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Citation: Hanson, Melissa C., Wuhbet Abraham, Monica P. Crespo, Stephanie H. Chen, Haipeng Liu, Greg Lee Szeto, Mikyung Kim, Ellis L. Reinherz, and Darrell J. Irvine. "Liposomal Vaccines Incorporating Molecular Adjuvants and Intrastructural T-Cell Help Promote the Immunogenicity of HIV Membrane-Proximal External Region Peptides." Vaccine 33, no. 7 (February 2015): 861–868. © 2014 Elsevier

As Published: http://dx.doi.org/10.1016/j.vaccine.2014.12.045

Publisher: Elsevier

Persistent URL: http://hdl.handle.net/1721.1/108190

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Published in final edited form as:
Vaccine. 2015 February 11; 33(7): 861–868. doi:10.1016/j.vaccine.2014.12.045.

Liposomal vaccines incorporating molecular adjuvants and intrastructural T-cell help promote the immunogenicity of HIV membrane-proximal external region peptides

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Abstract

An HIV vaccine capable of inducing high and durable levels of broadly neutralizing antibodies has thus far proven elusive. A promising antigen is the membrane-proximal external region (MPER) from gp41, a segment of the viral envelope recognized by a number of broadly neutralizing antibodies. Though an attractive vaccine target due to the linear nature of the epitope and its highly conserved sequence, MPER peptides are poorly immunogenic and may require display on membranes to achieve a physiological conformation matching the native virus. Here we systematically explored how the structure and composition of liposomes displaying MPER peptides impacts the strength and durability of humoral responses to this antigen as well as helper T-cell responses in mice. Administration of MPER peptides anchored to the surface of liposomes induced MPER-specific antibodies whereas MPER administered in oil-based emulsion adjuvants or alum did not, even when combined with Toll like receptor agonists. High-titer IgG responses to liposomal MPER required the inclusion of molecular adjuvants such as monophosphoryl lipid A. Anti-MPER humoral responses were further enhanced by incorporating high-T_m lipids in the

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vesicle bilayer and optimizing the MPER density to a mean distance of ~10–15 nm between peptides on the liposomes' surfaces. Encapsulation of helper epitopes within the vesicles allowed efficient “intrastructural” T-cell help, which promoted IgG responses to MPER while minimizing competing B-cell responses against the helper sequence. These results define several key properties of liposome formulations that promote durable, high-titer antibody responses against MPER peptides, which will be a prerequisite for a successful MPER-targeting vaccine.

Keywords

Liposome; adjuvant; HIV vaccine; lipid membrane; MPER; nanoparticle

1. Introduction

Development of a prophylactic HIV vaccine remains of critical importance for world health despite the many challenges facing this objective [1,2]. Based on studies in large animal models, induction of high and durable levels of broadly neutralizing antibody (BNAbs) should provide sterilizing immunity against HIV [3], although thus far, strategies to induce such antibodies by vaccination remain elusive [4]. The only proteins expressed on the surface of the HIV virion are the Env subunits gp120 and gp41, which form heterotrimeric spikes. The high mutation rate, low density of Env protein trimers on each viral particle, and the masking of highly conserved epitopes of the Env protein via glycosylation have all hindered efforts to develop an HIV vaccine [5,6].

The membrane proximal external region (MPER) of gp41 is a particularly attractive vaccine target because it is a highly conserved linear peptide to which a number of BNAbs (e.g., 2F5, 4E10, 10E8 and Z13e1) bind [7–11]. One critical factor in MPER-targeting vaccine design is that this sequence comes just before the transmembrane region of gp41, and the conformation of the peptide is directly modulated by interaction with lipids. When associated with the surface of lipid micelles or membranes, MPER peptides form two hydrophobic helices connected by a flexible hinge region, and this unique conformation of membrane-displayed MPER substantially influences its binding to 2F5 and 4E10 [12–14]. These findings have motivated strategies aiming to present MPER sequences in a defined helical structure [15,16]. For example, gp41 peptides have been incorporated as constrained epitopes within protein scaffolds [17–21] or displayed on virus-like particles (VLPs) [22] and although some formulations have been weakly immunogenic [23], other constructs have been able to produce 2F5-like antibodies with some neutralizing and ADCC activity after multiple booster immunizations [24].

Scaffolded epitopes can present a defined configuration but lack the surrounding lipid environment of the native MPER which may impact elicitation of BNAbs with 2F5 or 4E10-like characteristics. Thus, a second approach to promote a native conformation of gp41 epitopes has been to display MPER peptides on lipid vesicles. Although MPER peptides are weakly immunogenic, liposomal vaccine formulations have generated antibody responses [25] which can be enhanced via multiple booster immunizations [26,27], administration with complete Freund’s adjuvant [28], or through optimizing anchorage of lipid-conjugated MPER molecules in the lipid membrane [29,30].

