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Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses

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Vaccines based on recombinant proteins avoid the toxicity and antivector immunity associated with live vaccine (for example, viral) vectors, but their immunogenicity is poor, particularly for CD8$^+$ T-cell responses. Synthetic particles carrying antigens and adjuvant molecules have been developed to enhance subunit vaccines, but in general these materials have failed to elicit CD8$^+$ T-cell responses comparable to those for live vectors in preclinical animal models. Here, we describe interbilayer-crosslinked multilamellar vesicles formed by crosslinking headgroups of adjacent lipid bilayers within multilamellar vesicles. Interbilayer-crosslinked vesicles stably entrapped protein antigens in the vesicle core and lipid-based immunostimulatory molecules in the vesicle walls under extracellular conditions, but exhibited rapid release in the presence of endolysosomal lipases. We found that these antigen/adjuvant-carrying vesicles form an extremely potent whole-protein vaccine, eliciting endogenous T-cell and antibody responses comparable to those for the strongest vaccine vectors. These materials should enable a range of subunit vaccines and provide new possibilities for therapeutic protein delivery.

Currently licensed vaccine adjuvants (for example, aluminium hydroxide and the oil-in-water emulsion MF59) promote immunity by primarily eliciting humoral immune responses without stimulating cellular immunity[1,2]. As strong CD8$^+$ T cell (CD8T) responses may be required for vaccines against cancer or intracellular pathogens such as human immunodeficiency virus, malaria and hepatitis C, there is great interest in technologies to promote concerted humoral and cellular immune responses[3,4]. To this end, engineered live vaccine vectors such as non-replicating recombinant viruses have been developed[5–7], which can induce both robust antibody responses and massive expansion of functional antigen-specific CD8Ts in murine models. However, safety concerns with live vectors and antivector immunity can complicate development of live vector vaccines[7]. Pre-existing vector-specific immune responses have reduced the immunogenicity of live vector-based vaccines in clinical trials[8], and the immune response raised against live vectors following a priming immunization can render booster immunizations using the same vector problematic[7].

In contrast, non-living synthetic vaccines delivering defined antigens can be rationally designed to avoid antivector immunity[9]. Such 'subunit' vaccines are composed of one or a few selected recombinant proteins or polysaccharides normally present in the structure of the target pathogen. However, subunit vaccines elicit poor or non-existent CD8T responses, owing to the low efficiency of cross-presentation (the uptake and processing of extracellular antigen by immune cells for presentation on class I major histocompatibility complex molecules to naïve CD8Ts)[10]. To promote cross-presentation, synthetic particles loaded with protein antigens and defined immunostimulatory molecules have been used[11–17], mimicking in a reductionist fashion the cues provided to the immune system during infection by pathogens. Liposomes are particularly attractive materials for this application, owing to their low toxicity and immunogenicity, track record of safety in clinical use, ease of preparation, and proven manufacturability at commercial scales[18,19]. Lipid vesicles in the form of unilamellar, multilamellar or polymerized vesicles have been tested as vaccine-delivery materials, with some success[19–23]. Antigens entrapped in lipid vesicles are cross-presented *in vivo*[19,24,25], and liposomal protein vaccines have been shown to elicit protective T-cell-mediated antimicrobial and antitumour immune responses in small-animal models[23,26,27]. However, for diseases such as human immunodeficiency virus and cancer, it is currently believed that extremely potent T-cell responses (in concert with humoral immunity) will be required to control the virus/tumours, and therefore more potent T-cell vaccines are still sought[3,4].

A potential factor influencing the potency of lipid vesicles in vaccine delivery is their limited stability in the presence of serum components. For liposomal cargos that can be processed at high temperature or loaded by diffusion through preformed vesicle membranes, enhanced vesicle stability can be achieved by using high-melting-temperature lipids, especially when combined with cholesterol and/or PEGylation[28]. Uni- and multilamellar vesicles have also been stabilized by polymerizing reactive headgroups at the surface of bilayers[29], polymerizing reactive groups in phospholipid

