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## Design of Lipid Nanocapsule Delivery Vehicles for Multivalent Display of Recombinant Env Trimers in HIV Vaccination

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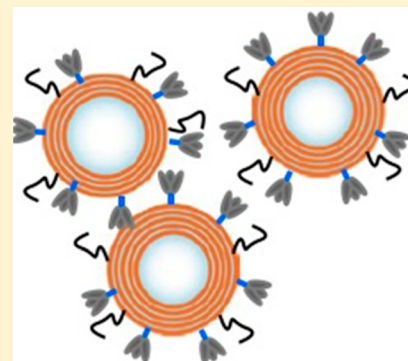
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**ABSTRACT:** Immunization strategies that elicit antibodies capable of neutralizing diverse virus strains will likely be an important part of a successful vaccine against HIV. However, strategies to promote robust humoral responses against the native intact HIV envelope trimer structure are lacking. We recently developed chemically cross-linked lipid nanocapsules as carriers of molecular adjuvants and encapsulated or surface-displayed antigens, which promoted follicular helper T-cell responses and elicited high-avidity, durable antibody responses to a candidate malaria antigen. To apply this system to the delivery of HIV antigens, Env gp140 trimers with terminal histags (gp140T-his) were anchored to the surface of lipid nanocapsules via Ni-NTA-functionalized lipids. Initial experiments revealed that the large (409 kDa), heavily glycosylated trimers were capable of extracting fluid phase lipids from the membranes of nanocapsules. Thus, liquid-ordered and/or gel-phase lipid compositions were required to stably anchor trimers to the particle membranes. Trimer-loaded nanocapsules combined with the clinically relevant adjuvant monophosphoryl lipid A primed high-titer antibody responses in mice at antigen doses ranging from 5  $\mu$ g to as low as 100 ng, whereas titers dropped more than 50-fold over the same dose range when soluble trimer was mixed with a strong oil-in-water adjuvant comparator. Nanocapsule immunization also broadened the number of distinct epitopes on the HIV trimer recognized by the antibody response. These results suggest that nanocapsules displaying HIV trimers in an oriented, multivalent presentation can promote key aspects of the humoral response against Env immunogens.



### INTRODUCTION

HIV ranks as one of the deadliest infectious diseases among adults and is the fourth leading cause of death worldwide. Globally, acquired immune deficiency syndrome induced by HIV infection has killed more than 25 million people (<http://www.unaids.org>). UNAIDS estimates that there are about 33 million people living with HIV/AIDS worldwide, including approximately 1.1 million people in the U.S. alone. Despite the availability of potent antiretroviral drug regimens,<sup>†</sup> these staggering statistics show that the epidemic continues unabated, underscoring the need for a potent and efficacious vaccine. Immunization strategies that elicit antibodies capable of neutralizing diverse strains of the virus will be an important part of a successful vaccine against HIV.

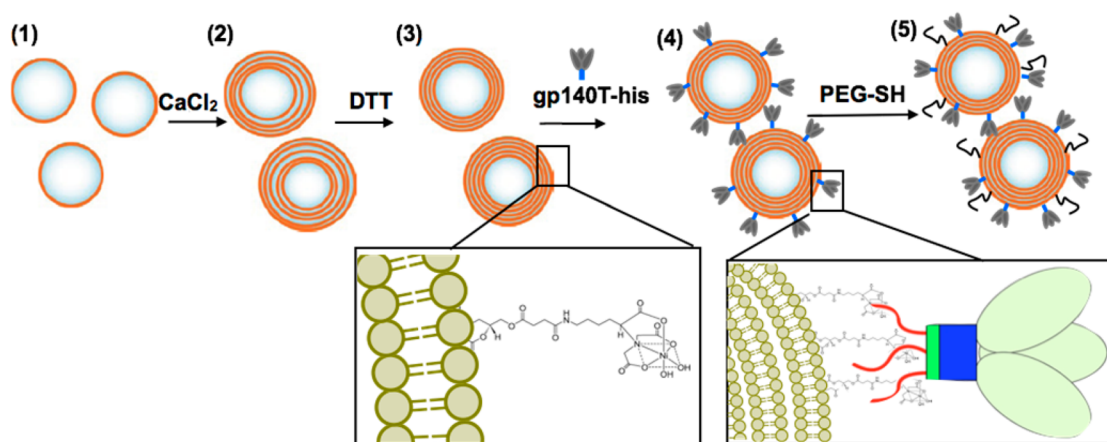
The HIV envelope glycoprotein trimer, Env, mediates virus attachment and fusion to host cells and consists of 2 subunits—the docking portion, gp120, and the transmembrane portion required for fusion, gp41.<sup>2,3</sup> Env is the only neutralizing target on the virus,<sup>4,5</sup> but current vaccine strategies have failed to elicit

the type of durable, high avidity, broadly neutralizing humoral responses that can confer sterilizing protection against diverse circulating strains of HIV. Most vaccine candidates targeting the envelope glycoprotein have used the monomer form of the docking subunit. Such monomeric gp120 vaccines have failed to induce broadly neutralizing antibodies in preclinical studies and clinical trials.<sup>6</sup> Further, antibodies elicited by monomeric gp120 bind epitopes that are poor neutralization targets and are potentially occluded on primary HIV isolates.<sup>7,8</sup> Several conserved targets for antibodies against the Env proteins are quaternary epitopes present only in the trimeric, glycosylated native form of the envelope spike.<sup>3,8,9</sup> Strategies to promote durable, high avidity antibody responses against the native intact trimer structure are lacking, and the production of stable gp120/gp41 trimers has proven difficult. However, the

