 2-week intervals. Serum samples were collected, inactivated at 56 °C for 30 min, and stored at −20 °C for ELISA and neutralization assays.

2.4. HIV-1 neutralization assay

The infectious molecular clone pNL4-3, p89.6, pMJ4 and p94UG114 were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Program. HIV-1 virus production and titration were performed with TZM-b1 cells to determine the neutralizing activity, as previously described [37]. In brief, 293FT cells were transfected with the infectious molecular clone plasmids using Lipofectamine 2000 (Invitrogen; Carlsbad, CA). At 12 h post-transfection, the culture medium was replaced with fresh medium and HIV-1 virus supernatant was harvested 36 h later. The virus was titrated onto TZM-b1 cells [38,39]. The neutralization assay was performed, as follows: Inactivated serum samples or antibodies were 3-fold serially diluted (1:20 to 1:4860) in complete Dulbecco's modified eagle medium (DMEM). Virus (50 μL; at 100 TCID₅₀), 15 μg/mL DEAE, and serum or antibodies were mixed and incubated at 37 °C for 1 h, and then added to 100 μL of prepared cells (1 × 10⁴ cells/well) in 96-well plates. Cells were further incubated at 37 °C with 5% CO₂ for 48 h, and then fixed with 0.2% glutaraldehyde and 1% formaldehyde, and stained with X-gal substrate

[37]. The HIV-infected cell spots were counted using an Immunospot Series Analyzer (Cellular Technology, Cleveland, OH). The half-maximal inhibitory dilutions (ID₅₀) or half-maximal inhibitory concentrations (IC₅₀) was calculated to represent the neutralizing activity.

2.5. Monoclonal antibody preparation

One week after the final immunization, splenic cells from mice immunized with CRM197-A-4E10 were harvested and fused with sp2/0 myeloma cells by PEG2000. The fused cells were seeded into 96-well plates containing 200 μL of RPMI-1640 medium supplemented with 20% fetal calf serum and hypoxanthine/aminopterin/ thymidine (HAT) for 1 week. The medium was replenished with fresh medium without HAT. The supernatants of the hybridomas were screened by ELISA to identify a specific reaction with the MPER peptide. Positive hybridoma cells were sub-cloned three times to obtain monoclonal antibodies. These cell lines were then injected into F1 mice to obtain ascites fluids. IgG isotype antibodies were precipitated with ammonium sulphate followed by protein A; IgM subtype antibodies were purified subsequently using phenyl and hydroxyapatite.

2.6. Alanine scanning mutagenesis

For the construction of alanine-replaced mutant protein, to eliminate the potential influence raised by CRM197-A antibodies in epitope