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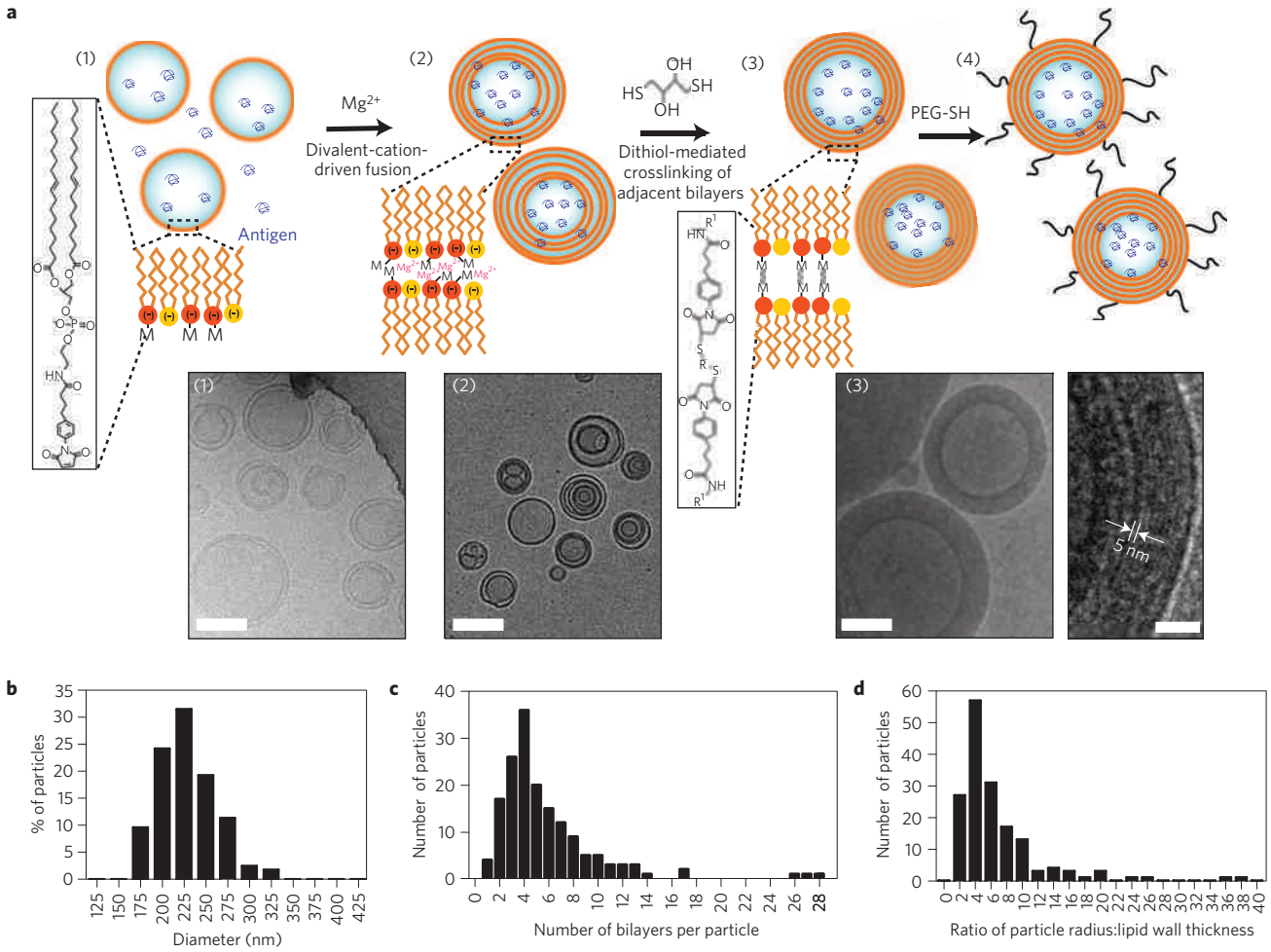

Figure 1 | Synthesis of ICMVs. a, Schematic illustration of ICMV synthesis and cryo-electron-microscope images: (1) anionic, maleimide-functionalized liposomes are prepared from dried lipid films, (2) divalent cations are added to induce fusion of liposomes and the formation of MLVs, (3) membrane-permeable dithiols are added, which crosslink maleimide lipids on apposed lipid bilayers in the vesicle walls, and (4) the resulting lipid particles are PEGylated with thiol-terminated PEG. Cryo-electron-microscope images from each step of the synthesis show (1) initial liposomes, (2) MLVs and (3) ICMVs with thick lipid walls. Scale bars = 100 nm. The right-hand image of (3) shows a zoomed image of an ICMV wall, where stacked bilayers are resolved as electron-dense striations; scale bar = 20 nm. **b**, ICMV particle-size histogram measured by dynamic light scattering. **c**,**d**, Histograms of ICMV properties from cryo-electron-microscope images show the number of lipid bilayers per particle (**c**) and the ratio of particle radius to lipid wall thickness (**d**). ($n = 165$ particles analysed.)