Considering the requirements of presenting MPER in a conformation-mimicking display on the virus and the need to enhance MPER immunogenicity, liposomal delivery of structure-guided peptide antigens is a particularly attractive vaccination strategy. The presentation of antigen in a nanoparticulate form increases lymphatic uptake, slows antigen clearance from lymph nodes, increases antigen capture by antigen presenting cells, and promotes B-cell receptor crosslinking [31,32]. Hydrophilic adjuvant compounds can be encapsulated in the interior of liposomes or anchored to the bilayer, while hydrophobic compounds can be embedded in the lipid bilayer. We recently reported that MPER anchored into a lipid membrane via a palmitoyl tail binds 2F5 and 4E10 and is more immunogenic than unmodified MPER absorbed onto lipid membrane surfaces [29], consistent with the findings of Watson et al. [30]. Here we evaluated the role of 3 key properties modulating the immunogenicity of MPER liposomal vaccines: liposome composition and size, inclusion of molecular adjuvants, and incorporation of intrastructural (within the same liposome) T-cell help.

2. Material and methods

2.1. Materials

Lipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phoshpo-(1′-rac-gylcerol) (DOPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)-2000] (DSPE-PEG-PDP) were purchased from Avanti Polar Lipids (Alabaster, AL). Solvents, bovine serum albumin (BSA) and monophosphoryl lipid A (MPLA, from *Salmonella enterica* serotype minnesota Re 595 cat. no. L6895) were purchased from Sigma Aldrich (St. Louis, MO). Ovalbumin (OVA) was purchased from Worthington Biochemical (Lakewood, NJ) and purified via detoxi-gel endotoxin removal columns (Pierce Biotechnology, Rockford, IL). Montanide ISA 51 VG was purchased from Seppic (Puteaux, France). AddaVax and cylic-di-GMP were purchased from Invivogen (San Diego, CA). Lipo-S-S-$(PEG)_4$-CpG (Lipid-PEG-CpG) was synthesized as previously described [33]. Briefly, CpG 1826 was synthesized using an ABI 394 DNA synthesizer (Applied Biosystems, Carlsbad, CA) on a 1.0 micromole scale, 5′ coupled to 4 repeats of DMT-hexaethyloxy-glycol phosphoramidite (Chemgenes, Wilmington, MA), thiol modifier C6 S-S (Glenres, Sterling, VA), and finally conjugated with lipid phosphoramidite (diacyl lipid phosphoramidite, synthesized according to published procedure [33]). After synthesis, lipid-PEG-CpG was purified by reverse phase HPLC. The $CD4^+$ T helper peptides LACK1 (ICFSPSLEHPIVVSGSWD) and HIV30 (RRNIIGDIRQAHCNISRAKW) and MPER peptide (ELDKWASLWNWFNITNWLWYIK) were synthesized at the Tufts University Core Facility (Boston, MA). MPER was purchased with either an N-terminal biotin (for ELISAs) or palmitoyl tail (for immunizations). For membrane-anchored DSPE-HIV30 conjugates, HIV30 was linked to DSPE-PEG-PDP via the cysteine residue of HIV30, by dissolving the peptide in DMF with 1.5 equivalents of DSPE-PEG-PDP and agitating at 25°C for 18 hours. The conjugate was then diluted in 10× deionized water, lyophilized into powder, and redissolved in deionized water. Peptide concentrations were determined by Direct Detect infrared spectroscopy analysis (EMD Millipore, Billerica, MA).

2.2. Liposome synthesis and characterization

A 4:1 molar ratio of DOPC:DOPG (for DOPC liposomes) or a 2:2:1 molar ratio of DMPC:DOPC:DOPG (for DMPC liposomes) in chloroform with or without 10 mol% of DSPE-PEG and palm-MPER added at a 1:200 MPER:lipid mole ratio was dried under nitrogen followed by incubation under vacuum at 25°C for 18 hr. Liposomes incorporating lipid-PEG-CpG, MPLA, or DSPE-HIV30 were prepared by including these components in the organic solution prior to drying lipid films. Lipids were hydrated with pH 7.4 PBS (containing 1 mg/mL LACK1 or 0.2 mg/mL HIV30 for soluble delivery of T-helper peptide) to a final concentration of 26.5 mM lipid and vortexed 30 seconds every 10 minutes for an hour. For 150 and 200 nm diameter liposomes, the resulting vesicles were passed through six freeze-thaw cycles between liquid nitrogen and a 37°C water bath followed by extrusion 21 times through 0.2μm or 0.4μm pore polycarbonate membranes (Whatman Inc, Sanford, ME), respectively. For 65 nm diameter liposomes, the lipid resuspension was subjected to 5 minutes of sonication alternating for 30 second periods between 10 watts and 3 watts output power on a Misonix XL-2000 probe sonicator. Efficiency of pMPER loading was determined by measuring fluorescence signal of liposomes loaded with a FITC-labeled pMPER before and after centrifugation via Airfuge (Beckman-Coulter). For c-di-GMP liposomes, a 38:38:19:5:0.95 molar ratio of DMPC:DOPC:DOPG:DSPE-PEG:c-di-GMP in chloroform was dried under nitrogen followed by incubation under vacuum at 25°C for 18 hr and following drying, the resulting lipid/c-di-GMP films were resuspended at 240 μg/mL of c-di-GMP in PBS and then freeze/thawed and extruded to form 150 nm liposomes. Unencapsulated c-di-GMP was removed by centrifugation of the liposomes via Airfuge (Beckman-Coulter) and quantification of c-di-GMP encapsulation efficiency was determined by UV absorption at 254 nm. Cryoelectron microscopy (Jeol 2100F TEM) and dynamic light scattering (Wyatt Dyna Pro Plate Reader II) were performed by the Swanson Biotechnology Center at the Koch Institute. For the calculation of the number of MPER peptides per liposomes, we utilized the following equation for the number of lipids (N_{Total}) per liposome, where a = surface area of a single phospholipid headgroup, h = bilayer width, and d = liposome diameter:

$$N_{Total} = \frac{4\pi\left[\left(\frac{d}{2}\right)^2\right] + 4\pi\left[\frac{d}{2} - h\right]^2}{a}$$

2.3. Mice and immunization protocol

Female BALB/c mice 6–7 weeks of age were purchased from Jackson Laboratories for all immunizations, and handled under federal, state, and local guidelines. Mice were immunized by injection of 100 μL MPER liposome solutions (40 μg MPER peptide) s.c. at the tail base, 50 μL on each flank, and boosted on days 21 and 42 with the same formulations. Where indicated, MPER liposomes included 17.5 μg MPLA or lipid-PEG-CpG as adjuvant. Alternatively, 100 uL cdGMP liposomes (5 μg cdGMP) were administered s.c. at the tail base, 50 uL per side, directly following MPER liposome injection. T-cell help was incorporated as 100 μg LACK1 peptide or 20 μg HIV30 peptide as indicated. Serum was collected weekly via retro-orbital bleeding for subsequent ELISA-based titer analyses. For

$CD4^+$ T-cell depletion studies, mice were injected i.p. with 0.3 mg anti-CD4 antibody (GK1.5, BioXCell) three days prior to, and 3 and 9 days after each immunization.

2.4. Immune responses analyses

For T-cell responses, mice were euthanized and 300,000 splenocytes in single cell suspensions were seeded into 96-well plates with or without 5 μM HIV30 peptide. Cells were incubated for 48 hours, and supernatants were collected for cytokine analysis using a Milliplex MAP mouse Th17 Magnetic Bead Kit from EMD Millipore (Billerica, MA) and the Bio-Plex 3D suspension array system from Bio-Rad (Hercules, CA). MPER-specific antibody levels were detected by ELISA: 96-well Nunc Polysorp plates (ThermoFisher) were coated with 25 μg/mL streptavidin (Jackson ImmunoResearch, West Grove, PA), blocked with 1% w/v BSA in PBS (BSA-PBS), washed with 0.05 Tween 20 in PBS, and incubated for 2 hours with 2 μg/mL biotin-MPER in BSA-PBS, washed, and then incubated for 2 hours with serially diluted serum samples. Following another washing step, the plates were incubated for 90 minutes with HRP-conjugated goat anti-mouse IgG (Bio-Rad) or anti-mouse IgM (Abcam) in BSA-PBS, washed, developed with TMB substrate, and read on a Tecan Infinite M200 Pro (Männedorf, Switzerland) absorbance plate reader. HIV30-specific antibody levels were detected via ELISA in a similar manner; plates were directly coated with 100 μg/mL of HIV30, blocked, washed, and incubated with serum and IgG-HRP as described above. Titers were defined as the inverse serum dilution giving an absorbance of 0.3. Frequencies of germinal center (GC) cells in inguinal and axillary lymph nodes were determined by flow cytometry analysis using a FACS Canto II (BD Biosciences) following staining with markers for GC cells (anti-B220, anti-GL-7, anti-IgD, peanut agglutinin (PNA) as well as 4′,6-diamidino-2-phenylindole (DAPI, for live-dead analysis). Data were analyzed using FlowJo software (TreeStar, Oregon, USA).

2.5 Histology

Inguinal lymph nodes were isolated from immunized mice and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA). 10 μm tissue sections were prepared by the Swanson Biotechnology Center at the Koch Institute, and imaged on a Zeiss LSM 510 confocal laser scanning microscope.

2.6 Statistics

Statistical analyses were performed using GraphPad Prism software. Comparisons of formulations over time employed two-way ANOVA tests and comparisons of multiple formulations at a single time point were performed using one-way ANOVA and Tukey's tests. Two-tailed unpaired t-tests were used to determine statistical significance between two experimental groups for all other data unless otherwise noted.