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**Figure 1.** Synthesis of ICMV lipid nanocapsules loaded with HIV env trimers. (1) Anionic maleimide-functionalized liposomes, including Ni-NTA-DGS are prepared from dried lipid films, (2) divalent cations are added to induce fusion of liposomes and the formation of MLVs, (3) membrane-permeable DTT is added, which cross-links maleimide lipids on apposed lipid bilayers in the vesicle walls at 37 °C, (4) post-vesicle recharging with NiCl<sub>2</sub>, followed by addition of gp140T-his, and (5) resulting lipid particles are PEGylated with thiol-terminated PEG.

ectodomain protein, gp140, has been used as an Env surrogate,<sup>8</sup> and use of soluble trimeric gp120 or trimeric gp140 in vaccines has shown better promise at inducing neutralizing antibodies relative to monomeric immunogens.<sup>8,10–15</sup> Recently, we described the preparation of stable and high-quality gp140 trimers (gp140T), which elicited higher titers of potent cross-clade neutralizing antibody responses compared to corresponding gp120 monomers for a diverse set of tier 1 and tier 2 viruses.<sup>8</sup>

Although soluble proteins mixed with adjuvants are most commonly used in vaccination, delivery of antigens arrayed on synthetic micro- and nanoparticles has shown clear advantages over immunization with soluble immunogens in preclinical animal models.<sup>16–18</sup> Particulate delivery promotes antigen uptake by antigen presenting cells (APCs), allows for physiological display of antigens on membranes, and provides multivalency, promoting B-cell triggering.<sup>19–22</sup> In addition, this approach allows for the simultaneous codelivery of antigens and adjuvants to B-cells and APCs, which can enhance vaccine efficacy even at low antigen doses, as compared to soluble antigens.<sup>23–27</sup>

We recently described the production of chemically cross-linked lipid nanocapsules termed ICMVs (interbilayer-cross-linked multilamellar vesicles) as carriers of molecular adjuvants and encapsulated or surface-displayed antigens, which promoted follicular helper T-cell responses and elicited high-avidity, durable antibody responses to a candidate *Plasmodium vivax* malaria antigen.<sup>19,23</sup> Here, we utilize this nanocapsule system to display membrane-anchored trimeric HIV Clade A gp140, mimicking native viral display of the envelope glycoprotein in a format where molecular adjuvants could be readily coloaded in the capsule walls or entrapped in the aqueous core. To stably anchor gp140T to the surfaces of these particles under conditions preserving the antigenic integrity of the trimers, we utilized noncovalent binding of polyhistidine-tagged proteins to nitrilotri-acetic acid (NTA)-headgroup lipids.<sup>28–30</sup> Because NTA-Ni(II)-His binding is site specific, the physical orientation of the protein on the nanoparticle surface is controlled. Compositions of ICMV capsules capable of stably anchoring the heavily glycosylated, half-megadalton trimers were determined, and the immunogenicity of trimer-conjugated nanocapsules compared to soluble trimers adju-

vanted with a very strong oil-in-water emulsion was determined in mice. Importantly, nanocapsules elicited strong class-switched antibody responses to a larger range of epitopes with a steadily increasing avidity of trimer-binding antibody over 90 days, which was not replicated by soluble protein vaccines. These results suggest that nanoparticles can be formulated to display large HIV Env trimers in an oriented, multivalent presentation, and can promote a durable and broad immune response against Env immunogens.

## RESULTS AND DISCUSSION

**Binding of High Molecular Weight gp140T-his to Low T<sub>m</sub>-Lipid Nanocapsules.** Generating a durable, potent broadly neutralizing antibody (BNAb) response is the ultimate goal of HIV vaccines targeting humoral immunity.<sup>31,32</sup> Potent circulating BNABs could potentially prevent the initial binding of HIV to its host cell, thereby ensuring sterilizing immunity. Early efforts at development of HIV vaccines exclusively employed monomeric forms of the envelope proteins gp120 (there is no stable monomeric form of gp41 available),<sup>33,34</sup> but such antigens lack quaternary epitopes found only in the trimeric form of the envelope spike, and further expose faces of the envelope proteins that are irrelevant to the intact native trimer.<sup>39</sup> Generation of stable trimers of Env for use as immunogens has proven challenging, but several groups have recently made progress in this area.<sup>8,10–15,35,36</sup> We recently successfully synthesized trimeric forms of the ectodomain of gp140 from sequences obtained from an array of primary isolates,<sup>8</sup> enabling the testing of such an antigen for vaccination. Initial immunization studies using soluble protein in adjuvant suggested an ability of these trimeric proteins to elicit broader HIV-neutralizing antibody responses compared to monomeric gp120 proteins.<sup>8,14</sup>

Nanoparticles have been shown to enhance humoral vaccine responses as compared to their soluble counterparts, in terms of quantity, quality, and breadth of response.<sup>19,27,37</sup> For lipid-enveloped pathogens, the use of lipid-based nanoparticles further provides the ability to mimic antigen display on the lipid membrane of the target virus. To this end, we utilized lipid nanocapsules we recently described, ICMVs, where the capsule walls are composed of stacked lipid bilayers chemically “stapled” bilayer-to-bilayer via covalent cross-links between