acyl tails[20,29] or polymerizing hydrophobic monomers adsorbed into the hydrophobic interior of membranes[30]. Common to each of these approaches is the concept of polymerizing components in the plane of the bilayer. However, finding polymerization chemistries that can be carried out in mild conditions compatible with vaccine antigens is challenging[20].

Here we describe a new class of lipid drug carriers, interbilayer-crosslinked multilamellar vesicles (ICMVs), formed by stabilizing multilamellar vesicles with short covalent crosslinks linking lipid headgroups across the opposing faces of adjacent tightly stacked bilayers within the vesicle walls. ICMVs encapsulated and stably retained high levels of proteins, releasing entrapped cargo very slowly when exposed to serum (over 30 days) compared with simple liposomes or multilamellar vesicles (MLVs) of the same lipid composition. However, these vesicles were quickly degraded in the presence of lipases normally found at high levels within intracellular compartments[31]. Using this new vesicle structure to co-entrap high levels of a model protein antigen (ovalbumin, OVA) and a lipid-like immunostimulatory ligand (monophosphoryl lipid A, MPLA), we carried out immunization studies in mice and found that ICMVs elicited robust antibody titres ~1,000 times greater than simple liposomes and ~10 times greater than MLVs of identical lipid compositions. Unlike live vectors, which are often only effective for a single injection administered to a vector-naïve individual[7], these synthetic vesicles triggered steadily increasing humoral and CD8T responses following repeated administrations, with antigen-specific T cells expanding to a peak of nearly 30% of the total CD8Ts in blood following a prime and two booster immunizations. These new materials may thus open the door to subunit vaccines that are both safe and highly effective for generating both humoral and cellular immunity.

We introduced covalent crosslinks between functionalized lipid headgroups of adjacent, apposed bilayers within preformed MLVs to form ICMVs (Fig. 1a): in a typical synthesis, dried phospholipid films containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), anionic 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) and the anionic maleimide-headgroup lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(p-maleimidophenyl) butyramide (MPB) in a 4:1:5 molar ratio were hydrated and sonicated to form simple liposomes (step (1)). Divalent cations (for example, Mg^{2+}) were added to the liposomes to induce vesicle fusion and the

Table 1 | Particle characterization at each step of ICMV synthesis.

Synthesis step (Fig. 1a)	1	2	3	4
Samples	Liposomes	Mg^{2+}-fused MLVs	ICMVs	PEGylated ICMVs
Hydrodynamic diameter* (nm)	192±39	220±26	244±17	263±20
Polydispersity index	0.385±0.11	0.217±0.053	0.223±0.11	0.183±0.025
Zeta potential (mV)	−0.141±0.44	−0.151±0.67	−0.415±0.33	−2.34±0.44
Diameter after 7 days at 4 °C (nm)	N/A	N/A	1,610±570	265±27
Diameter after lyophilization (nm)	N/A	N/A	N/A	2,960±1,800
Diameter after lyophilization with 3% sucrose (nm)	N/A	N/A	N/A	269±41
Fraction of lipid surface exposed†	0.37±0.023	0.15±0.025	0.19±0.010	N/A

*Measured by dynamic light scattering. †The fraction of lipid exposed on the external surface of vesicles decreased after interbilayer crosslinking as measured by a lamellarity assay[36]. All values given as mean±s.d.

formation of MLVs as reported previously[32] (step (2)). To introduce crosslinks between adjacent bilayers in the MLVs, dithiothreitol (DTT) was then added to the vesicle suspension to act as a membrane-permeable crosslinker, forming a covalent linkage between maleimide headgroups of apposed membranes brought into proximity by the cation salt bridges formed between vesicle layers (step (3)). PEGylation is a well-known strategy to increase the serum stability and blood circulation half-life of lipid vesicles[18]. Thus, as a final step, the vesicles were washed and residual maleimide groups exposed on the external surfaces of the particles were capped with thiol-terminated polyethylene glycol (PEG) (step (4)).