3. Results & Discussion

3.1. MPER delivered on liposomes is immunogenic while traditional aluminum- or oil-based adjuvants elicit no response to MPER peptides

We first compared several candidate lipophilic formulations as potential adjuvants for MPER peptides. Liposomes incorporating palmitoylated MPER (pMPER) were prepared by

lipid rehydration and extrusion; the hydrophobic pMPER was incorporated into PEGylated and non-PEGylated liposomes with efficiencies of 95±2.3% and 94±4.8%, respectively. Cryoelectron microscopy imaging of 150 nm diam. liposomes revealed the presence of unilamellar and multilamellar vesicles and incorporation of pMPER had no impact on liposome structure (Fig. 1A). In addition to liposomes, water/oil emulsions are classical adjuvants [34], which could potentially adsorb the hydrophobic pMPER to promote uptake by immune cells. We thus compared the immunogenicity of pMPER carried by liposomes, an oil-in-water emulsion (AddaVax), or a water-in-oil emulsion (Montanide), and also compared to the traditional adjuvant alum. To provide further adjuvant activity, the TLR-4 agonist monophosphoryl lipid A (MPLA) was added to all of the formulations (liposomes, AddaVax, and Montanide) in this experiment as a molecular adjuvant [35]. Strikingly, even in the presence of MPLA, only liposomes primed anti-MPER IgG responses in all animals (Fig. 1B). Immunization with liposomes carrying lipid-anchored MPER and helper peptide without additional molecular adjuvants elicited a detectable but very weak immune response, but MPER liposomes adjuvanted with several different molecular danger signals–MPLA, lipid-PEG-CpG, or co-administered liposomes containing the STING agonist cyclic-di-GMP (cdGMP) [36–38]–all primed 600–4600-fold higher anti-MPER titers (Fig. 1C). Note that to separately control the MPER and cdGMP doses it was necessary to co-deliver cdGMP in separate liposomes. Based on these data showing equivalence of MPLA and CpG and elevated responses elicited by the cyclic dinculeotides, we opted to focus on MPLA (as representative of an agonist already used in licensed vaccines) and cdGMP (encapsulated in separate liposomes) as adjuvants for further studies. Antibody responses raised by these MPER liposome vaccines supplemented with helper peptides appeared to be classically T-help-dependent, as IgG titers following immunization were reduced by >90% in $CD4^+$ T-cell-depleted animals (Supplementary Fig. 1).

3.2. Magnitude of MPER-specific antibody responses is controlled by liposome physical properties

Poly(ethylene glycol) (PEG) coatings are routinely used in drug delivery to limit liposome aggregation/fusion and enhance their penetration through tissue [39], and thus might promote trafficking of MPER liposomes to draining lymph nodes. However, because the MPER sequence embeds into the lipid membrane, PEGylation of MPER liposomes would also be expected to hinder antigen-specific B-cell engagement with the peptide. PEGylation has variously been reported to facilitate draining to lymph nodes [40] as well as to have no impact on lymph node draining [41]. We found that four hours after immunization with liposomes co-labeled with the lipid dye DiD and encapsulated AlexaFluor-conjugated ovalbumin (AF-OVA), both PEGylated and non-PEGylated liposomes were readily detected in both the primary (inguinal) and secondary (axillary) draining lymph nodes via IVIS-based fluorescent imaging of whole lymph nodes and confocal microscopy of histological lymph node sections (Fig. 2A–B). DiD (green) and AF-OVA (red) signals were co-localized in the subcapsular sinus of inguinal lymph nodes, suggesting that liposomes were still intact four hours post-immunization (Fig. 2A). Quantification of AF-OVA signal detected from whole lymph nodes via IVIS indicated that PEGylated liposomes trended toward increased accumulation in lymph nodes relative to non-PEGylated vesicles, but this did not reach

statistical significance (Fig. 2B). However, this slight enhancement of vesicle trafficking achieved by PEGylation had no impact on immunogenicity (Fig. 2C).

Particle size plays a key role in vaccine responses, though optimal dimensions are likely dependent on particle composition and the type of immune responses desired [42]. In parallel to the liposome draining analyses, vesicles with mean diameters of 203±27 nm, 150±12 nm, or 64.5±5 nm were tested as MPER/MPLA delivery vehicles. Interestingly, immunization with the smallest 65 nm liposomes induced 20-fold and 15-fold lower MPER-specific IgG titers than 150 nm and 200 nm liposomes, respectively (Fig. 2D). This inverse relationship between immunogenicity and liposome size is in agreement with prior studies of flexible liposomes in the size range of 40 – 400 nm, where vesicle size was inversely correlated with drainage from the injection site but positively correlated with retention in draining lymph nodes [43].

The immunogenicity of protein or hapten antigens delivered by liposomes has been shown previously to be inversely proportional to membrane fluidity [44–46], and we expected a similar trend due to more stable anchoring of pMPER to vesicles with more rigid bilayers. In agreement with these predictions, immunization of mice with low-fluidity 2:2:1 DMPC:DOPC:DOPG liposomes (Average T_m = 6.7°C) or fully-fluid 4:1 DOPC:DOPG liposomes (Average T_m = −8.1C) showed that DMPC-containing vesicles elicited an average 6.2-fold higher titer than DOPC-only liposomes (Fig. 2E). We sought to test liposome compositions with higher mean melting temperatures, but found that pMPER peptides were unstable in lipid membranes with melting temperatures above 37°C (data not shown).