lipid headgroups of adjacent bilayers.<sup>23</sup> We hypothesized that the enhanced stability of ICMVs relative to traditional liposomes would make them more effective for delivery of surface-displayed trimer antigens, and additionally could provide the option of incorporating high levels of molecular adjuvants in the capsule walls or aqueous particle cores. To provide oriented display of gp140 trimers on the surface of these lipid nanocapsules, we took advantage of the 6 histidine residues at the base of each “leg” of the trimer (originally incorporated to facilitate purification of the protein), and utilized these to multivalently anchor gp140T to Ni-NTA-functionalized lipids incorporated in the particle bilayers (Figure 1). Based on our previous successful nanocapsule vaccines carrying malaria antigens or ovalbumin<sup>23</sup> we first prepared ICMVs comprising the low- $T_m$  lipids DOPC, MPB (maleimide-headgroup lipid for interbilayer cross-links), and Ni-NTA-DGS (Ni-NTA-headgroup lipids for histidine-protein capture) in a 4:5:1 mol ratio (Figure 1 step 1). Lipid capsules were formed by fusing small unilamellar DOPC/MPB/Ni-NTA-DGS vesicles via addition of calcium (step 2), followed by addition of DTT as a membrane-permeable dithiol to introduce interbilayer cross-links at 37 °C (step 3). Ni-NTA-ICMVs containing 5% Ni-NTA-DGS were synthesized with yields similar to DOPC/MPB-only capsules, and had a mean size of  $374 \pm 27$  nm diameter by dynamic light scattering. To anchor protein to the nanocapsules, fluorescently tagged gp140 trimer with histags (gp140T-his) was incubated with Ni-NTA-ICMVs at 4 °C for 18 h (step 4), followed by a final PEGylation step using thiol-terminated PEG to cap any remaining maleimide groups at the surfaces of the particles (step 5). As controls, ICMVs loaded with his-tagged GFP were constructed in parallel. Although ICMVs showed effective binding of his-tagged GFP, the high molecular weight trimer molecules showed a low efficiency of binding to the particles at any protein/nanocapsule ratio tested (Table 1). This low level of

**Table 1. Binding of GFP-his and gp140T-his to Low- $T_m$  Lipid ICMV Nanocapsules**

	HIV gp140 trimer - his			GFP-his
molecular weight		408 kDaA		28 kDaA
conc. of protein added (ug/mL)	50	75	150	150
% protein bound to particle	$6.5 \pm 1.8$	$6.0 \pm 2.7$	$7.3 \pm 1.5$	$54 \pm 5$

binding was observed despite calculations of available NTA groups and capsule surface area suggesting that these ~375-nm-diameter capsules should be capable of accommodating ~570 trimers per particle, and therefore have a maximal capacity of ~250  $\mu$ g trimer, a 1.7-fold excess of theoretical capacity over the total amount of trimer added. ICMVs lacking Ni-NTA lipid showed low GFP-his binding (<5% protein binding), indicating his-tag specificity of the protein capture by the particles. The effective binding of his-GFP confirmed the functionality of Ni-NTA groups displayed at the surfaces of ICMVs.

The efficiency of gp140T-his protein binding was only modestly increased by post-insertion of additional Ni-NTA-DGS into the capsule surfaces (Figure 2A). The low concentration of DTT used to cross-link the lipid nanocapsule bilayers induced some reduction of NTA-bound Ni (evidenced by a change in color of the particle pellets from blue toward brown), but this mild reduction did not affect his-GFP binding and recharging of nickel on the chelator groups of the particles

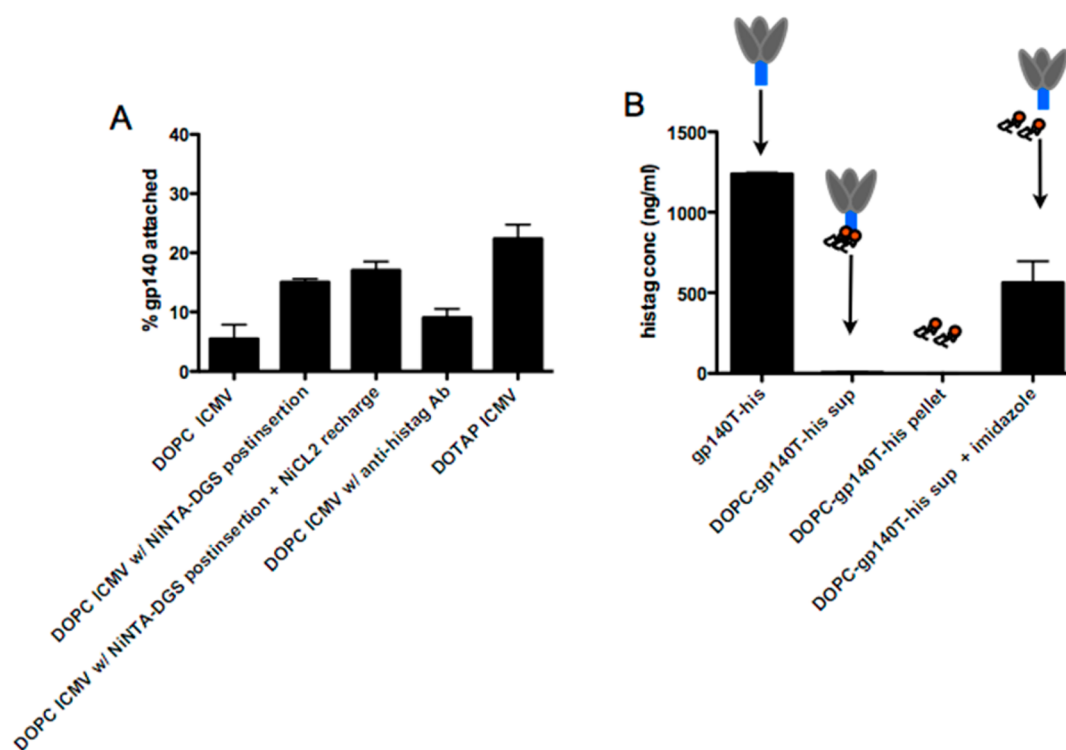
just prior to protein addition failed to improve trimer loading (Figure 2A). Notably, we also found that attempting to load trimers via an alternative noncovalent strategy, such as using an anti-histag Ab to capture the trimers or using electrostatic adsorption of trimers to cationic ICMVs formed with DOTAP lipids (and thus sacrificing control over protein orientation), also failed to give yields of more than 20% of added trimer bound to the capsules (Figure 2A).