The diameter/polydispersity of the particles will determine the cell types capable of internalizing these particles[33], whereas the number of bilayers comprising the vesicle walls would be expected to impact the stability of the vesicles and their ability to retain/slowly release cargos in the presence of serum. To evaluate these properties and better understand the process of ICMV formation, we characterized the products at each step of the synthesis. The initial liposomes formed by sonication (step (1)) had hydrodynamic diameters of ∼190 nm, and the size increased slightly to ∼240 nm following Mg^{2+}-mediated vesicle fusion (step (2)) and subsequent DTT 'stapling' of the bilayers (step (3), Table 1). The resulting ICMVs showed a unimodal, relatively narrow size distribution (comparable to that of common lipid-vesicle or polymer-nanoparticle preparations[12,21]), and there was no evidence for gross aggregation of particles during the crosslinking step from dynamic light scattering or cryo-electron microscopy (Fig. 1a,b, and Table 1). Addition of thiol-terminated PEG to DTT-treated vesicles quenched remaining detectable maleimide groups on the surfaces of MLVs and introduced PEG chains on ∼2 mol% of the surface-exposed lipids of ICMVs without significantly altering particle diameters (Supplementary Fig. S1a,b and Table 1). PEGylated ICMVs stored at 4 °C or 37 °C in PBS remained stable over 7 days, and they were amenable to lyophilization with 3% sucrose added as an excipient[34], highlighting their compatibility with long-term storage conditions (Table 1 and data not shown). We imaged the initial liposomes, Mg^{2+}-fused MLVs and final ICMVs by cryo-electron microscopy (Fig. 1a), and saw that crosslinking with DTT led to the formation of vesicles with thick multilamellar walls composed of tightly stacked bilayers resolved as ∼4–5 nm electron-dense striations. The median number of bilayers per particle was 4.4 (interquartile range 3.3–6.9), and the median particle-radius-to-lipid-wall-thickness ratio was 3.8 (interquartile range 2.4–6.8; Fig. 1c,d). Interestingly, the majority of ICMVs had vesicle walls composed of concentric bilayers, although a few examples of ICMVs with surface defects in the form of incomplete external lipid layers could also be found (Supplementary Fig. S2). Consistent with the increased lamellarity of the vesicles following cation-mediated fusion and DTT crosslinking observed by electron microscopy imaging, the fraction of lipids exposed on the external surfaces of the vesicles decreased in steps (2) and (3) of the synthesis, as measured by a bulk dye-quenching lamellarity assay[35] (Table 1). Chemical evidence for crosslinking between the maleimide lipids following DTT treatment was found in thin-layer chromatography and matrix-assisted laser desorption/ionization time-of-flight measurements on ICMVs (Supplementary Fig. S3 and data not shown). Importantly, both the particle size and individual lamellarity distributions were unimodal, with less than 3% contaminating unilamellar vesicles and no large aggregates, which could skew the functional properties (for example, protein release) of the particles. The ICMVs have a size that should be avidly taken up by monocytes and dendritic cells[36], and a crosslinked multilamellar wall structure that will stabilize protein entrapment compared with traditional unilamellar or multilamellar liposomes.

Analysis of the conditions required to form stable vesicles provided insight into the mechanisms of ICMV formation. Following interbilayer crosslinking, ICMVs could be collected by centrifuging at 14,000 *g* for 4 min ('low-speed conditions'), whereas simple liposomes or MLVs of identical lipid composition required ultracentrifugation to pellet. Using the mass of particles collected by low-speed centrifugation as a surrogate measure of crosslinked vesicle yield, we found that both divalent-cation-mediated fusion (Fig. 1a step (2), either Mg^{2+} or Ca^{2+}) and DTT treatment (step (3)) were required for ICMV formation (Table 2; entries 1–3). Precursor vesicles treated with either Mg^{2+} or DTT alone even at ×10 molar excess relative to maleimide groups did not generate significant yields of particles (Table 2, entries 4–6). In addition, at least 25 mol% MPB was required to form ICMVs (Table 1, entries 1, 7–9); the high level of reactive headgroups required for substantial ICMV yield may reflect competition between intra- (between headgroups on the same bilayer) and interbilayer crosslink formation. DTT could be replaced with DPDPB, another membrane-permeable dithiol, but not with 2 kDa PEG-dithiol (Supplementary Fig. S4 and Table 2 entries 10, 11). ICMVs could also be formed using only DOPC and MPB lipids, or using cationic 1,2-dioleoyl-3-(trimethylammonium) propane in place of DOPG (data not shown). As an alternative method to form ICMVs, maleimide-dithiol crosslinking could be replaced with bio-orthogonal click chemistry, employing alkyne-terminated lipids for vesicle formation and diazides for crosslinking[37,38] (Supplementary Fig. S5). Thus, interbilayer crosslinking for the formation of stabilized vesicles is a general strategy that can be adapted to other lipid/crosslinker chemistries. On the basis of their high synthetic yield and colloidal stability, we chose to focus on PEGylated ICMVs with a lipid composition of DOPC:DOPG:MPB in a 4:1:5 molar ratio for further testing as protein/vaccine delivery vehicles.