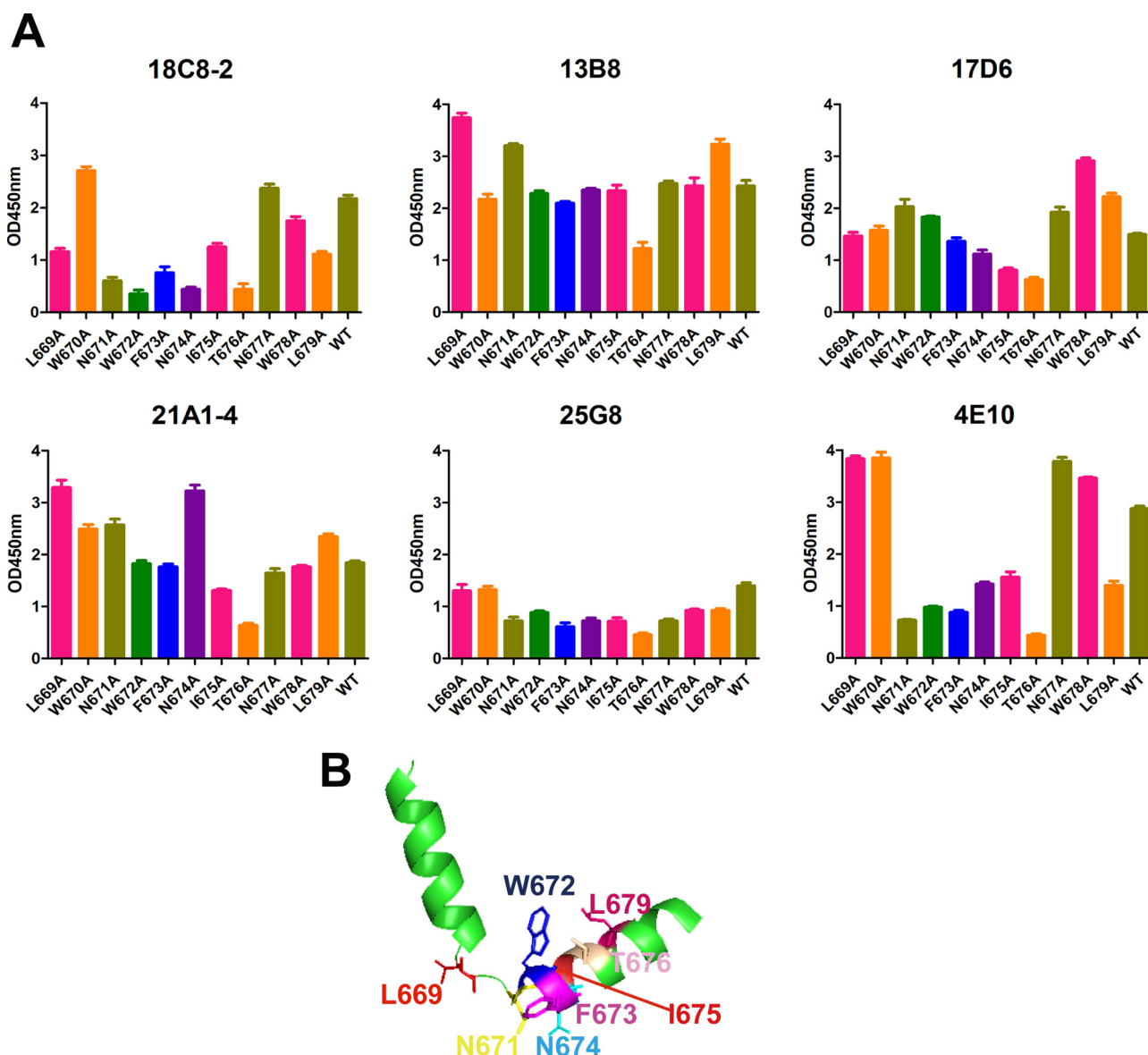


Fig. 4. Alanine scanning mutagenesis was used to map the binding sites of antibodies. (A) ELISA analysis of antibody-binding residues. The epitope (LWNWFNI-TNWL) was fused onto Cholera Toxin B-Subunit (CTB) vector and single mutation fusion proteins were expressed and purified. Mutated proteins were coated into the wells of a 96-well plate at 100 ng/well, and all the antibodies were diluted at 4 μ g/mL to react with the mutated proteins. The binding results are shown as OD values. Wild-type (WT) is the fused protein without any mutation (control). (B) The binding sites of antibodies on the MPER peptide. These sites were obtained from the alanine scanning mutagenesis assay. The mutations that significantly reduce antibody binding is indicated by different colors.

mapping, CTB was used as the otherwise scaffold to fuse 4E10 epitope. The alanine mutants were introduced to the 4E10 epitope in CTB-4E10 construct using the QuikChange site-directed mutagenesis system (Agilent, Stratagene) and confirmed by DNA sequencing. Then, the expression and purification process were conducted same as the above-mentioned.

2.7. Molecular modeling and molecular docking

A structural model of the mAb 18C8-2 variable region (as a 4E10-like representative) was built by the homology module of Discovery Studio 2016 software (Accelrys, San Diego, CA). Briefly, the variable sequences (VH and VL) of 18C8-2 were blast-searched separately for the modeling template having the highest sequence identity with the target sequence. The amino acid sequences of the VH and VL regions of 18C8-2 were inputted into the software and loop grafting and side-chain modeling were chosen for the calculations. The obtained model was

further refined by minimizing the energy approach incorporated into the DS software. Subsequently, we docked the 18C8-2 Fab model onto the crystal structure of MPER (PDB no. 1TZG) using the Zdock module with prior knowledge of the key residues involved in the interaction of 18C8-2 and MPER. The top-scoring predictions were refined using the RDOCK suite. All refined predictions were re-ranked and the best orientation was selected as the final complex model. All maps rendering the complex structures in this study were prepared by the program PyMol (<http://www.pymol.org/>).