To understand the relative failure of trimer loading we examined the state of the histidine tags on Alexafluor-labeled gp140T-his proteins before and after attempted ICMV loading, to rule out possible proteolytic cleavage of the anchoring residues. Alexafluor-labeled gp140T-his was added to Ni-NTA-ICMVs and incubated for 8 h, then particles were centrifuged and separated from the supernatant. ELISA detection of the histidine tags on the protein was performed on the supernatants, protein recovered from detergent-lysed ICMV capsules, and control neat gp140T-his protein. As shown in Figure 2B, histidines were detected on the control protein as expected. In addition, low binding of the trimer to particles yielded low levels of histag detection following pelleting and lysis of the particles with Triton (DOPC-gp140T-his pellet). Unexpectedly, however, trimer histags also failed to be detected in the particle/trimer supernatants (~4.42 ng/mL, Figure 2B), despite the presence of substantial amounts of trimer protein in these sups as detected by fluorescence from the Alexafluor label. However, when imidazole, which competes with histidine for Ni-NTA binding, was added to the supernatants, histidines of the trimer protein were again detected by ELISA (Figure 2B “+ imidazole”). This result suggested that trimer protein in the supernatant had extracted Ni-NTA lipids from the nanocapsules into solution, and these lipids remained bound to the trimer and blocked access of the his-tags to detection antibodies in the ELISA measurements.

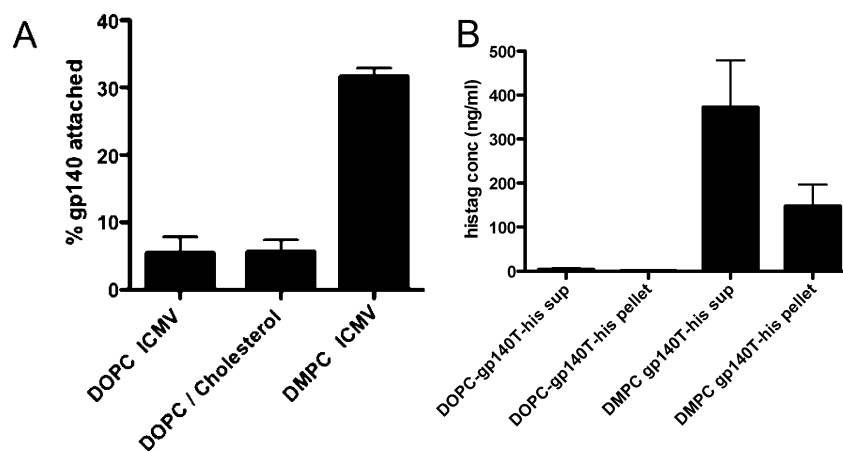
#### Anchoring of gp140T-his to High- $T_m$ Lipid Capsules.

The use of noncovalent chemical conjugation methods to display antigen on particles has been shown previously to enhance T-cell and antibody responses to subunit vaccines.<sup>29,30,38</sup> Of note, such binding of proteins to the surface of liposomes has typically involved proteins of substantially lower molecular weight than the env trimer. The synthetic gp140 trimer has a molecular weight of 409 kDa; we hypothesized that this large molecular weight combined with the heavy glycosylation of the protein trimer was responsible for extraction of bound Ni-NTA lipids from the fluid phase bilayer of the capsule surfaces. In order to increase the conjugation efficiency of the large trimer to the particles while continuing to use the site-specific Ni-NTA metal chelation method, we tested stabilizing lipid anchorage in low- $T_m$  DOPC-containing ICMVs by addition of cholesterol, or alternatively exchanged DOPC for a higher  $T_m$  lipid, DMPC ( $T_m$  23 °C). As shown in Figure 3A, incorporation of cholesterol into DOPC nanocapsules failed to stabilize trimer anchoring. However, DMPC lipid nanocapsules showed a substantial increase in gp140T-his binding, with a 9-fold increase in stable trimer binding over DOPC capsules (Figure 3A). Following incubation with Ni-NTA-bearing DMPC capsules, unbound trimer protein remaining in the supernatant retained accessible his-tags by ELISA analysis, consistent with the idea that lipid was not being extracted from the higher- $T_m$  lipid particles by the trimers (Figure 3B). Despite the modest total binding efficiency of 35%, this degree of protein loading translates to approximately 160 trimers per nanocapsule, giving a mean





**Figure 2.** HIV gp140T-his trimer binding to low- $T_m$  lipid ICMVs. (A) Percentages of gp140T-his bound by ICMVs prepared from distinct compositions chosen to enhance trimer loading. Various formulations to stabilize the protein on the nanoparticle vesicle (B) Accessibility of trimer histags following incubation of gp140T-his with Ni-NTA-ICMVs was assessed by measuring the concentration of histags by ELISA.

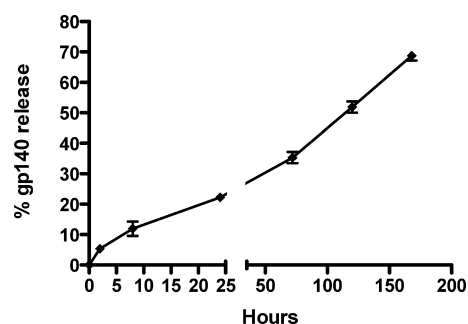


**Figure 3.** HIV env trimer conjugation to ICMVs stabilized by incorporation of cholesterol or high- $T_m$  lipids. (A) Percentage of added gp140T-his bound to ICMVs of different compositions. (B) Histag ELISA to determine the availability of histidine residues following attachment to high  $T_m$  lipid vesicles.

spacing between trimers on the particle surfaces of 33 nm. To determine the stability of multivalent Ni-NTA anchoring of trimers to the DMPC particles, we measured trimer release from ICMVs over 7 days at 37 °C. The protein bound stably to ICMVs and was released slowly over a period of 7 days in the presence of 10% serum (Figure 4). Given their successful anchoring of Env trimers, we focused on DMPC lipid nanocapsules for subsequent *in vivo* immunization studies.