To test the suitability of ICMVs for protein delivery, we examined the entrapment of several globular proteins: SIV-gag, a simian analogue of human immunodeficiency virus vaccine

Table 2 | ICMV particle yield with varying synthesis conditions.

Entry	Lipid composition (molar ratio)*	Cation†	Crosslinker‡	Yield (%)§
1	DOPC/DOPG/MPB (40:10:50)	$MgCl_2$	DTT	45
2	DOPC/DOPG/MPB (40:10:50)	$CaCl_2$	DTT	50
3	DOPC/DOPG/MPB (40:10:50)	20 mM NaCl	DTT	0
4	DOPC/DOPG/MPB (40:10:50)	$MgCl_2$	-	0
5	DOPC/DOPG/MPB (40:10:50)	-	DTT	7
6	DOPC/DOPG/MPB (40:10:50)	-	15 mM DTT	4
7	DOPC/DOPG/MPB (60:15:25)	$MgCl_2$	DTT	15
8	DOPC/DOPG/MPB (72:18:10)	$MgCl_2$	DTT	0
9	DOPC/DOPG (80:20)	$MgCl_2$	DTT	0
10	DOPC/DOPG/MPB (40:10:50)	$MgCl_2$	DPDPB‖	48
11	DOPC/DOPG/MPB (40:10:50)	$MgCl_2$	HS-PEG-HS¶	3

*Hydrated with 10 mM bis-tris propane at pH 7.0. †At 10 mM unless noted otherwise. ‡At 1.5 mM unless noted otherwise. §Percentage of lipid mass recovered after synthesis and centrifugation at 14,000 *g* for 4 min. ‖1,4-Di-[3′-(2′-pyridyldithio)-propionamido]butane (molecular mass 482). ¶Molecular mass 2000.