3. Results

3.1. Construction and characterization of fusion proteins

To enhance MPER immunogenicity, we fused HIV-1 MPER neutralizing epitopes to the C-terminus of CRM197-A, an intramolecular adjuvant, using a GGGSGGGSGGGSG linker, and generated two

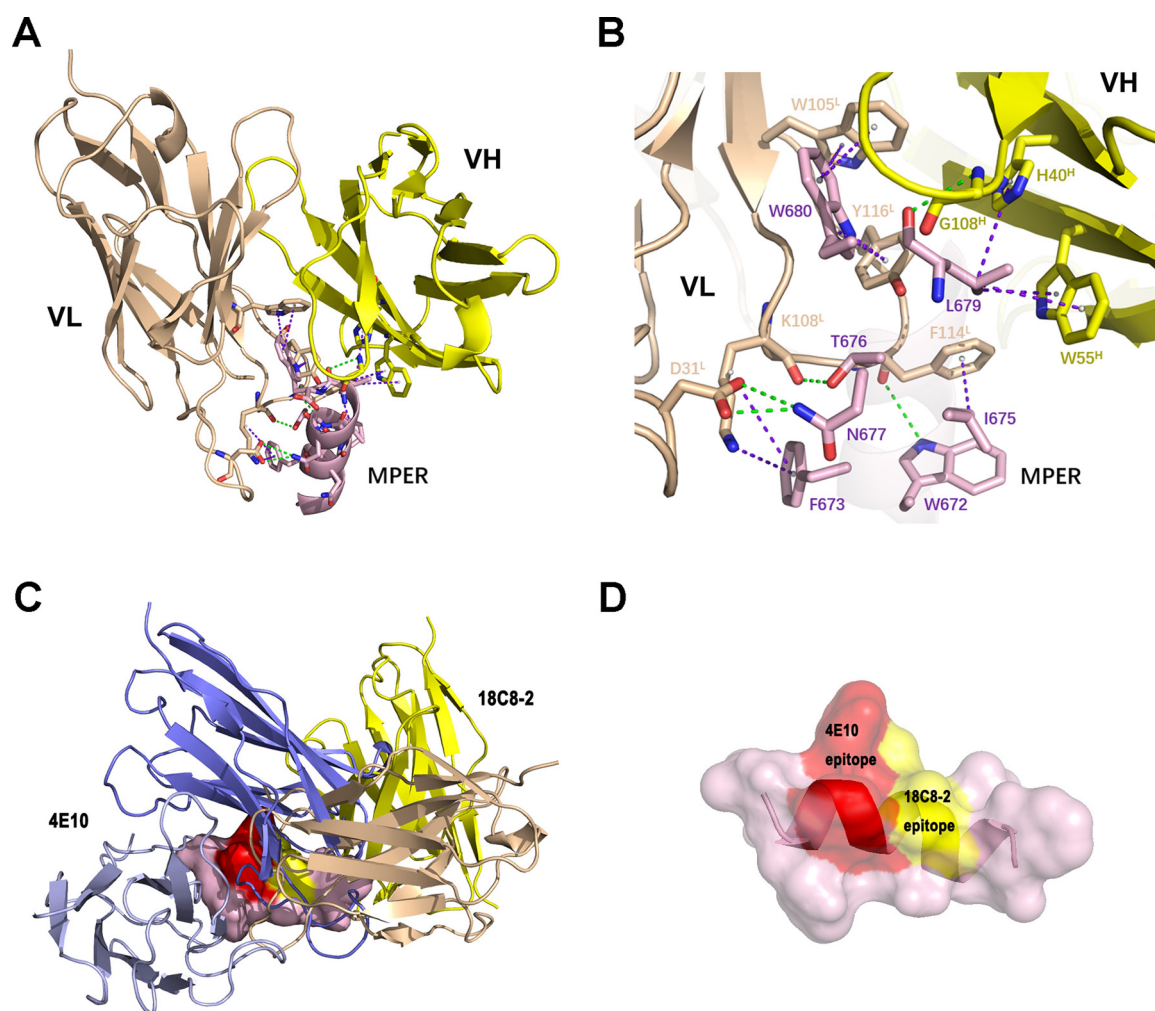


Fig. 5. Cartoon model of the 18C8-2 Fab-MPER complex. (A) Overall model of the 18C8-2 Fab-MPER complex. (B) The interface of the 18C8-2 Fab-MPER complex. The green dotted line indicates a hydrogen bond and the purple dotted line represents a Pi (π) bond. L (wheat) and H (yellow) represent the light and heavy chains of Fab. MPER is light pink. (C) Comparison of 18C8-2 with 4E10 in the recognition of the MPER. (D) Binding epitopes in MPER recognized by 18C8-2 and 4E10. The 4E10 epitope is red whereas the 18C8-2 epitope contains both red and yellow areas.

fusion proteins, designated CRM197-A-2F5 and CRM197-A-4E10 (Fig. 1A, 1B). The fusion proteins expressed as inclusion bodies in *E. coli*. Following purification, the fusion proteins were observed to migrate at ~30 kDa on 12% reduced SDS-PAGE (Fig. 1C). Immunoblotting with 2F5 and 4E10 antibodies showed the positive reactivity and confirmed the expression of the fusion proteins (Fig. 1C), with CRM197-A protein used as a negative control. Using ELISA, we found that the CRM197-A-2F5 and CRM197-A-4E10 proteins had good reactivity with the human 2F5 and 4E10 antibodies, respectively. Overall, these results indicated that the 2F5 and 4E10 epitopes, fused with CRM197-A, showed faithful antigenicity (Fig. 1D).

3.2. Immunization and immunogenicity analysis

