Reorienting our view of particle-based adjuvants for subunit vaccines

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Despite a myriad of advances in the understanding and development of vaccine formulations, safe and effective vaccines have yet to be discovered for many pathogens. An excellent example of such is the malarial parasite *Plasmodium vivax*. Not only does this parasite transition between both extracellular and intracellular states during infection, but it can remain dormant in the liver and have greater transmission potential with lower titers than its more notorious counterpart, *Plasmodium falciparum* (1). As a consequence, it is important for a candidate vaccine to elicit both cellular (Th1) and humoral (Th2) immune responses that are potent and long-lived. Although vaccine antigens have been identified for the malarial sporozoites (2), the resulting immune responses elicited are short-lived and limited in scope, which is not uncommon for subunit vaccines that do not contain all of the components of a live-attenuated vaccine (3). For this reason, the malarial subunit vaccine is a hallmark example of a formulation that will require an appropriate adjuvant capable of boosting the most relevant immune responses to be effective. In PNAS, Moon et al. (4) describe a unique, lipid-based nanoparticle adjuvant (called an interbilayer-crosslinked multilamellar vesicle, or ICMV) that could not only be a promising candidate for prophylaxis against *P. vivax* but may even provide clues to how protective immunity to malaria is acquired.

Nanoparticle adjuvants seem to be well suited for making this particularly challenging vaccine formulation effective. Indeed, nano- and microparticles are particularly flexible adjuvants that can serve as a point source for antigen retention and release in a sustained or even triggered fashion (5–7). Furthermore, as shown in the study by Moon et al. (4), synthetic particles can also be engineered to exhibit repetitive orientation of antigen on the surface. The multivalency of this surface antigen presentation has the potential to generate B-cell receptor crosslinking and enhanced activation, a phenomenon that has likely been acquired through evolution to recognize the repetitive nature of surface antigen on live pathogens (8). The result of this synthetic, multivalent presentation of subunit antigen on a nanoparticle surface is almost a full order of magnitude increase in antibody titers compared with using ICMVs that only encapsulate and release antigen but do not orient the antigen on the surface (4). Multivalent display also seems to lead to a more balanced Th1/Th2 response and may also play some role in the expansion of antigen-specific, follicular helper T cells, which are important in developing B-cell memory. Furthermore, achieving comparable antibody responses (with any of the conventional adjuvants used in this study) required 10 times the amount of unoriented, soluble antigen compared with particle-based orientation of that same antigen. Even then, responses were short-lived compared with those elicited by multivalent display of the antigen on a particle surface. It even seems that the majority of germinal centers (where B-cell responses are initiated) nucleate directly adjacent (within 100 μm) to the particle deposition centers in the draining lymph nodes. All of these observations seem consistent with the mechanisms of a particle-based adjuvant that would orient pathogen-based immunological cues around a point source and maintain persistent presentation, effectively mimicking how a "particle" or piece of sporozoite would be recognized (albeit in a circumscribed capacity).

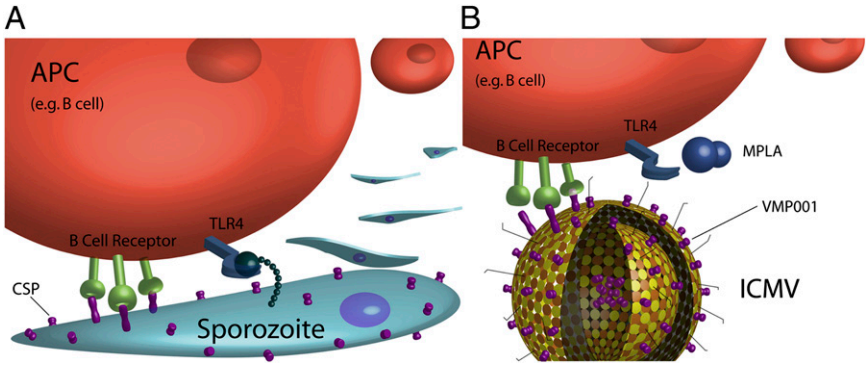

Fig. 1. Multivalent display of antigen and TLR4 agonist to an antigen presenting cell (APC) such as a B cell by a malarial sporozoite and a nanoparticle/adjuvant formulation. (*A*) *P. vivax* displays circumsporozoite proteins (CSP) prominently on its surface, as well as structures that likely serve as TLR4 agonists (12, 13). Clustering of B-cell receptors is achieved as a result of the natural, repetitive display of the CSP. This combination of persistent, multivalent antigen presentation in context with particular, parasite-associated "danger signals" would be recognized by the immune system in a way that would produce immune responses that are well-suited to combat the parasite. (*B*) Synthetic ICMVs [as described in PNAS (4)] can be designed to display a subunit CSP antigen (VMP001) through both sustained release and multivalent presentation on their surface. When administered along with the TLR4 agonist MPLA, these nanoparticles produce an immune response that is better suited to combat malaria than when antigen and conventional adjuvant are delivered alone.

Another fascinating observation is that oriented presentation of antigen on the particle surface seems to confer some diversity in terms of the specificity of antibodies produced (i.e., what regions of the sporozoite should be bound by antibodies). In effect, the nanoparticle adjuvant leads to the production not only of antibodies that bind to epitopes previously identified to be required for malarial protection, but also antibodies for the region I domain that effectively could deter sporozoite internalization by hepatocytes. It is unclear exactly how the nanoparticle adjuvant produces this effect, but it is speculated that lower-avidity B cells are provided with an opportunity to compete for activation when antigen is displayed multivalently on a particle surface (4). The ability of various adjuvants to determine the diversity of epitope recognition has also been seen in the past with $CD4^+$ T-cell responses, with depot/particle-based presentation being suggested as

Author contributions: S.R.L. wrote the paper.

The author declares no conflict of interest.

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a necessary condition (9). Regardless, the result of vaccination with the ICMVs with surface-bound antigen is a multimodal immune response that would block multiple stages of a parasite's attempt to infect its host.

The choice of "conventional" adjuvant (monophophoryl lipid A, or MPLA) used in this study is also quite interesting. This particular pathogen-associated molecular pattern is a chemically modified derivative of bacterial lipopolysaccharide (10) that binds to the innate immune activating receptor TLR4. These types of "danger signals" can provide an additional piece of information to effectively inform the immune system "how" it should mount a defense. Unlike other conventional adjuvants, such as Montanide (incomplete Freund's adjuvant) and alum (aluminum hydroxide), which have both been traditionally suggested to aid in antigen depot and release (11), the MPLA danger signal may be more appropriate in the context of a malaria vaccine because TLR4 is likely activated by malarial surface moieties during sporozoite infection (12) (Fig. 1*A*). As further evidence, a prior study suggests that TLR4 activation is a critical event in the development of protection from malarial infection, with polymorphisms in TLR4 being responsible for significantly increased risk of severe malaria in children (13).

Taken collectively, these observations may have greater implications than simply suggesting a viable vaccine formulation for *P. vivax*. Specifically, the particle-based formulation put forth by Moon et al. in PNAS (4) represents an interesting form of adjuvancy whereby the individual components are rationally "reorganized" to mimic the natural presentation of the individual components of a target pathogen, resulting in more appropriate immune responses. In other words, synthetic particles can serve as a template on which a pathogen-derived "packet of information" can be "encoded" through some combination of sustained presence at a point source, oriented presentation on the surface, and/or addition of relevant danger signals (Fig. 1*B*). Once encoded into the particle, this "packet of information" would be "decoded" by the immune system through recognition mechanisms that have been naturally selected to provide life-saving protection against natural pathogen particles. Beyond presentation of various chemical stimuli, even the geometry and aspect ratio of the particle itself may be recognized as important information by the immune system (14). Overall, synthetic particles are particularly attractive candidates for the next generation of rationally designed adjuvants, given their flexibility in terms of what can be encoded by the engineer.