**Humoral Response to gp140T-ICMV Vaccines.** To determine the humoral response generated by the gp140T-his proteins displayed on ICMV lipid nanocapsules (gp140T-ICMVs), in pilot studies we compared ICMVs to soluble trimer. C57Bl/6 mice were immunized with various doses of gp140T-ICMVs admixed with 5  $\mu$ g of the Toll-like receptor-4

agonist MPLA, a clinically relevant molecular adjuvant.<sup>39,40</sup> Although MPLA can be readily loaded in the membranes of the ICMV capsules,<sup>22,23</sup> we previously found that simply admixing this adjuvant with ICMVs gave strong humoral responses,<sup>19</sup> and thus we used this approach of simply admixing MPLA and ICMVs to keep the formulations as simple as possible. For comparison to a strong experimental adjuvant for mouse humoral responses, soluble trimer was admixed with SAS (Sigma Adjuvant System) adjuvant, an oil-in-water adjuvant containing MPLA and trehalose-6,6'-dimycolate that elicits extremely strong antibody responses in small-animal models but is too toxic for human use. Sera were collected from vaccinated mice over time for analysis of titer and avidity of gp140T-specific antibodies. As shown in Figure 5A, soluble

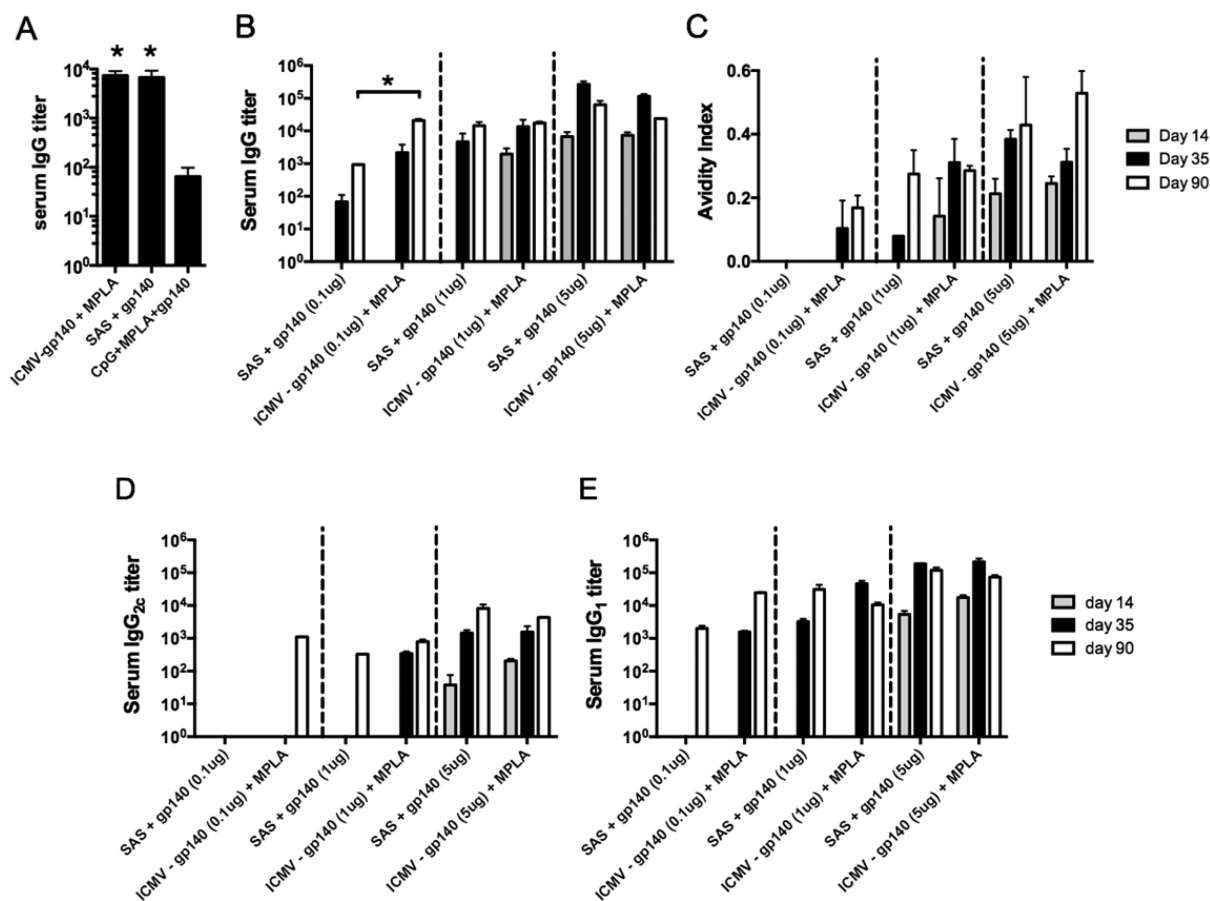


**Figure 4.** Stability of HIV env trimer anchoring on Ni-NTA-ICMV lipid nanocapsules. Trimer release from ICMVs over time in the presence of medium with 10% serum at 37 °C was measured by spectrofluorimetry.