BALB/C mice were immunized with fusion proteins via a prime-boost immunization scheme. To evaluate the immunogenicity of the fusion proteins, we tested the antisera taken from mice at 8 weeks after the first immunization using ELISA. Both CRM197-A-2F5 and CRM197-A-4E10 induced strong antibody titers (10^6), indicating good immunogenicity of the fusion proteins in BALB/c mice (Fig. 2A). Next, to further assess the specific anti-MPER reactivity of the antisera, we examined whether the antisera could react with the whole gp41 extracellular domain protein (containing MPER) produced in HEK293FT cells. We found that the antisera from all mice exhibited the specific

reactivity with the gp41 extracellular domain protein at a 100-fold dilution (Fig. 2B). These results indicated that both CRM197-A-2F5 and CRM197-A-4E10 proteins elicited HIV epitope-specific antibody responses in BALB/c mice.

Subsequently, we used TZM-b1 neutralization assay to determine the neutralizing activity of the antisera against four HIV-1 viral strains: HIV_{NL4-3} (clade B), HIV_{89.6} (clade B), HIV_{MJ4} (clade C) and HIV_{94UG114} (clade D). The neutralizing ID₅₀ value showed that the antisera from CRM197-A-4E10 could neutralize HIV_{NL4-3}, HIV_{89.6}, and HIV_{94UG114}, whereas antisera from CRM197-A-2F5 only neutralized HIV_{NL4-3} in Fig. 2C. The pre-immune serum did not have any detectable neutralizing activity against the same virus. Thus, the antisera from CRM197-A-4E10 had broader neutralizing activity than did CRM197-A-2F5. There was no neutralizing activity against HIV_{MJ4} from either antiserum. These results suggest that the CRM197-A-4E10 fusion proteins can induce cross-clade neutralizing antibodies against the HIV MPER epitope.