Synthetic particles are particularly attractive candidates for the next generation of rationally designed adjuvants.

Presently, combinations of synthetic particles and well-characterized conventional adjuvants (such as the combination put forth by Moon et al.) may be a superb way to engineer subunit vaccine formulations to tackle a wide variety of other pathogens for which safe and effective vaccines are currently unavailable. Most certainly, our ever-increasing understanding of how our own bodies recognize pathogens will be the key to unlock even more advanced adjuvant formulations that can be increasingly more specific for producing a response to a given challenge. However, until synthetic adjuvants can be engineered to fully encode the necessary information to produce robust and customized immune responses, these synthetic, particle-based formulations with intentionally selected conventional adjuvants seem to be one step closer to tricking the body into believing that it has been exposed to the real thing.

ACKNOWLEDGMENTS. I thank Mintai Peter Hwang for help with graphic design of the figure. Work on biomimetic particles and drug delivery in my laboratory is supported by National Institutes of Health Grants 1R01DE021058-01 A1 and 5KL2 RR024154 02, National Science Foundation Grant 0941260, the Arnold and Mabel Beckman Foundation, and the Wallace H. Coulter Foundation.

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Enhancing humoral responses to a malaria antigen with nanoparticle vaccines that expand T_{fh} cells and promote germinal center induction

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Edited by Melody A. Swartz, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland, and accepted by the Editorial Board November 15, 2011 (received for review August 10, 2011)

For subunit vaccines, adjuvants play a key role in shaping immunological memory. Nanoparticle (NP) delivery systems for antigens and/or molecular danger signals are promising adjuvants capable of promoting both cellular and humoral immune responses, but in most cases the mechanisms of action of these materials are poorly understood. Here, we studied the immune response elicited by NPs composed of multilamellar "stapled" lipid vesicles carrying a recombinant *Plasmodium vivax* circumsporozoite antigen, VMP001, both entrapped in the aqueous core and anchored to the lipid bilayer surfaces. Immunization with these particles and monophosphoryl lipid A (MPLA), a US Food and Drug Administration–approved immunostimulatory agonist for Toll-like receptor-4, promoted high-titer, high-avidity antibody responses against VMP001, lasting more than 1 y in mice at 10-fold lower doses than conventional adjuvants. Compared to soluble VMP001 mixed with MPLA, VMP001-NPs promoted broader humoral responses, targeting multiple epitopes of the protein and a more balanced Th1/Th2 cytokine profile from antigen-specific T cells. To begin to understand the underlying mechanisms, we examined components of the B-cell response and found that NPs promoted robust germinal center (GC) formation at low doses of antigen where no GC induction occurred with soluble protein immunization, and that GCs nucleated near depots of NPs accumulating in the draining lymph nodes over time. In parallel, NP vaccination enhanced the expansion of antigen-specific follicular helper T cells (T_{fh}), compared to vaccinations with soluble VMP001 or alum. Thus, NP vaccines may be a promising strategy to enhance the durability, breadth, and potency of humoral immunity by enhancing key elements of the B-cell response.

Outside of sub-Saharan Africa, *Plasmodium vivax* is the most frequent cause of recurring malaria and infects 130–390 million people each year, representing approximately 50% of all malaria cases globally (1). Although not as virulent as *Plasmodium falciparum*, *P. vivax* has a long dormant liver stage, lasting months in some cases, and poses a significant threat to the global health (2). The circumsporozoite protein (CSP) is the most prevalent protein in *Plasmodium* sporozoites (3) and has been the target of clinical vaccine trials for *P. falciparum* (4). To date, however, there have been limited attempts to advance vaccines for *P. vivax*. We recently developed VMP001, a recombinant protein antigen composed of CSP core sequences derived from two widespread isolates of *P. vivax* (5, 6). Although VMP001 mixed with either alum or Montanide elicits antigen-specific antibody responses (5, 6), adjuvants capable of eliciting high-affinity antibodies against protective regions within CSP, which may lead to opsonization of sporozoites (7) or inhibition of their entry into hepatocytes (8), are of great interest.

Recently, antigens and adjuvants delivered by synthetic nanoparticles (NPs) have emerged as promising vaccine formulations. NPs may allow co-delivery of antigen and immunostimulatory molecules to the same intracellular compartment in antigen-presenting cells (APCs) (9) and promote cross-presentation of antigens, enhancing $CD8^+$ T-cell expansion and functionality (10–12). NP delivery of antigens and adjuvant molecules such as Toll-like receptor (TLR) agonists also has been shown to promote humoral immune responses (13, 14). However, a greater understanding of the mechanisms by which synthetic particles enhance immunity will be critical to maximize the potential of NP vaccine strategies.

We recently reported the design of a unique class of lipid-based NPs, interbilayer-crosslinked multilamellar vesicles (ICMVs), possessing many favorable characteristics for vaccine delivery (10). ICMVs, synthesized by forming covalent cross-links across lipid layers within multilayered lipid vesicles, are stable in the extracellular milieu following injection and retain entrapped proteins until the particles are internalized into intracellular compartments, thereby increasing the delivery of antigen to APCs in draining lymph nodes (dLNs). Immunization with ICMVs generated strong humoral and cellular immune responses to the model antigen ovalbumin (OVA) (10). In these studies, OVA was entrapped in the aqueous core of ICMVs. However, because cross-linking of B-cell receptors (BCRs) and B-cell stimulation are facilitated by structurally repetitive antigens, such as in viral and bacterial membranes (15, 16), we hypothesized that multivalent display of antigen on the surfaces of ICMVs would enhance the humoral response.

Here, we tested the efficacy of ICMVs for delivery of the VMP001 malaria antigen, and exploited available terminal cysteine groups in the protein to both encapsulate VMP001 in the core of the NPs and anchor a fraction of the protein to the lipid membranes of the vesicle walls, creating VMP001-ICMVs. Vaccination with VMP001-ICMVs dramatically enhanced both the quantitative strength of the antibody response and qualitative breadth and isotype bias relative to the conventional adjuvants, such as MPLA, alum, or Montanide. ICMV vaccination also promoted robust GC formation with the majority of GCs nucleated adjacent to depots of ICMVs accumulating in dLNs. Furthermore, ICMVs induced antigen-specific T_{fh} cells, surpassing the

Author contributions: J.J.M. and D.J.I. designed research; J.J.M., H.S., and A.V.L. performed research; C.F.O. and A.Y. contributed new reagents/analytic tools; J.J.M., H.S., A.V.L., and D.J.I. analyzed data; and J.J.M. and D.J.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.A.S. is a guest editor invited by the Editorial Board.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1112648109/-/DCSupplemental.

levels induced by 10-fold greater doses of antigen adsorbed to alum. Thus, NP vaccines have the capacity to enhance the breadth, avidity, and durability of humoral responses by promoting multiple key stages of antibody development.