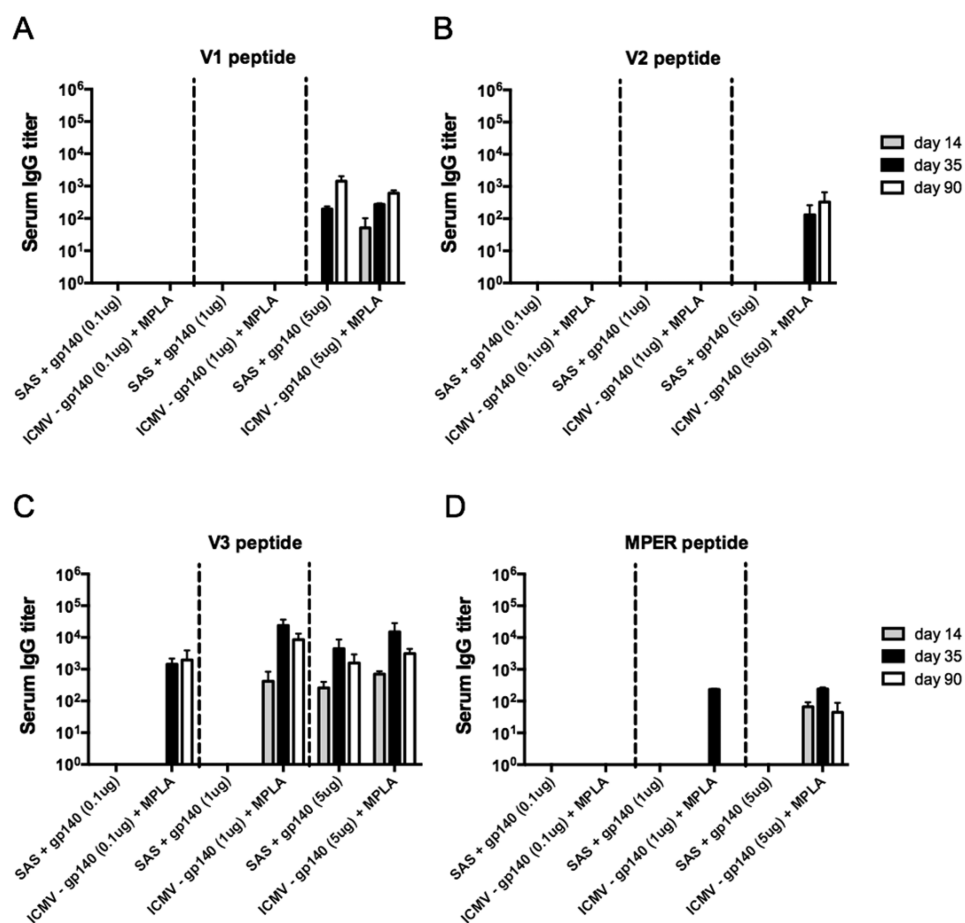
trimer elicited much weaker humoral responses than gp140T-ICMV or gp140T mixed with SAS, even when adjuvanted by multiple TLR agonists (MPLA alone as adjuvant elicited even weaker responses, not shown). We thus focused on comparing ICMVs to the strong oil-in-water emulsion adjuvant. Both soluble trimer in SAS and ICMV vaccinations were capable of eliciting robust trimer-specific antibody responses at antigen doses  $\geq 1 \mu\text{g}$  (Figure 5B). However, gp140T-ICMVs appeared

to elicit a more potent response despite the use of a less inflammatory adjuvant, as evidenced by readily detectable anti-gp140T antibody after a single priming injection with  $1 \mu\text{g}$  of antigen on ICMVs (day 14) compared to no response detected for the soluble antigen/SAS group until post-boost (day 35). Further, ICMV vaccines at all 3 doses tested eventually achieved essentially identical titers by 3 months post-priming, while soluble trimer in SAS showed a 67-fold decrease in titer as the antigen dose was reduced from  $5 \mu\text{g}$  to  $0.1 \mu\text{g}$  ( $p = 0.025$ , Figure 5B). Thus,  $1 \mu\text{g}$  of gp140T in SAS elicited the same IgG titer as a 10-fold lower dose of the antigen bound to ICMVs at 3 months. Similarly, the avidity of anti-gp140T sera decayed more quickly as a function of antigen dose for soluble trimer in SAS compared to the nanocapsule vaccines (Figure 5C). At higher antigen doses, both SAS and ICMVs showed similar titers at day 90, but we did not follow these responses longer to determine if the long-term durability of responses was comparable.

Analysis of IgG isotypes revealed that the ICMV vaccines elicited a more Th1/Th2-balanced humoral response, with both IgG<sub>2c</sub> and IgG<sub>1</sub> env-specific antibodies elicited earlier and to higher titers at the  $1$  or  $0.1 \mu\text{g}$  antigen doses compared to antigen in SAS (Figure 5D, E). Thus, ICMV delivery of oriented, membrane-bound trimers enabled strong, Th1/Th2-



**Figure 5.** Humoral immune responses elicited by gp140T-ICMVs. (A) C57Bl/6 mice ( $n = 3/\text{group}$ ) were immunized subcutaneously at day 0 with  $5 \mu\text{g}$  gp140T-his either in soluble form mixed with SAS or in soluble form mixed with MPLA ( $5 \mu\text{g}$ ) and CpG ( $10 \mu\text{g}$ ), or bound to ICMVs mixed with  $5 \mu\text{g}$  MPLA. Shown are total gp140-specific IgG titers at day 14. (B–E) C57Bl/6 mice ( $n = 3/\text{group}$ ) were immunized subcutaneously at day 0 and day 21 with  $5 \mu\text{g}$ ,  $1 \mu\text{g}$ , or  $0.1 \mu\text{g}$  gp140T-his either in soluble form mixed with  $5 \mu\text{L}$  of SAS or bound to ICMVs mixed with  $5 \mu\text{g}$  of MPLA. ELISA analysis of sera was carried out over time to assess total gp140T-specific IgG (B), avidity (C), IgG<sub>2c</sub> (D), and IgG<sub>1</sub> (E). \*,  $P < 0.05$ . All values are reported as mean  $\pm$  s.e.m.