3.3. Generation and characterization of monoclonal antibodies

To further evaluate the specific epitope responses after immunization with the fusion proteins, hybridomas generated from a CRM197-A-4E10-immunized mouse with the highest cross-clade neutralizing activity were screened by ELISA using the MPER peptide as a probe. We

acquired a total of 5 monoclonal antibodies: 4 IgM and 1 IgG subtypes. Their sequence alignment showed in Fig. 3A. Obviously, the length of complementarity determining region 3 (CDR3) in heavy chain is shorter than that of human mAb 4E10. We tested the binding activity of the antibodies to the gp41 extracellular domain protein containing the MPER and compared the binding against that measured with 4E10. We found that 21A1-4 had better reactivity than 4E10; 18C8-2 had similar reactivity; and 13B8, 17D6 and 25G8 had weaker reactivity (Fig. 3B). Next, we used a native virion-based neutralization assay to determine the neutralizing activity of these antibodies. Notably, the IgG-type antibody (18C8-2) showed cross-clade neutralizing activity, whereas the IgM antibodies (17D6 and 25G8) weakly neutralized the HIV_{89.6} strain (Fig. 3C). The sequence alignment of 4E10 epitope in four HIV-1 viruses showed that N671 and D674 of HIV_{89.6} strain is different from that of other three viruses, indicating two crucial neutralization sites for 17D6 and 25G8 (Supplementary Fig. S1). The other antibodies (13B8 and 21A1-4) had binding activity with MPER epitope but no neutralizing activity. These results suggested the CRM197-A-4E10 fusion protein as a potential and effective immunogen to elicit neutralizing antibodies.

3.4. Alanine scanning mutagenesis to analyze key recognition sites

To further define the key amino acid residues required for antibody recognition, we conducted alanine scanning mutagenesis of the 4E10 epitope. In ELISA analysis, we found the monoclonal antibodies had weak reactivity with the CRM197-A vector protein (Supplementary Fig. S2), suggested that the antibodies epitopes involve several amino acid residues located in the CRM197-A. To avoid the interference of CRM197-A vector on epitope mapping, we fused the 4E10 epitope to another Cholera Toxin B-Subunit (CTB) vector [40–42], and measured the reactivity of the resultant proteins to the 5 mAbs using ELISA (Fig. 4A). In the reactivity profile, we found that residue T676 is critical for the binding of all antibodies to MPER. Residues N671, W672, F673, N674, I675, T676 and L679 are also involved in the binding of mAb 4E10 to MPER, consistent with a previous report [16]. Mutations in residues L669 and W678 also affected the binding of 18C8-2 (Fig. 4A). Overall, mAb 18C8-2 shared a similar reaction profile to that of mAb 4E10, but were remarkably different to those of clade-specific and non-neutralizing antibodies. These findings suggest that mAb 18C8-2 recognize an epitope overlapping with the 4E10 epitope, as shown in the MPER structure (Fig. 4B, Supplementary Table 1).

3.5. Structural modeling of 18C8-2 Fab in complex with the MPER peptide

To further map the epitope defined by 4E10-like antibodies, we chose 18C8-2 as a representative antibody to molecularly model the antibody:MPER complex; 18C8-2 was selected as it had the best neutralizing potency and a broad antiviral activity. The initial structural model of the 18C8-2 Fab was generated using the Modeler module in the Discovery Studio (DS) software (see Methods). In the complex model, the 18C8-2 antibody mainly recognized the MPER peptide by π (π) bonding and hydrogen bonding (Fig. 5A, Supplementary Table 2). Specifically, the molecular interaction between the 18C8-2 Fab fragment and the MPER was mediated by intermolecular π bonds between the D31^L, W105^L, Y116^L, F114^L, H40^H, W55^H side-chains of the 18C8-2 heavy chains, and the F673, N677, W680, I675, L679 side-chains of MPER. Hydrogen bonds were formed by the D31^L, K108^L, Y116^L, F114^L, G108^H side-chains of 18C8-2 and the N677, T676, W672, L679 side-chains of MPER, respectively (Fig. 5B). Compared with 4E10, 18C8-2 has a different binding orientation. The model also indicates that 18C8-2 binding covers more amino acid residues than does that of 4E10 (Fig. 5C, D).

4. Discussion

Although the current anti-retroviral therapy (ART) has turned HIV-1

infection from a lethal disease to a chronic and manageable one, the development of HIV-1 vaccine is still urgent and full of challenging. The RV144 vaccine was the first vaccine to show 31.2% protection from HIV-1 infection over a period of 42 months, which encouraged the development of HIV-1 vaccine [43]. In recent years, abundant structures of bNAbs isolated from the HIV-1 infected individuals have been solved through x-ray crystallography and cryo-EM and provided valuable structural information for the immunogen design. Recently, Xu et al. exploited an iterative structure-based optimization to design FP-bearing immunogens, demonstrating the elicitation of FP specific neutralizing antibodies [44]. Thus, the epitope-based approach to generate effective bNAbs seems to be promising.