Results

Design of ICMV NPs Combining Membrane Display and Encapsulation of Soluble Antigen. The synthesis of antigen-loaded ICMVs proceeds in three steps: formation of anionic maleimide (MAL)-functionalized vesicles by hydration of lipid films in the presence of antigen, fusion of vesicles via divalent cations, and finally "stapling" of the vesicles by addition of membrane-permeable dithiols that cross-link lipid headgroups bilayer-to-bilayer (Fig. S1) (10). We previously showed that immunization with the TLR4 agonist MPLA and ICMVs entrapping OVA in their aqueous core provided substantially enhanced antibody responses against the protein, compared to soluble protein/MPLA vaccinations. To determine whether the effectiveness of ICMVs could be further enhanced by combining aqueous encapsulation with anchoring of a fraction of the antigen to the membranes of the particles, we exploited the MAL groups in ICMV precursor vesicles as sites for both cross-linking of the bilayers and for conjugation to free cysteines of the malaria antigen VMP001 (Fig. S1 and Fig. 1*A*). We thus formed ICMVs in the presence of VMP001 containing cysteines at the N and C termini. In a typical synthesis, a total of 45 ± 8.2 μg protein was incorporated in ICMVs per mg of lipids with $50 \pm 9.1\%$ loading efficiency. PEGylated VMP001-loaded ICMVs (VMP001-ICMVs) had hydrodynamic diameters of 180 ± 14 nm and a relatively narrow size distribution with polydispersity index of 0.29 ± 0.021.

To assess whether VMP001 was linked to the membranes of ICMV particles, we probed for surface-accessible VMP001 bound to the NPs using antibodies (Abs) against the C-terminal his-tag of the VMP001 protein. Particles incubated with fluorophore-conjugated anti-his-tag Abs showed Ab binding to VMP001-ICMVs, but not to OVA-ICMVs or VMP001-ICMVs treated with trypsin to digest surface-bound protein (Fig. 1*B*). Blockade of MAL-mediated coupling by capping the free thiols of VMP001 with ethyl maleimide before particle synthesis (ctVMP001-ICMVs) did not affect protein loading efficiency significantly ($44 \pm 8.5\%$, $p = 0.45$), but eliminated anti-his-tag Ab binding, suggesting that VMP001 display on the particle surfaces was due to conjugation with MAL lipid headgroups rather than by surface adsorption. In agreement with these bulk measurements, confocal micrographs of fluorescently tagged VMP001-ICMVs showed lipid colocalization with anti-his-tag Ab staining, which was absent for OVA-ICMVs, trypsin-treated VMP001-ICMVs, or ctVMP001-ICMVs (Fig. 1*C*).

To test the impact of membrane conjugation on the immunogenicity of ICMVs, mice were immunized with VMP001-ICMVs, where antigen was both membrane-conjugated and encapsulated, or with ctVMP001-ICMVs, where antigen was only encapsulated in a soluble state in the particle interior. Mice in each group were primed and then boosted on day 21 with a total of 1 μg VMP001 and 0.1 μg MPLA. Measurement of resulting serum VMP001-specific IgG titers showed that membrane display of the antigen clearly increased the potency of ICMV vaccination, as homologous VMP001-ICMV immunization elicited IgG titers approximately 9-fold greater than homologous ctVMP001-ICMV immunization ($p < 0.05$, Fig. 1*D*). Heterologous immunizations with VMP001-ICMVs and ctVMP001-ICMVs revealed that vaccination with antigen bound on particle membranes during the prime rather than boost was more critical to elicit high IgG titers (Fig. 1*D*).

VMP001-ICMV Vaccination Induces Durable Antibody Responses at 10-Fold Lower Doses of Antigen than Soluble Protein with Traditional Adjuvants. Immunization of C57Bl/6 mice with VMP001-ICMVs mixed with MPLA as a molecular adjuvant elicited durable, high titers of serum anti-VMP001 IgG, sustained for more than 1 y following a prime and boost with as little as 100 ng of the malaria antigen (Fig. 2*A*). In contrast, vaccines composed of soluble VMP001 mixed with MPLA or adjuvanted with Montanide or alum required at least 10-fold more protein to elicit a response, and exhibited waning titers over time (Fig. 2*A*). VMP001-ICMV + MPLA vaccination induced antigen-specific IgG_1 and IgG_{2c} isotype responses, while soluble protein + MPLA elicited only Th2-skewed IgG_1 antibodies (Fig. 2 *B* and *C*). Priming of a more balanced Th1/Th2 antibody response correlated with the enhanced production of IFN-γ and TNF-α by splenocytes from NP-immunized mice, compared to soluble protein vaccination (Fig. 2*D*). VMP001-ICMVs incubated with sera from naïve,

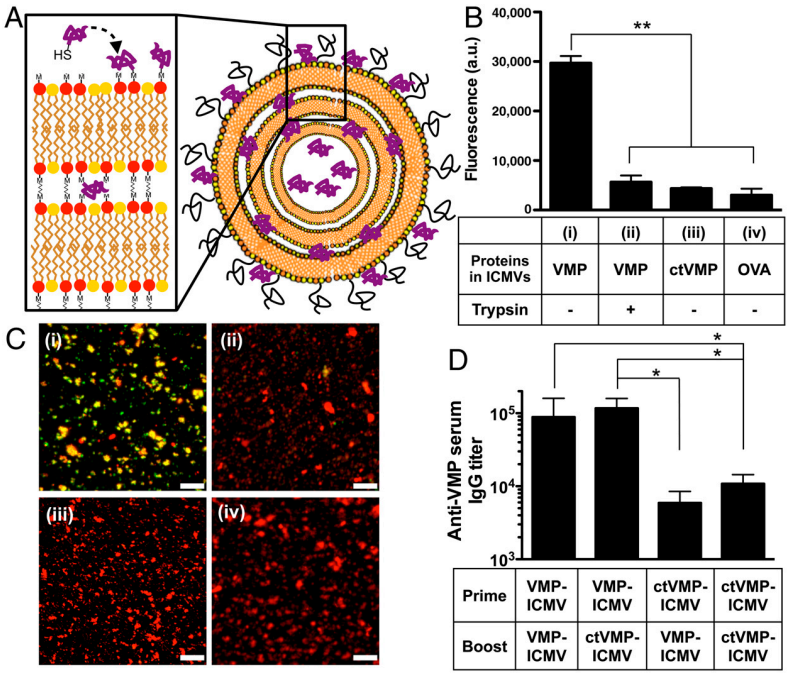

Fig. 1. ICMVs with surface-conjugated VMP001 induce potent humoral immune responses. (*A*) Schematic illustration of VMP001-loaded ICMVs: VMP001 is surface-displayed on ICMVs via coupling of cysteine residues with MAL-functionalized lipids. (*B* and *C*) (*i*) VMP001 bound on membranes of ICMVs was detected by staining with anti-his-tag Ab (recognizing the C terminus of the antigen) followed by a labeled secondary Ab; bound Abs (green) on particles (lipids, red) was detected by (*B*) a fluorescence plate reader or (*C*) confocal microscopy (colocalization of Ab with lipids appears yellow). Controls included (*ii*) ICMVs treated with trypsin to digest surface-exposed antigens, or loaded with capped-thiol VMP001 [(*iii*) ctVMP001] or (*iv*) OVA. (*D*) C57Bl/6 mice were immunized with either VMP001-ICMVs or ctVMP001-ICMVs in different combinations (prime day 0, boost day 21; 1 μg VMP and 0.1 μg MPLA). Anti-VMP001 IgG sera titers were measured on day 50 by ELISA. Scale bars, 10 μm. Data represent mean ± SEM of two to three independent experiments conducted with $n = 3$.