3.3. Humoral responses are maximized by high-density MPER display on vesicles

Multivalent surface-display of antigen on particulates is known to enhance antibody responses, presumably by promoting BCR aggregation [47,48]. To understand how the surface density of antigen impacts pMPER immunogenicity, we immunized mice with a fixed total dose of antigen delivered on liposomes carrying varying quantities of pMPER per liposome. Serum antibody titers post-boosting indicated that 200 pMPER per liposome initially generated the highest MPER-specific IgG levels, but densities ranging from 40 to 1000 pMPER per liposome were all capable of generating high titers by day 50 (Fig. 3A). Interestingly, the highest antigen density tested, 2000 MPER/liposome, elicited substantially lower titers, suggesting that the optimal mean distance between peptides on the liposome surface is approximately 7 – 17 nm (Fig. 3B).

3.4. Immunization with soluble or lipid-anchored T-helper peptide reduces off-target antibody responses

Preliminary studies suggested that exogenous helper epitopes enhanced the humoral response against MPER peptides in balb/c mice [29], and thus our immunizations included either the model H-2^d-restricted LACK1 helper peptide (derived from Leishmania [49]) or HIV30, a gp120-derived peptide known to be presented by both H-2^d in balb/c mice and HLAs of humans [50]. In order to maximize $CD4^+$ T-cell help through co-delivery of MPER and helper epitopes to B-cells while avoiding off-target antibody responses against the

helper peptide, we tested three different methods for incorporating helper peptides into liposomes (Fig. 4A): Palmitoylated HIV30 anchored to the lipid bilayer ("palm HIV30"); HIV30 anchored through a disulfide linkage to a PEG tether on the bilayer ("uncleaved HIV30"); or disulfide/PEG-linked HIV30 cleaved from the external surfaces of the vesicles, leaving it incorporated only on the interior of the liposomes ("cleaved HIV30"). The latter formulations were obtained by exposing uncleaved HIV30 liposomes to the membrane-impermeable reducing agent TCEP, followed by removal of free peptide. We first compared the T-cell priming capacity of these 3 modes of intrastructural HIV30 co-delivery, vs. soluble HIV30 mixed with liposomes. Splenocytes from animals immunized with all 3 forms of MPER/HIV30 liposomes and restimulated *ex vivo* with HIV30 peptide secreted IL-4 (Fig. 4B) and IL-5 (Fig. 4C), but liposomes containing PEG-anchored HIV30 (cleaved or uncleaved) generated stronger TNF-α responses(Fig. 4D). Minimal T-cell responses were detectable against the MPER peptide itself (data not shown).

We next confirmed that HIV30 could enhance antibody responses to MPER, using the cleaved HIV30 form to co-incorporate the helper peptide into MPER liposomes. As shown in Fig. 5A, induction of anti-MPER IgG following immunization with MPER liposomes adjuvanted by co-delivered cdGMP liposomes was dependent on the presence of HIV30, confirming importance of the T-helper epitope. To compare the relative effectiveness of the 3 modes of helper epitope incorporation, mice were immunized with pMPER liposomes containing palm HIV30, uncleaved HIV30, cleaved HIV30, or pMPER liposomes co-administered with unencapsulated soluble HIV30; each vaccine was adjuvanted with cdGMP liposomes. While all 4 formulations elicited strong anti-MPER titers at 7 days post-boost, soluble HIV30 and cleaved HIV30 minimized off-target anti-HIV30 IgG titers (Fig. 5B). Flow cytometry-based assessment of germinal center B cell populations at 7 days post-boost indicated that all 4 formulations primed substantial germinal center B-cell populations (Fig. 5C). These results suggest that cleaved HIV30 offers the optimal balance between strong humoral MPER-specific responses, weak off-target antibody generation and strong induction of Th_1- and Th_2-associated cytokines.

4. Conclusions

MPER is a highly hydrophobic sequence near the transmembrane domain of gp41, which exhibits substantially enhanced immunogenicity when displayed on the surface of liposomes. In order to design a potent MPER vaccine, we systematically optimized three critical elements of a liposomal MPER vaccine: liposome properties, adjuvant incorporation, and T-cell help. The present studies demonstrate that humoral responses to liposomal MPER vaccines are impacted by all three of these components. Importantly, anti-MPER antibody responses are potently adjuvanted by the inclusion of TLR-4, TLR-9, or STING agonists, and are maximized by incorporation of high surface densities of MPER and the inclusion of high melting temperature lipids. The data suggest that 150 nm diameter, 2:2:1 DMPC:DOPC:DOPG liposomes adjuvanted with MPLA or cdGMP and incorporating encapsulated T-cell helper epitopes achieve strong and durable antibody responses against gp41 MPER peptides, while limiting competing humoral responses against the helper sequence itself. Our studies of the biochemistry of antigen recognition carried out in parallel to the formulation studies described here showed that the MPER peptides tested here do not