**Figure 6.** Nanocapsule-delivered trimer increases the breadth of the env-specific humoral response. ELISA analysis of sera was carried out over time to assess titers of IgG reactive with linear peptide epitopes derived from gp140: shown are IgG responses against the V1 (A), V2 (B), and V3 (C) loops, or the MPER (D). All values are reported as mean  $\pm$  s.e.m.

balanced humoral responses to be achieved at low antigen doses where a highly inflammatory oil-in-water adjuvant was ineffective.

**Immune Sera Recognition of gp140T Epitopes Following Nanocapsule Vaccination.** We hypothesized that presentation of HIV envelope trimers presented in a physiological orientation on the surface of lipid nanocapsules might promote breadth of the humoral response, by enabling a more diverse pool of B-cells to be recruited into the immune response via multivalent engagement of particle-bound trimers. Following immunization, we tested the presence of binding antibodies to several conserved linear peptide epitopes of the trimer—the variable loops V1, V2, V3, and the conserved Membrane-Proximal External Region (MPER). As shown in Figure 6A–D, soluble trimer in SAS elicited responses against the V3 peptide and these sera bound the V1 epitope at the highest (5  $\mu$ g) immunization dose, but no binding was detected to V2 or the MPER sequence. By contrast, ICMVs elicited responses not only against V3 and V1, but also against the V2 peptide and a weak response against the MPER segment at the highest antigen dose (Figure 6A–D). Thus, nanocapsule delivery of the trimer increased the breadth of responses elicited against this key target of the humoral response against HIV.

## CONCLUSIONS

In conclusion, we have shown that particle display of a large, complex HIV trimer, gp140, required the use of high- $T_m$  lipids to enable stable tethering to lipid nanocapsules. Importantly, immunization with trimers anchored to ICMV lipid nanocapsules elicited a significantly stronger humoral response compared to soluble protein in a strong oil-in-water emulsion adjuvant, with a balanced production of multiple isotypes of env-specific antibodies. Importantly, we have shown that the use of the particulate vaccine increased the breadth of the antibody response, as evidenced by binding antibodies produced to several conserved targets on the envelope structure, including the MPER sequence of gp41. Induction of virus-neutralizing antibodies was not assessed here due to the high background of mouse serum in standard neutralizing assays, but future work will assess neutralizing titers in other small animal models (e.g., guinea pigs). Nanoparticles can thus be used to display large HIV trimers in an oriented, multivalent presentation and can promote key aspects of the humoral response against Env immunogens.

## EXPERIMENTAL SECTION

**Recombinant Gp140T-his Trimer Production.** Expression of the subtype A 92UG037.8 gp140 trimer was previously described.<sup>8,14</sup> Briefly, a stably transfected 293T cell line was expanded to confluence in DMEM supplemented with 10% FBS and subsequently media exchanged to Freestyle 293

expression media (Invitrogen). Cell supernatants were collected 96 h after media exchange and subjected to standard Ni-NTA (Qiagen) affinity chromatography followed by Superose 6 (GE Healthcare) size exclusion chromatography in 25 mM TRIS (pH 7.5) plus 150 mM NaCl. Fractions containing the purified protein were subjected to SDS-PAGE electrophoresis in order to monitor purity before fractions were pooled, concentrated, and flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Synthesis of ICMVs.** Interbilayer cross-linked multilamellar vesicles (ICMVs) have been previously described.<sup>19,23</sup> Here, modifications were made to the procedure used by Moon et al. to accommodate conjugation of the large molecular weight gp140 trimers. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used as received. Briefly, low- $T_m$  lipids (1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC): 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)-iminodiacetic acid)succinyl] nickel salt (Ni-NTA-DGS): 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimidophenyl) butyramide] (MPB) in a 4:1:5 molar ratio), or high- $T_m$  lipids (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC):Ni-NTA-DGS:MPB 4:1:5 molar ratio) in chloroform were dried into a thin film (total  $1.26\ \mu\text{mol}$  lipid) in glass vials by evaporating the organic solvent under vacuum overnight. In some experiments, cationic low- $T_m$  ICMVs were prepared using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP):DOPC:MPB at an 4:1:5 mol ratio. For the formation of low- $T_m$  ICMVs, lipid films were rehydrated in 10 mM bis-tris propane pH 7.0 for 1 h at  $25^{\circ}\text{C}$ , followed by sonication at a constant power of 5 W for 5 min on ice (Misonix Microson XL probe tip sonicator, Farmingdale, NY). For high- $T_m$  ICMVs, liposome formation was carried out at a constant temperature of  $30^{\circ}\text{C}$  (DMPC  $T_m = 23^{\circ}\text{C}$ ) with rigorous vortexing every 10 min, and then sonicated at a constant power of 5 W for 5 min at  $30^{\circ}\text{C}$ . Next,  $\text{CaCl}_2$  (final conc. of 10 mM) was added to induce fusion of the liposomes to form multilamellar vesicles (MLV), followed by 1.5 mM DTT (maleimide/DTT molar ratio of 2:1) and incubation of the vesicles for 1 h at  $37^{\circ}\text{C}$  for lipid cross-linking. The resulting ICMV lipid nanocapsules were recovered by centrifugation at  $14\,000 \times g$  for 4 min, and washed twice with deionized water. Since DTT is known to reduce metal ions, the ICMVs were incubated for 5 min with 100 mM  $\text{NiCl}_2$  at  $25^{\circ}\text{C}$  to recharge the Ni-NTA groups, followed by washing. In some of the low- $T_m$  preparations, 5 mol % cholesterol was included to enhance bilayer stability, or additional Ni-NTA-DGS lipids were post-inserted by incubation of ICMVs ( $1.26\ \mu\text{mol}$  lipids) with 300 nM Ni-NTA-DGS in PBS at  $25^{\circ}\text{C}$  for 10 min.