VMP001-ICMV-, or OVA-ICMV-immunized mice had detectable binding only to VMP001-ICMV sera, indicating minimal cross-reactivity of elicited IgG to nonprotein components of ICMVs; i.e., maleimide, lipids, or PEG (Fig. S2). Notably, VMP001-ICMVs reconstituted in saline from lyophilized powders maintained their original particle diameter and size distribution (diameter of 200 nm ± 11, polydispersity index of 0.23± 0.05), and elicited similar humoral responses, suggesting that freeze-dried materials could be used to enhance the storage life of ICMV vaccines (Fig. S3).

Enhanced Immunogenicity of NP-Formulated Antigen Enables Dose Sparing of MPLA. Strategies to reduce the dose of potent immunostimulatory adjuvants such as MPLA or other TLR agonists are attractive, to lower the risk of reactogenicity or other side effects that could hamper the safety of vaccine candidates. To determine whether the enhanced immunogenicity of ICMVs would permit dose sparing of MPLA, we titrated down the dose of MPLA with a fixed dose of VMP001 antigen (1 μg) in soluble or ICMV form. Strikingly, the peak IgG response was comparable for 25 μg or 1 μg MPLA given with VMP001-ICMVs, and MPLA doses as low as 100 ng elicited IgG titers comparable to a 250-fold greater dose of MPLA given with soluble protein (Fig. 2*E*).

NP Delivery Increases the Breadth and Avidity of the Humoral Responses. Several studies thus far have suggested that a humoral response against the Type I repeat in *P. vivax* CSP, specifically toward the AGDR motif within the VK210 sequence, may confer protective immunity against sporozoites (17–19). Thus, to evaluate the quality of the antibody response raised against VMP001, we assessed the avidity and epitope specificity of sera from immunized animals. ICMV vaccinations elicited IgG responses with up to 4.3-fold higher avidity than soluble protein immunization (VMP001-ICMV vs. VMP001 at 1 μg, $p < 0.05$) (Fig. 3*A*). Sera from mice immunized with VMP001-ICMVs bound the Type I repeat sequence with significantly higher titers, and explicitly recognized the AGDR motif, while sera from soluble protein immunizations did not recognize this fragment (Fig. 3*B*). ICMV vaccination also generated antibodies capable of recognizing the Region I domain, which may inhibit sporozoite invasion into hepatocytes by blocking receptor-ligand interactions during parasite entry (8). In contrast, the C-terminal fragment of VMP001 and the Region II domain, which share sequence homology to endogenous thrombospondin (20), were not recognized by antibodies elicited with either vaccine, suggesting lack of activation of self-reactive B cells. Taken together, these results suggest that NP vaccination generates antibody responses that are more durable and have higher avidity than those elicited by traditional adjuvants even using 10-fold less antigen, and elicit broader humoral responses with the capacity to recognize the sporozoite domains thought to be critical in protective immunity against infection.

Enhanced Antigen Delivery and Germinal Center Formation Triggered by ICMVs. Durable and high-affinity humoral responses elicited by ICMVs suggest that NP vaccines may effectively stimulate formation of GCs, where activated B cells proliferate, undergo immunoglobulin isotype-switching and somatic hypermutation, and eventually form memory B cells that can rapidly differentiate into plasma cells on re-exposure to antigen (21, 22). To investigate GC induction and its anatomic relationship with NP delivery to dLNs, we immunized mice with fluorophore-conjugated OVA (soluble or encapsulated in fluorescent ICMVs) and MPLA, and carried out histological analyses of dLNs at serial time points. Soluble OVA was detected in the dLNs within 4 h, but was rapidly cleared within 24 h (Fig. 4*A*). In contrast, OVA-loaded ICMVs were detected at the subcapsular sinus (SCS) of dLNs by 24 h, with continued accumulation over the next 2 wk, depositing a large amount of antigen beneath the SCS (Fig. 4*A*). Flow cytometry and histological analysis of OVA^+ cells on 1 or 4 d post NP injection showed that the major APC population acquiring particles were LN-resident macrophages, although OVA^+ $CD11b^+$ $CD11c^+$ DCs were also detected (Fig. S4), suggesting that both free draining and cell-mediated transport of particles to the dLN contributed at both time points.

OVA-ICMV immunizations elicited prominent GC formation, and notably, in the majority of cases, GCs were nucleated within 100 μm of ICMV-draining sites in dLNs (Fig. 4 *A* and *B*). Immunization with VMP001-ICMVs also induced GCs; compared with soluble VMP001+MPLA immunizations, NPs induced a significantly enhanced frequency and 8-fold increase in the absolute number of isotype class-switched GC B cells ($B220^+IgD^{low}$ $GL\text{-}7^+PNA^+$) in dLNs by day 14 ($p < 0.05$, Fig. 4 *C* and *D*). His-

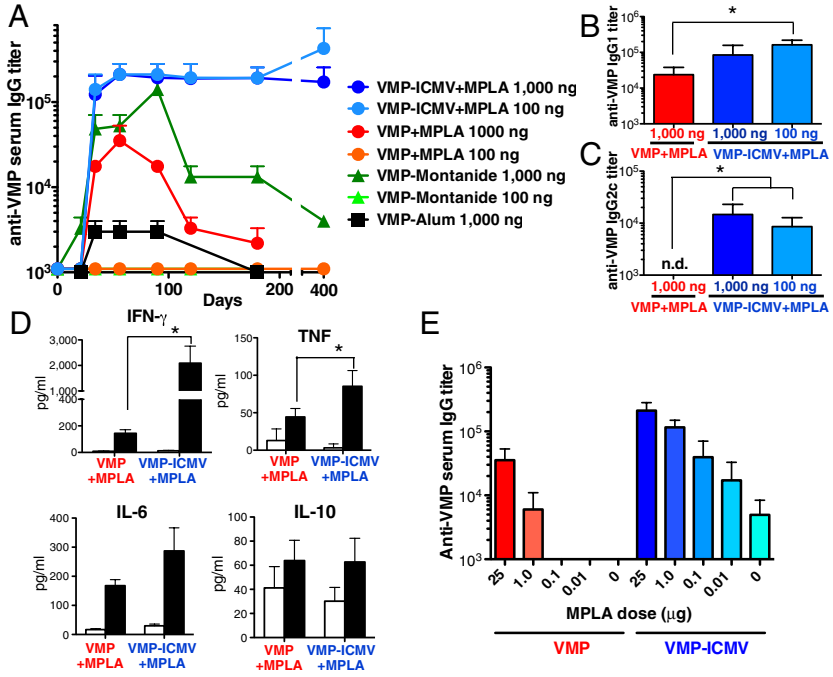

Fig. 2. VMP001-ICMV vaccines elicit robust, durable antibody titers with significantly reduced antigen/adjuvant doses. C57Bl/6 mice were immunized s.c. on day 0 and day 21 with the indicated doses of VMP001 in ICMVs mixed with 25 μg MPLA, or as soluble proteins mixed with either 25 μg MPLA, Montanide ISA-50, or alum. (*A*) Anti-VMP001 IgG sera titers were assessed over time by ELISA. Anti-VMP001 IgG sera were further characterized on day 90 for (*B*) IgG_1 and (*C*) IgG_{2c} titers (n.d., not detected). (*D*) Splenocytes isolated 7 d after priming and boosting with 1 μg VMP001 and 0.1 μg MPLA were stimulated with PBS (white bars) or 2 μg/mL VMP001-ICMVs (black bars) ex vivo, and the cell media were analyzed on day 2 for the concentrations of cytokines. (*E*) Mice were immunized with titrated amounts of MPLA mixed with 1 μg of VMP001 in either soluble or ICMV formulations. Shown are anti-VMP001 IgG sera titers measured by ELISA on d 50. Data represent the mean ± SEM of two independent experiments with $n = 3$–4 per group.