elicit neutralizing antibodies against HIV [29]. Epitope mapping suggested this is due to an immunodominant response to residues at the free end of the peptides, which preliminary data suggests can be alleviated by incorporation of transmembrane segments of gp41 [29]; ongoing studies are testing such structures. Irrespective of these immunogen structure issues, the results reported here provide a clear guide to enhancing the immunogenicity of these and other novel MPER constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Koch Institute Swanson Biotechnology Center for technical support. This work was supported in part by the Bill & Melinda Gates Foundation and the NIH (AI091693). DJI is an investigator of the Howard Hughes Medical Institute.

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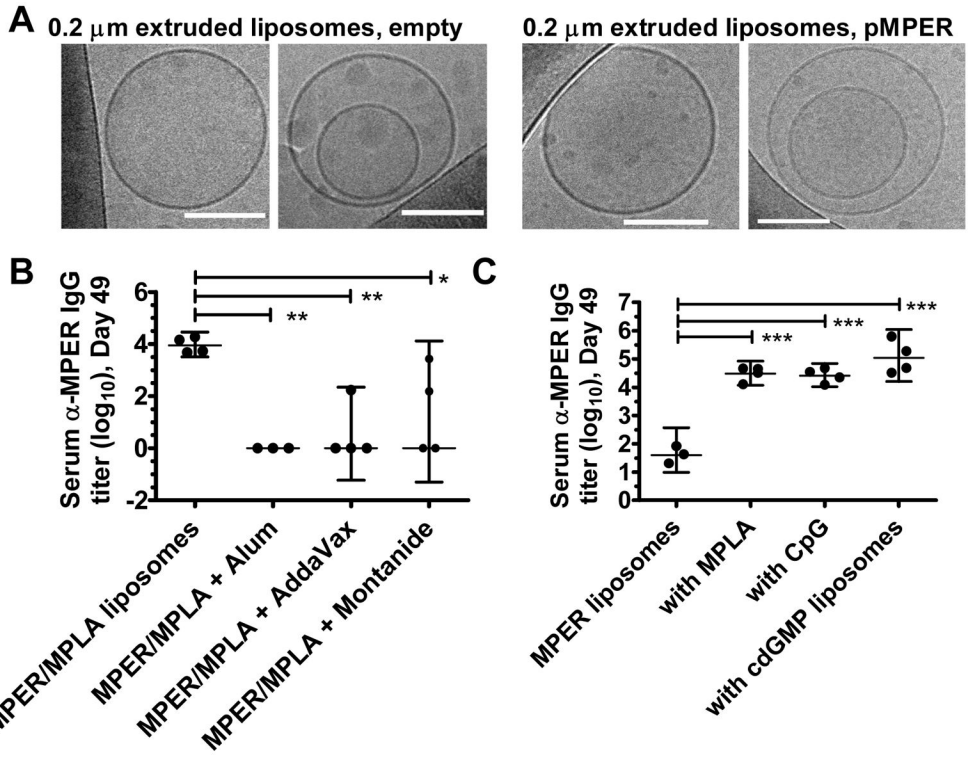

Fig. 1.

Humoral responses against MPER peptides are promoted by liposomal delivery and molecular adjuvants. (A) Representative cryoelectron microscopy images of 150 nm mean diameter DOPC/DOPG liposomes with or without incorporated pMPER, scale bars 100 nm. (B–C) Groups of balb/c mice (*n*=3–4/group) were immunized on days 0, 21, and 42 with 40 μg pMPER peptide. (B) Anti-MPER serum IgG titers following immunization with pMPER delivered by liposomes, alum, or oil-based emulsions together with 100 μg soluble LACK1 helper peptide and 17.5 μg MPLA per injection. (C) Serum anti-MPER IgG titers following immunization with pMPER on 150 nm diameter liposomes adjuvanted with MPLA, lipid-PEG-CpG, or co-delivered cyclic-di-GMP (cdGMP) liposomes. To provide $CD4^+$ T-cell help, formulations contained 20 μg of encapsulated HIV30. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as determined by ANOVA followed by Tukey's multiple comparison test.