**In Vitro Protein Attachment and Assessment of Trimer Stability.** HIV env proteins were anchored to low- $T_m$  ICMV capsules by incubating Ni-NTA-bearing ICMVs ( $1.26\ \mu\text{mol}$  lipids) with gp140T-his ( $200\ \mu\text{g}/\text{mL}$ ) in PBS at  $25^{\circ}\text{C}$  for 8 h. To anchor to high- $T_m$  ICMVs, gp140T-his was incubated with DMPC-containing ICMVs at  $4^{\circ}\text{C}$  to ensure binding while DMPC remained in the gel state. In some experiments, ICMVs were loaded with GFP-his (Invitrogen, Carlsbad, CA) as an irrelevant antigen control. Following incubation, ICMVs were washed 3 $\times$  with deionized water to remove unbound trimer protein, then PEGylated by incubation with 2 kDa PEG-SH (Laysan Bio, Arab, AL) in a 1.5-fold molar excess of PEG-SH to maleimide groups for 1 h at  $37^{\circ}\text{C}$ . The resulting particles were washed 3 $\times$  with deionized water, then stored in PBS at  $4^{\circ}\text{C}$  until use. As an alternative strategy for gp140T-his protein binding, electrostatic adsorption of trimers

to cationic ICMVs formed with DOTAP lipids was also tested; DOTAP-containing ICMVs were incubated with gp140T-his as described above for 4 h at  $25^{\circ}\text{C}$ , followed by washing to remove unbound protein. As a final alternative, the gp140T protein was instead bound to ICMV capsules using an anti-histag Ab (GenScript, Piscataway, NJ) anchored to the ICMV lipids through hinge thiols: Briefly, anti-histag antibody with exposed free hinge region thiols was prepared by mixing anti-histag ( $12\text{--}15\ \text{mg}/\text{mL}$ ) with a 25 $\times$  molar excess of DTT for 20 min at  $25^{\circ}\text{C}$  in the presence of 10 mM EDTA in PBS. The mildly reduced anti-histag was passed through a desalting column to remove DTT, and then immediately mixed with maleimide-functionalized ICMV capsules at a ratio of 1 mg Ab:2.5 mmol nanocapsules for covalent coupling in the presence of 10 mM EDTA. The maleimide-thiol reaction was allowed to proceed for at least 10 h at  $25^{\circ}\text{C}$ , followed by multiple washes with PBS, to remove unbound antibody.

To assess efficiency of gp140T-his protein attachment to the surface of ICMVs, gp140T-his was labeled with Alexa-Fluor 647 (AF647) (Invitrogen, Carlsbad, CA) for direct fluorometric quantification of bound protein. Release of gp140T-his labeled with AF647 from ICMVs was quantified in PBS supplemented with 10% fetal calf serum at  $37^{\circ}\text{C}$ . At regular intervals, supernatants were removed for quantification of fluorescence, and an equal volume of fresh medium was replaced for continued monitoring of protein release. Residual gp140T-his remaining at the end of the time-course was determined by disruption of the ICMV particles with 1% Triton X-100 treatment and measuring released protein by fluorescence spectrophotometry.

**Anti-histag ELISA.** To qualify the presence of the histidine residue tag on gp140T-his proteins, an enzyme-linked immunosorbent assay (Cell Biolabs, Inc., San Diego, CA) analysis of the concentration of histags was performed on solutions of neat AF647-labeled gp140T-his or Alexafluor-labeled protein recovered from ICMVs. Total protein concentration was measured in parallel via AF647 fluorescence, for concurrent protein and histidine tag detection.

**Vaccination Studies.** Animals were cared for following National Institutes of Health, state, and local guidelines following an MIT IACUC-approved protocol. Groups of 6–8-week old C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were immunized subcutaneously at the tail base with the various indicated doses of gp140T-his (either soluble form or loaded on ICMVs) mixed with the TLR4 agonist monophosphoryl lipid A (MPLA, Invivogen, San Diego, CA), or in some cases CpG 1826 DNA ( $10\ \mu\text{g}$ , Invivogen). As a standard, mice were immunized with soluble gp140T-his mixed with SAS adjuvant (Sigma, St. Louis, MO), a strong experimental adjuvant. Mice were immunized twice, 3 weeks apart, and anti-gp140T-his IgG titers, defined as the dilution of sera at which the 450 nm OD reading was 0.5, were determined by ELISA analysis of sera from immunized mice. For antibody avidity analysis, low-affinity antibodies were eluted by the addition of 6 M urea for 10 min at  $25^{\circ}\text{C}$  following serum incubation. The antibody titers (450 nm OD reading) obtained with and without addition of urea were then used to calculate the IgG avidity values.

**Statistics.** Statistical analysis was performed with Graphpad Prism (La Jolla, CA). Data sets were analyzed using one-way analysis of variance (ANOVA). *p*-Values less than 0.05 were considered statistically significant. All values are reported as mean  $\pm$  s.e.m.



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## Notes

The authors declare the following competing financial interest(s): DJI is an inventor on patents related to ICMV lipid capsules and is a co-founder of a company developing the ICMV technology for vaccines.

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