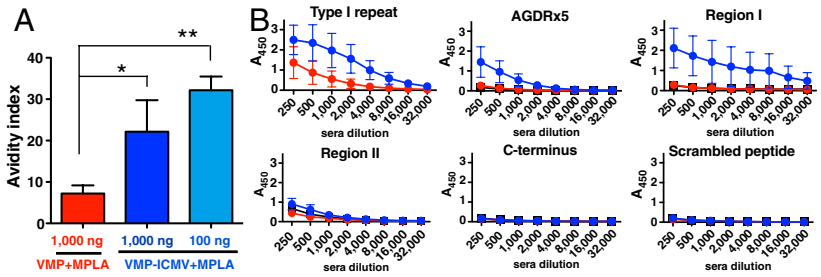

Fig. 3. VMP001-ICMV immunization elicits antibodies with high avidity and broader specificity than soluble protein vaccination. C57Bl/6 mice were immunized with VMP001-ICMVs or soluble VMP001 (25 μg MPLA with 0.1 or 1 μg protein) as in Fig. 2 and avidity and specificity of sera were analyzed on day 90. (*A*) Avidity index of sera from immunized mice binding to whole VMP001 protein. (*B*) Anti-VMP001 IgG antibodies elicited with VMP001-ICMVs + MPLA (blue) or VMP001 + MPLA (red) were further analyzed for binding to fragments of VMP001, including the Type I insert, AGDRx5, Region I, Region II, C terminus, or a scrambled peptide negative control. Data represent the mean ± SEM of two independent experiments conducted with $n = 3$.

tological analysis revealed 2–5 GCs per dLN in mice immunized with 100 ng VMP001-ICMV+MPLA, in contrast to their complete absence in the VMP001+MPLA immunization groups (Fig. 4 *E* and *F*). Notably, as observed with OVA, VMP001-ICMVs promoted GCs in close proximity to the particle-draining sites, with approximately 75% of GCs observed directly adjacent to NP deposits (Fig. 4 *B* and *G*).

ICMV Vaccination Enhances the Generation of Antigen-Specific T_{fh} Cells. Strong humoral immune responses, characterized by GC formation and long-lived plasma and memory B cells, are dependent on help provided by $CD4^+$ T_{fh} cells (23, 24). To determine whether ICMVs amplify the humoral response in part by enhanced $CD4^+$ T-cell differentiation, we turned to a model system to trace antigen-specific T helper responses: TCR-transgenic $CD45.2^+$ OT-II $CD4^+$ T cells recognizing OVA peptides were adoptively transferred into $CD45.1^+$ recipient mice that were subsequently immunized with 1 μg MPLA mixed with 10 μg soluble OVA or OVA-ICMVs. As an additional comparison, mice were also immunized with the traditional adjuvant alum and a 10-fold greater dose of OVA (100 μg). Eight days post priming, soluble protein immunization induced 6.3-fold expansion of OT-II $CD4^+$ T cells in spleens compared to mice treated with PBS after adoptive transfer; in contrast, OVA-ICMV vaccination induced a 21-fold expansion of the transferred cells, compared with PBS controls ($p < 0.05$, Fig. 5*A*). Alum also induced robust expansion of OVA-specific T cells (23-fold increase, compared to PBS, $p < 0.05$). Notably, OVA-ICMV vaccination promoted differentiation of OT-II $CD4^+$ T cells toward T_{fh} phenotypes ($CXCR5^+PD\text{-}1^+$), leading to a substantially increased frequency of antigen-specific T_{fh} cells, compared to the other immunization regimens (Fig. 5 *B* and *C*). Taken together, these results suggest that the enhanced humoral responses elicited by ICMVs compared to soluble vaccines or other traditional adjuvants are a product of enhanced GC formation and increased expansion/differentiation of antigen-specific $CD4^+$ T cells toward T_{fh} phenotypes.

Discussion

Strategies to enhance the efficacy of recombinant protein subunit vaccines without sacrificing safety are of great interest, due to the weaker magnitude and durability of immune responses elicited

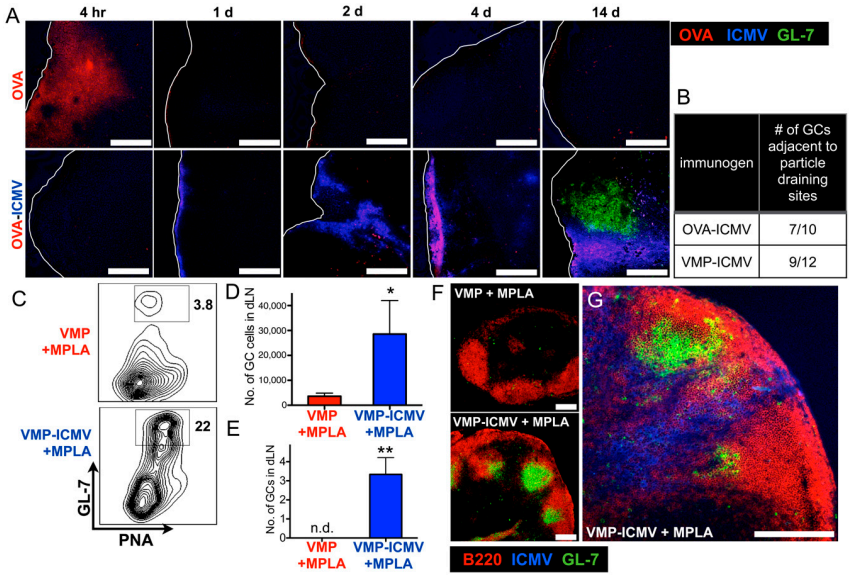

Fig. 4. ICMV immunization sustains antigen deposition in dLNs and induces germinal center (GC) formation. (*A* and *B*) C57Bl/6 mice were immunized with 100 μg fluorophore-conjugated OVA (shown in red) either in soluble or ICMV (shown in blue) formulations with 5 μg MPLA, and dLNs excised at indicated time points were examined by immunohistochemical analysis. (*A*) Representative confocal sections of dLNs; pink signals indicate colocalized OVA/ICMV lipids. Day 14 sections were stained with anti-GL-7 to identify GCs. (*B*) Enumeration of GCs located within 100 μm of ICMV-draining sites in dLNs. (*C–G*) C57Bl/6 mice were immunized with 1 μg VMP001 and 0.1 μg MPLA in either soluble or ICMV formulations, and dLNs were analyzed for GC formation on day 14. Shown are representative flow cytometry scatter plots of (*C*) isotype-switched GC B cells ($GL\text{-}7^+PNA^+$), gated on $B220^+IgD^{low}$ populations, and (*D*) their absolute numbers in dLNs. (*E*) Number of GCs observed in sections from whole dLNs ($n = 4$), cryosectioned and stained with anti-B220 and anti-GL-7. (*F* and *G*) Representative confocal micrographs of dLNs from immunized animals. Scale bars, 200 μm. Data represent the mean ± SEM of two to three independent experiments conducted with $n = 2–3$.

by subunit vaccines relative to more potent live attenuated or recombinant vectors. This is particularly relevant in the context of malaria, where short-lived immunity has been observed in the field trials of candidate subunit vaccines (4, 25). Analogous to the development of recombinant live viruses or bacteria as vectors to deliver nucleic acid-encoded antigens, synthetic NPs are emerging as promising vectors for the delivery of protein subunit antigens. Like live vectors, NPs can serve multiple roles in vaccination, carrying antigen into target tissue sites, controlling the antigen uptake by immune cells, and co-delivering "danger" signals that provide critical cues for the development of early effectors and late memory (10–14). Here using ICMVs as a lipid-based NP vector, we assessed the impact of NP delivery on immune responses elicited by a candidate *P. vivax* subunit vaccine, and particularly focused on defining qualitative and quantitative aspects of the humoral response and biological pathways contributing to this response.