In addition to FP epitope, the linear and conserved MPER is the other major target for immunogen design [45]. The MPER is involved in the virus-infecting process and may be an ideal target for HIV-1 vaccine development. Despite many efforts, only few MPER-based immunogens showed the antibody elicitation with low potency and limited breadth. Ofek and colleagues previously outlined the utility of scaffolding proteins to display target epitopes for the elicitation of structure-specific antibodies [24]. In order to improve the MPER-based vaccine design, more aspects should be considered. Others groups have also shown that the immune system tends to recognize flexible regions in an epitope-display scaffold [46]. In this study, we utilized CRM197-A as a vector to display the 2F5 and 4E10 epitopes, joined at the C-terminus of CRM197-A by a flexible (GGGGG)₃ linker, which allowed epitope-preferred folding. The purified fusion proteins, CRM197-A-2F5 and CRM197-A-4E10, showed good reactivity with the mAb 2F5 and 4E10 in ELISA analysis. In immunized BALB/C mice, the immune serum had an antibody titer of $\sim 10^6$, and could bind to the MPER on the whole gp41 extracellular domain protein produced in 293FT cells. We found that the fusion proteins also elicited polyclonal serum capable of neutralizing different HIV-1 clades. Serum from immunization with the 4E10 fusion protein offered better neutralization than that from 2F5, possibly due to their different epitope on the MPER. Thus, exploitation on more available broad neutralization epitopes is of importance to epitope-based vaccine design.

Five monoclonal antibodies were screened via ELISA using the MPER peptide as a probe. Most of the antibodies were IgM subtype, with only one IgG antibody. We found that all of the IgM antibodies reacted better with the MPER peptide than did the IgG antibody (data not shown), whereas only the IgG-subtype antibody 18C8-2 showed cross-clade neutralization against the HIV-1 viruses. Among the antibodies, they bound to similar sites as 4E10, whereas 18C8-2 had a neutralization potency lower than that of 4E10 [17]. The 13B8 and 21A1-4 antibody was unable to neutralize the HIV-1 virus, even though it recognized the sites important for 4E10 binding, suggesting that other residues may be necessary for antibody neutralization. There may be other reasons for the weaker neutralizing ability of the antibodies: 1) Because all of the antibodies are from mice, they have a shorter CDRH3 (9 aa) of the heavy chain as compared with human antibodies [47]. 2) These antibodies don't react with the part of the membrane involved in antibody recognition and neutralization. 3) Others have shown that the longer CDRH3 enhances the neutralization activity via additional interactions with the viral lipid membrane rather than exclusively binding to the epitopes [48–50]. Besides, we found that 18C8-2 antibody mainly interacts with MPER through light chain in the structure model, whereas heavy chain dominates the epitope recognizing in 4E10, 2F5 and 10E8 structures (Supplementary Table 2). It may also explain the lower neutralizing activity of 18C8-2 than that of 4E10.

In summary, numerous studies have used MPER transplantation onto surface loops of various protein scaffolds to elicit bNAbs [23,24,51–54]. In each case, the MPER fusion proteins induced antibodies that could bind to the MPER epitopes, with several also eliciting weak neutralizing activities. However, none of these previous studies have been able to elicit 4E10-like monoclonal antibodies that not only bind to the 4E10 epitope but can also neutralize the HIV-1 virus. Thus,

our results provide important insight for an MPER-based HIV-1 vaccine design, and offer evidence to support the use of MPER as a promising HIV-1 immunogen candidate. Furthermore, an optimization on immunization schemes for CRM197-A-MPER proteins may benefit for the elicitation of neutralizing antibodies with more potent activity and wider breadth. Moreover, multiple tandem epitope repeats, conservative epitope sequence and polyvalent vaccination strategies could be taken into consideration aiming to produce considerable titer of HIV-1 bNAbs.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.imlet.2019.07.004>.

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