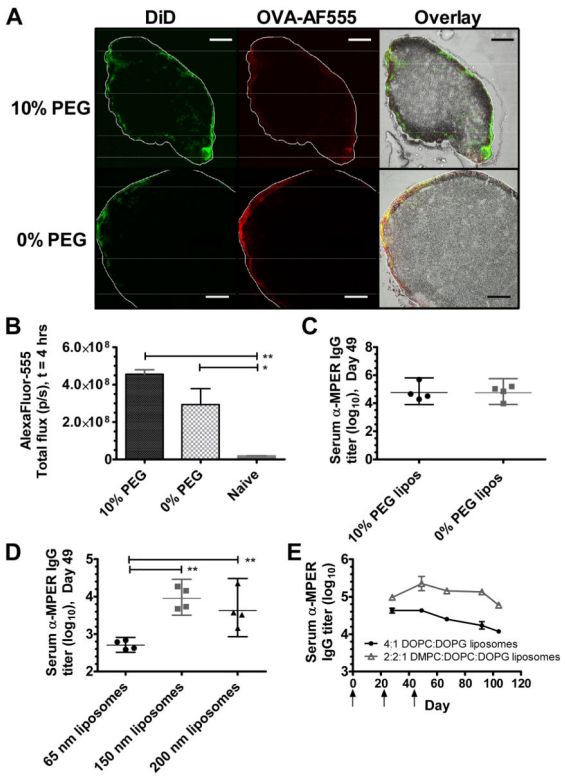

Fig. 2.

Anti-MPER humoral responses are shaped by liposome composition and particle size. (A–B) Balb/c mice (*n*=3/group) were injected with 150 nm diameter pMPER liposomes with or without 10 mol% PEG-DSPE containing DiD lipid and OVA-AlexaFlour-555, followed by histological sectioning (red = OVA-AF555, green = DiD, 10× magnification, scale bar = 200 μm) of inguinal nodes (A) and IVIS-based fluorescence quantification of axillary and inguinal nodes at 4 hours post-immunization (B). *, $p < 0.05$; **, $p < 0.01$ as determined by Dunnett's test. (C) Anti-MPER titers at day 49 for balb/c mice (*n*=3/group) immunized as in (A) with pMPER/MPLA liposomes and LACK1 helper peptide on days 0, 21, and 42. (D) Serum anti-MPER IgG titers at one week after the third immunization in mice immunized with 65, 150 or 200 nm diameter PEGylated pMPER /MPLA liposomes and LACK1 helper peptide (** = p value < .01). (E) Mice were immunized as in (D) with 150 nm diameter PEGylated liposomes with a bilayer composition comprised of 4:1 DOPC:DOPG lipids or 2:2:1 DMPC:DOPC:DOPG lipids and serum MPER titers were followed over time; $p < 0.0001$ by two-way ANOVA.

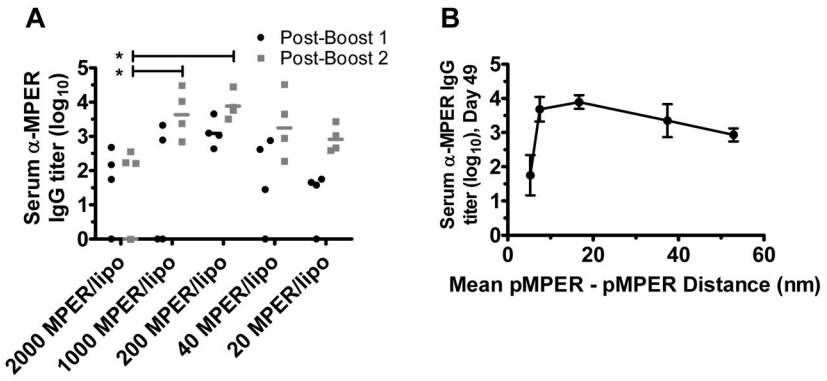

Fig. 3.

Anti-MPER humoral responses are maximized by liposomes carrying peptide with a mean spacing near 10 nm. Groups of balb/c mice (*n*=4/group) were immunized with PEGylated MPER/MPLA liposomes bearing 20–2000 peptides per vesicle on average, mixed with LACK1 helper peptide. (A) Dependence of serum anti-MPER IgG titers on the density of antigen per liposome. (B) Correlation of serum anti-MPER IgG titer and the mean distance between pMPER antigens on the liposome surfaces. *, $p < 0.05$ as determined by ANOVA followed by Tukey's multiple comparison test.