We first optimized antigen incorporation into ICMVs with the goal of promoting the antibody response. Our original NP design was based on the entrapment of soluble antigen in the core of these multilamellar "stapled" lipid vesicles (10). Although T-cell responses rely on recognition of antigen fragments following intracellular antigen processing by APCs, antibody responses are ultimately linked to B-cell recognition of intact antigen via cell surface BCRs. Therefore, we hypothesized that particles with antigens anchored to their surfaces, mimicking the multivalent display of epitopes on the surfaces of pathogens, may facilitate cross-linking of BCRs and enhance the activation of B cells for stronger humoral immune responses, compared to soluble bolus injection of antigens (15, 16). To achieve such antigen surface-display, we exploited available cysteine residues in VMP001 and utilized maleimide groups in ICMV precursor vesicles for the dual purpose of both "stapling" the vesicle walls together in the presence of cross-linker and anchoring a fraction of the antigen to the vesicle membranes. Notably, we found that ICMVs incorporating VMP001 by both entrapment in the core and anchoring to the membranes enhanced the antigen-specific antibody response by 9-fold relative to NPs that contained protein only in the particle core (Fig. 1*D*).

VMP001-ICMVs adjuvanted with MPLA elicited strong antibody titers, surpassing the response induced by soluble VMP001 injected with MPLA or the conventional adjuvants alum or Montanide, and maintaining high titers for more than 1 y in mice. ICMV vaccines required 10-fold and 250-fold less antigen and MPLA, respectively, to produce similar antibody titers as soluble formulations. In addition to these quantitative effects on the antibody response, VMP001-ICMV immunization also changed the qualitative nature of the antibody response, promoting a more balanced Th1/Th2 response (both in terms of T-cell cytokine production and antibody isotypes) and broadening the antibody targets within VMP001, including epitopes implicated in protection against *Plasmodium* (5). We previously reported that immunization with Freund's adjuvant, a very strong but toxic adjuvant for small-animal experimental immunization, could elicit responses against multiple epitopes within VMP001 (5). Here we have made the important advance of achieving broad antibody responses using clinically relevant adjuvant materials. Previous studies using recombinant proteins or multipeptide constructs to target multiple vivax CSP regions showed evidence of immunodominance with some peptide combinations, with antibody responses focused on only a single epitope (19) or responses that failed to bind the critical protective AGDR sequence (26). Although the precise mechanism underlying NP-mediated broadening of the antibody response is the subject of further studies beyond the scope of this first report, we speculate that multivalent display of VMP001 on ICMVs may stimulate a more diverse set of naïve B cells, by allowing some lower-avidity B cells to compete for the antigen.

In order to gain a deeper mechanistic understanding of how NP-mediated delivery of antigen generates high-affinity, long-lasting antibody responses, we examined antigen distribution in dLNs following ICMV immunizations and subsequent B- and T-cell differentiation. Whereas soluble OVA injected s.c. drained to dLNs and was rapidly cleared within 24 h, OVA-loaded ICMVs drained to the SCS in dLNs after 24 h and accumulated over the next 2 wk (Fig. 4*A*). The ability of particles in the 200–300 nm size range to drain to LNs is consistent with recent work characterizing the transport of similarly sized synthetic or viral particles to LNs (27, 28), and other studies have reported that synthetic particles can continue to drain from s.c. injection sites to dLNs for at least 8 d (29). GC formation tended to occur directly adjacent to NP accumulation sites, suggesting that increased deposition/retention of antigen drives B-cell responses locally in the tissue. Indeed, we have shown previously that ICMV vaccines effectively increased antigen delivery to dLNs, enhancing antigen presentation by DCs, compared with soluble protein immunizations (10). Prolonged antigen presentation mediated by ICMV vaccines may have also contributed to enhanced development of $CD4^+$ T_{fh} cells (Fig. 5 *B* and *C*) (24), which provide critical cytokines and signals required to initiate somatic hypermutation and affinity maturation for B cells (23).

In summary, ICMV lipid NP vectors enhanced a range of quantitative and qualitative features of the immune response to the VMP001 CSP antigen, suggesting their utility in recombinant protein subunit vaccination. Enhanced humoral responses correlated with enhanced GC formation and induction of antigen-specific T_{fh} cells. Here we focused on vaccination using a single TLR agonist, MPLA, and future studies will be needed to determine if other clinically relevant molecular adjuvants combined with the current NP vaccine may allow further amplification of humoral

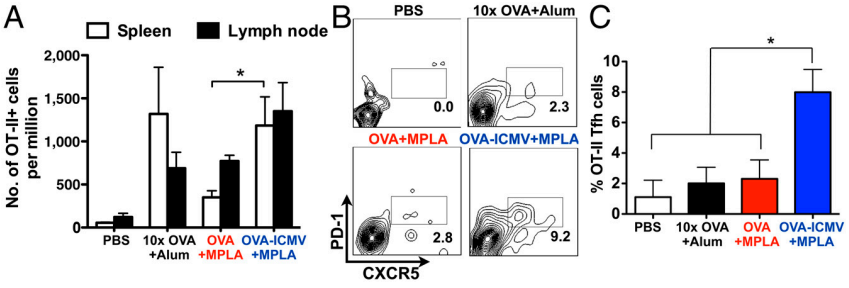

Fig. 5. ICMV immunization enhances follicular helper T-cell expansion. (*A–C*) One day after adoptive transfer of 10^5 $CD45.2^+CD4^+$OT-II T cells i.v. into naïve $CD45.1^+$ C57Bl/6 mice, recipient mice were immunized s.c. with 100 μg OVA in alum or 10 μg OVA and 1 μg MPLA in either soluble or ICMV formulations. (*A*) On day 8 after immunization, $CD45.2^+CD4^+$OT-II T cells were enumerated from spleens and dLNs. (*B* and *C*) $CD45.2^+CD4^+$OT-II T cells from spleens were further analyzed for expression of T_{fh} markers ($CXCR5^+PD\text{-}1^+$). (*B*) Representative flow cytometry scatter plots, gated on $CD45.2^+CD4^+$OT-II T cells in spleens, are shown with the percentages of $CXCR5^+PD\text{-}1^+$ populations, and (*C*) the percentage of OT-II cells with T_{fh} phenotypes were enumerated. Data represent mean ± SEM of two independent experiments conducted with $n = 3$.

immune responses in a synergistic manner, thus enhancing the protective efficacy of the NP vaccine.