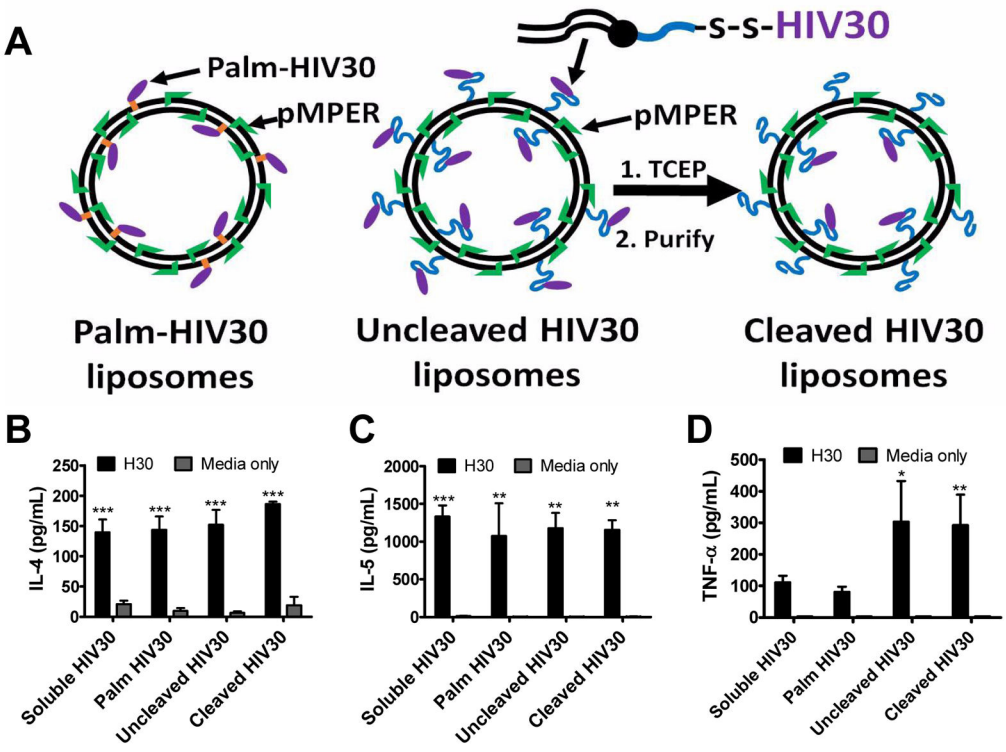

Fig. 4.

Liposomes carrying encapsulated bilayer-anchored helper peptide stimulate both Th1 and Th2 cytokine production from antigen-specific T-cells. (A) Schematic of 3 forms of T-helper epitope incorporation in MPER liposomes. (B–D) Groups of balb/c mice (*n*=3/group) were immunized as in Fig. 1 with 150 nm diameter pMPER liposomes containing the T-helper peptide HIV30 either in soluble form or incorporated into the MPER liposomes via a palmitoyl anchor or DSPE-PEG-S-S-HIV30 (cleaved or uncleaved) and all groups were adjuvanted by mixing with cdGMP liposomes. On day 49, splenocytes were isolated and restimulated *ex vivo* for 48 hours in the presence of 5 μM HIV30 peptide and concentrations of (B) IL-4, (C) IL-5, and (D) TNF-α, were determined via bead-based ELISA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for HIV30 vs. media only restimulation conditions as determined by two-way ANOVA and Bonferonni post-test.

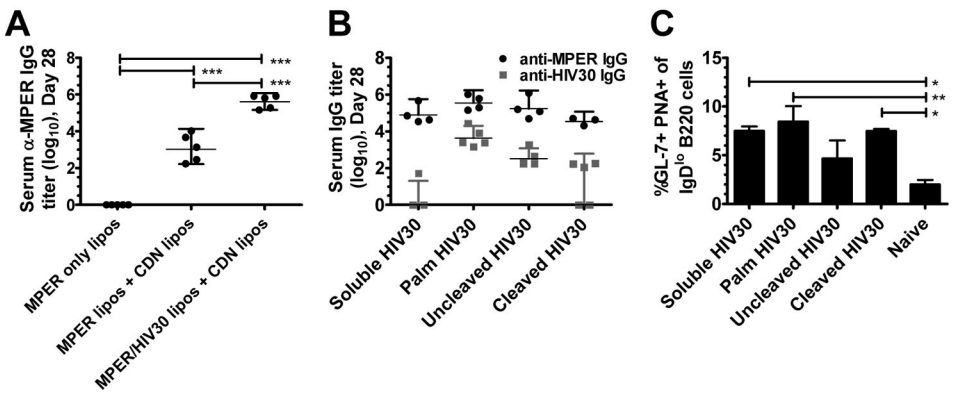

Fig. 5.

Liposomes with surface-displayed pMPER and encapsulated HIV30 promote strong B-cell responses against MPER while minimizing off-target responses against the helper epitope. (A) Balb/c mice (*n*=5/group) were immunized with cdGMP liposomes mixed with 150 nm diam. pMPER liposomes with or without cleaved DSPE-PEG-S-S-HIV30 helper epitope on days 0 and 21, and serum IgG titers were measured on day 28. ***, p value < 0.001 (B–C) Balb/c mice (*n*=3–4/group) were immunized as in Fig. 1 with 150nm MPER liposomes containing the T-helper peptide HIV30 either in soluble form or incorporated into the MPER liposomes via a palmitoyl anchor or DSPE-PEG-S-S-HIV30 (cleaved or uncleaved); all groups were adjuvanted with co-delivered cdGMP liposomes. Shown are serum anti-MPER and anti-HIV30 IgG titers at seven days post-boost 1 (B) and frequencies of germinal center B-cells in draining lymph nodes at 7 days post-boost 2 (C). *, $p < 0.05$; **, $p < 0.01$ as determined by Dunnett's comparison to control test.