Materials and Methods

Synthesis of ICMVs Loaded with VMP001. VMP001 was prepared as previously described (5, 6). Synthesis of ICMVs was performed as described previously with slight modifications (10). Briefly, dried films of 1.26 μmol of lipids [DOPC:DOPG:MPB (1,2-dioleoyl-*sn*-glycero-3-phosphoethanol amine-N-[4-(p- maleimidophenyl) butyramide) at 4:1:5 mol ratio (Avanti Polar Lipids)] were rehydrated in 20 mM bis-tris propane at pH 7.0 with 50 μg VMP001 or ovalbumin (Worthington) for 1 h with vortexing every 10 min, and sonicated in alternating power cycles of 6 W and 3 W in 30 s intervals for 5 min on ice (Misonix Microson XL probe tip sonicator). DTT and Ca^{2+} were then sequentially added at final concentrations of 3 mM and 40 mM, respectively, and incubated for 1 h at 37 °C to form ICMVs. The particles were recovered by centrifugation, washed twice, and incubated with 10 mg/mL 2 kDa PEG-thiol (Laysan Bio) for 30 min at 37 °C. For some experiments, capped-thiol VMP001 (ctVMP001) was prepared by incubating 0.5 mg VMP001 with 3.6 mM TCEP for 2 h at 25 °C, followed by incubation with 40 mM ethyl-maleimide (Pierce) at 37 °C for 2 h. The extent of thiol protection was measured to be >98% by Ellman's assay. Characterization of ICMVs is described in *SI Materials and Methods*.

Immunizations. Animals were cared for following National Institutes of Health, state, and local guidelines. Groups of 6- to 10-wk-old female C57Bl/6 mice (Jackson Laboratories) were immunized s.c. at the tail base with VMP001-ICMVs and indicated doses of MPLA (Sigma-Aldrich) in 100 μL PBS on day 0 and 21. Control immunizations with soluble VMP001 were performed using MPLA, Montanide ISA-50 V2 (Seppic), or alum (Imject alum, Pierce) at an adjuvant:protein solution volume ratio of 1:1 following the manufacturer's instructions. Characterization of humoral responses, germinal center formation, and follicular helper T-cell analysis is described in *SI Materials and Methods*.

Statistical Analysis. Datasets were analyzed using one- or two-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for multiple comparisons with Prism 5.0 (GraphPad Software). *P* values less than 0.05 were considered statistically significant, and marked with one asterisk. *P* values less than 0.01 were marked with two asterisks. All values are reported as mean ± SEM.

ACKNOWLEDGMENTS. This work was supported in part by the Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University; the Gates Foundation; the Department of Defense (Contract W911NF-07-D-0004); and National Institutes of Health (AI095109 and 1U19AI091693). D.J.I. is an investigator of the Howard Hughes Medical Institute. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Supporting Information

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SI Materials and Methods

Characterization of ICMVs. Particle sizes were determined by dynamic light scattering using a 90Plus/ZetaPals particle size (Brookhaven Instruments). VMP001 encapsulation in ICMVs was quantified by a fluorescence-based peptide assay (LavaPep, Gel Company) and confirmed by SDS-PAGE. In some studies, surface-exposed VMP001 was removed from ICMVs by trypsin treatment (40 μg/mL) at 37 °C for 10 min. Surface-exposed VMP001 was detected with either polyclonal goat anti-his-tag antibody (Abcam) or anti-VMP001 sera obtained from mice 120 d after immunizations with VMP001-ICMVs, followed by incubation with either Alexa-fluor 488 labeled anti-goat IgG or anti-mouse IgG (BD), respectively, and detection in a fluorescence microplate reader (Infinite200Pro, Tecan Group) or confocal microscopy (LSM510, Carl Zeiss).

Characterization of Anti-VMP001 Humoral Responses. Sera were analyzed for IgG, IgG_1, and IgG_{2c} antibodies by ELISA using VMP001-coated plates (6). Anti-VMP001 IgG titers were defined as the lowest serum dilution at which the ELISA OD reading was ≥0.5. Avidity measurements were performed by incubating plates with 6 M urea for 10 min at 20 °C to remove weakly bound IgG prior to addition of detection Ab (30). The avidity index was defined as (IgG titer with 6 M urea/IgG titer without urea) X 100. For epitope analysis, strepavidin-coated plates (Pierce) were incubated with 50 μg/mL peptide fragments derived from VMP001; Type I repeat (GDRAAGQPAGDRADGQPA), AGDRx5 (AGDRAGDRAGDRAGDRAGDR), Region I (NPRENKLKQP), Region II (EWTPCSVTCGVGVRVRRR), C terminus (PNEKSVKEYLDK), or a scrambled control (KPLDVEKNSEY), each with an N-terminal biotin-GSSSG spacer. Splenocytes isolated 7 d after boost vaccinations were restimulated ex vivo with either PBS or VMP001-ICMV at 2 μg/mL, and on day 2, cytokine levels in the culture supernatants were measured by flow cytometry (Cytometric Bead Array, BD).

Germinal Center and Follicular Helper T-Cell Analysis. Mice were immunized s.c. with 5 μg of MPLA and 100 μg of OVA-alexa fluor 555 in either soluble or ICMV formulations (lipids labeled with DiD). At serial time points, dLNs were isolated, flash-frozen, cryosectioned, and examined by confocal microscopy. To identify cell types associated with particles, dLNs were isolated on day 1 or 4, stained with anti-CD11b, anti-CD11c, anti-CD169, anti-CD205, and F4/80, and analyzed by flow cytometry. To assess GC formation, dLNs were isolated on day 14 from mice immunized with 1 μg of unlabeled soluble VMP001 or VMP001-ICMVs (both with 0.1 μg MPLA), stained with anti-B220, anti-IgD, anti-GL-7 (BD), peanut agglutinin (Vectorlabs), and analyzed by flow cytometry or confocal microscopy. To monitor antigen-specific $CD4^+$ T cells, 10^5 OVA-specific $CD4^+$ T cells from OT-II ($CD45.2^+$) mice were adoptively transferred i.v. into recipient congenic $CD45.1^+$ mice. After 1 d, recipient mice were immunized s.c. with 10 μg of OVA in either soluble or ICMV formulations, both with 1 μg MPLA. A second comparison group of mice were immunized s.c. with 100 μg OVA in alum. The frequencies of OVA-specific $CD4^+$ T cells and their phenotypes in spleens and dLNs were determined by flow cytometry analysis, following staining with DAPI, anti-CD4, anti-CD45.2, anti-PD-1, and anti-CXCR5 (BD).

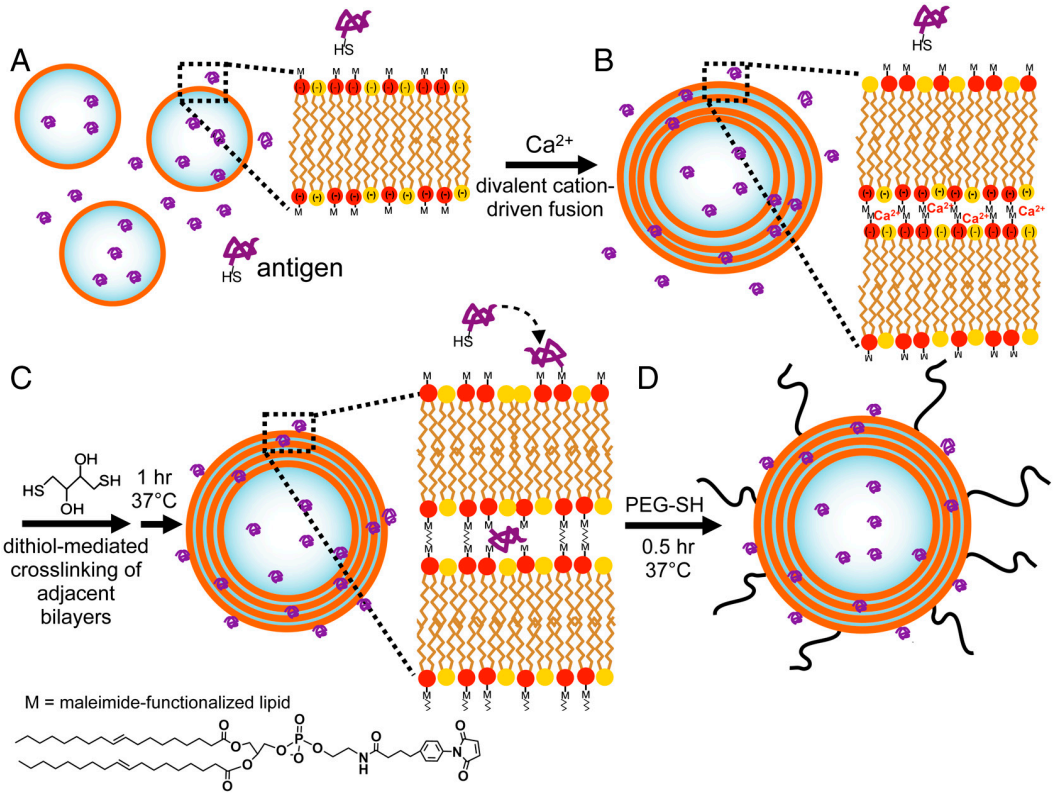

Fig. S1. Synthesis of VMP001-loaded interbilayer-crosslinked multilamellar vesicles (ICMVs). VMP001 is surface-displayed on ICMVs via reaction of its cysteine residues with maleimide (MAL)-functionalized lipids. (*A*) Anionic, maleimide-functionalized liposomes are prepared from dried lipid films in the presence of VMP001, (*B*) divalent cations are added to induce fusion of liposomes into multilamellar vesicles, (*C*) membrane-permeable dithiols are added, and (*D*) the resulting lipid particles are PEGylated with thiol-terminated PEG.

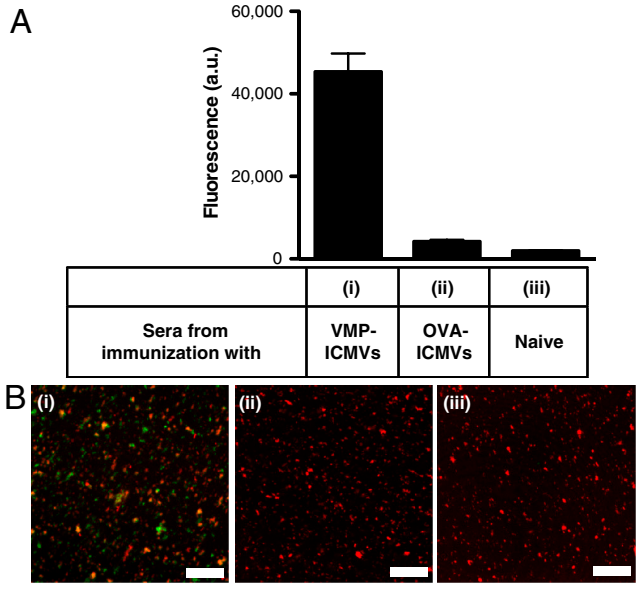

Fig. S2. Minimal cross-reactivity of sera from VMP001-ICMV immunizations to nonprotein components of ICMVs. VMP001-ICMVs were incubated with sera from naïve, VMP001-ICMV-, or OVA-ICMV-immunized mice, and IgG antibodies bound to the particles (shown in red) were detected with Alexa Fluor 488-conjugated anti-mouse IgG (shown in green), followed by (*A*) fluorescence spectrometry measurements and (*B*) confocal microscopy. Preferential binding of VMP001-ICMVs with sera from VMP001-ICMV-immunized mice indicates minimal cross-reactivity of elicited IgG to nonprotein components of ICMVs. Scale bars, 10 μm. Data represent mean ± SEM of three independent experiments conducted with $n = 3$.

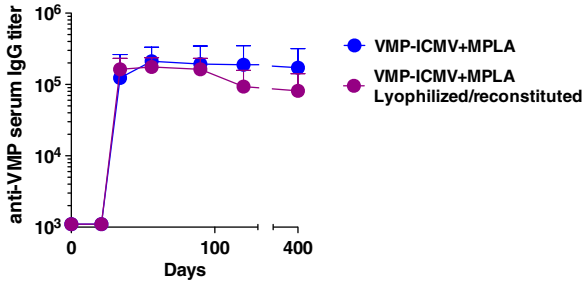

Fig. S3. VMP001-ICMV vaccines reconstituted from lyophilized powder elicit similar antibody titers as fresh NPs. C57Bl/6 mice were immunized s.c. on day 0 and day 21 with 25 μg MPLA and 1 μg VMP001-loaded ICMVs either synthesized on the day of administration or reconstituted from lyophilized powder after storage at −20 °C for 3 wk with 6.7% sucrose as a cryoprotectant. Anti-VMP001 IgG sera titers were assessed over time by ELISA.

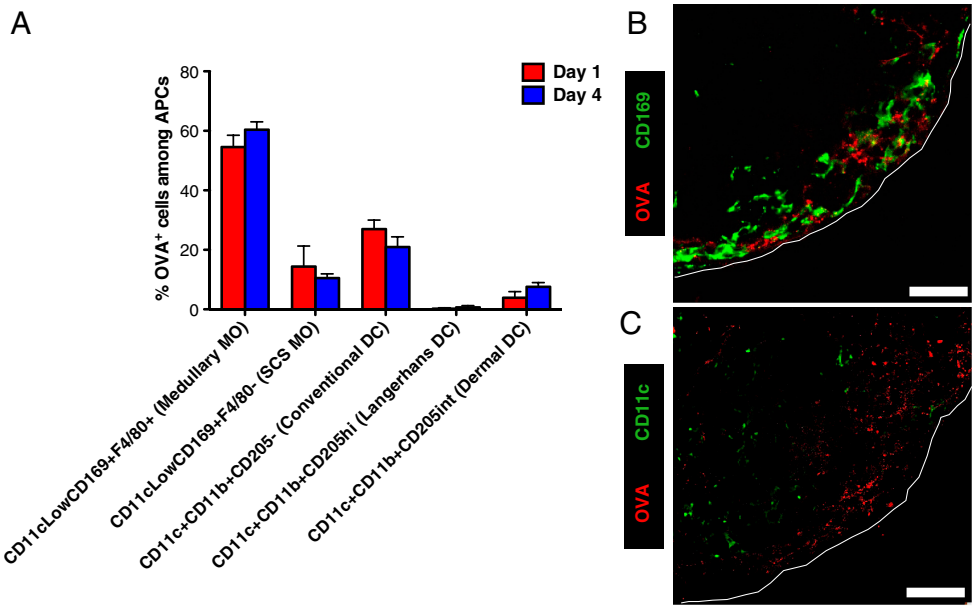

Fig. S4. ICMVs are deposited at subcapsular sinus layers with macrophages in draining lymph nodes. (*A–C*) C57Bl/6 mice were immunized with 100 μg fluorophore-conjugated OVA-ICMVs with 5 μg monophosphoryl lipid A (MPLA), and the relative contribution of different APC subsets in draining lymph nodes (dLNs) to the total OVA^+ antigen presenting cell population were quantified on days 1 and 4 by staining dLN cells with anti-CD11c, -CD11b, -CD169, -CD205, and -F4/80, followed by analysis with flow cytometry. (*B*) dLNs excised on day 1 were examined by immunohistochemical analysis after staining with (*B*) SCS macrophage marker, anti-CD169, or (*C*) DC marker, CD11c. Representative confocal sections of dLNs are shown. Scale bars, 20 μm.