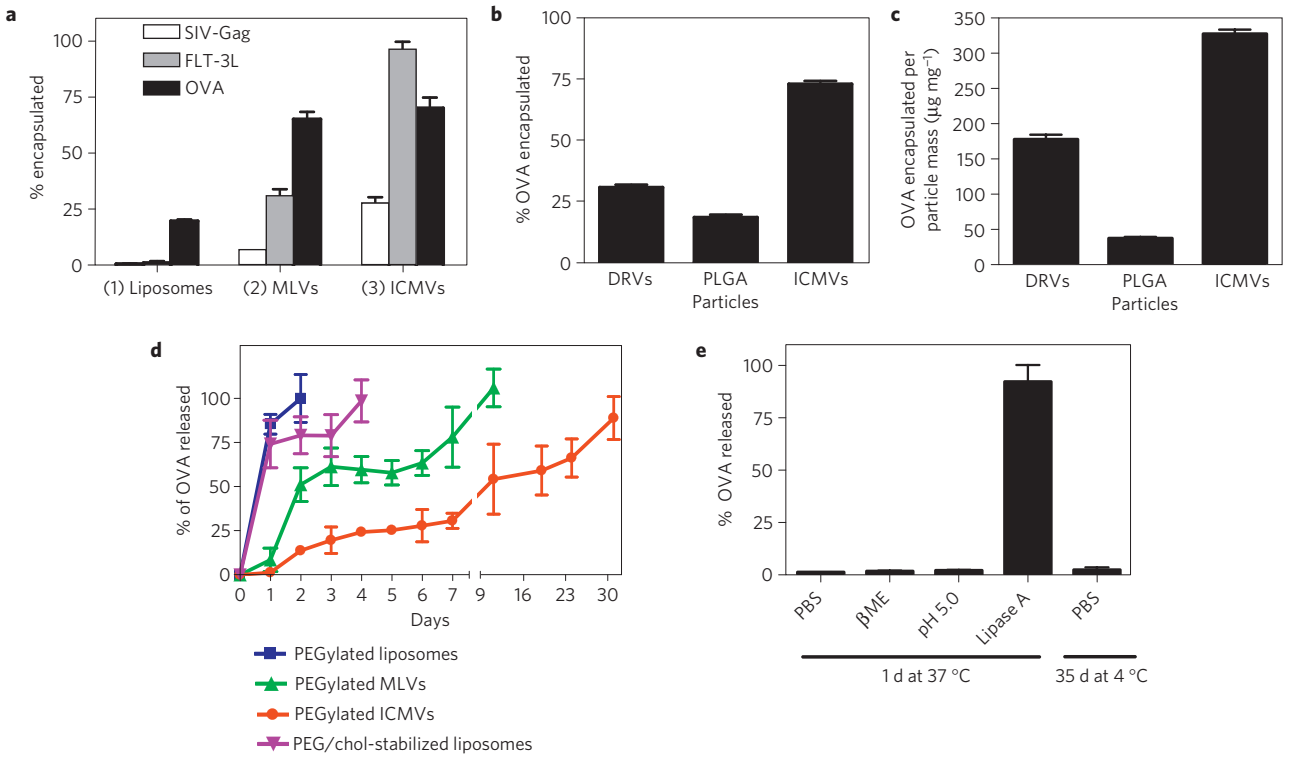

Figure 2 | Protein encapsulation and release from ICMVs. a, Encapsulation efficiency of the globular proteins SIV-gag, FLT-3L or OVA in lipid vesicles collected at each step of ICMV synthesis. **b**,**c**, Comparison of OVA encapsulation efficiency (**b**) and total protein loading per particle mass (**c**) in ICMVs versus DRVs or PLGA nanoparticles. **d**, Kinetics of OVA release from simple liposomes, MLVs or ICMVs (all with base lipid composition 4:5:1 DOPC:MPB:DOPG) incubated in RPMI medium with 10% serum at 37 °C measured over 30 d *in vitro*. Also shown for comparison are release kinetics for liposomes stabilized with cholesterol and PEG-lipid (38:57:5 DOPC:chol:PEG-DOPE). **e**, Release of OVA from ICMVs was measured in buffers simulating different aspects of the endolysomal environment: reducing buffer, 100 mM β-mercaptoethanol (β ME) in PBS; acidic buffer, 50 mM sodium citrate pH 5.0; lipase-containing buffer, 500 ng ml^{-1} lipase A in Hank's buffered saline solution. Data represent the mean ± s.e.m. of at least three experiments with $n = 3$.

antigen; FLT-3L, a therapeutic cytokine, and OVA, a model vaccine antigen. Protein encapsulation was achieved by rehydrating dried lipids with protein solutions in step (1) of the synthesis (Fig. 1a). The amount of encapsulated protein increased at each step of the ICMV preparation (Fig. 2a), which may reflect extra protein entrapment occurring as vesicle fusion occurs in both steps (2) and (3). Protein entrapment in ICMVs was not mediated by conjugation of thiols on the cargo proteins with the maleimide-functionalized lipid vesicles, as OVA prereduced with tris(2-carboxyethyl)phosphine and treated with ethyl maleimide to block all thiol groups on the protein was encapsulated in ICMVs at levels similar to those for unmodified protein (76.1 ± 6.3% versus 83.3 ± 8.4% for capped-thiol versus unmodified OVA; $p = 0.17$). We also confirmed that disulphide linkages in model protein cargos were not reduced by the DTT crosslinker during the vesicle-formation process and that ICMV encapsulation did not trigger protein aggregation (Supplementary Fig. S6). To directly compare the efficiency and quantity of protein loading achieved with ICMVs with two of the most common types of drug-delivery vehicle[39–41], we compared encapsulation of OVA in liposomes, poly(lactic-co-glycolic acid) (PLGA) nanoparticles and ICMVs: using a model stable lipid composition consisting of phosphocholine, PEG-lipid

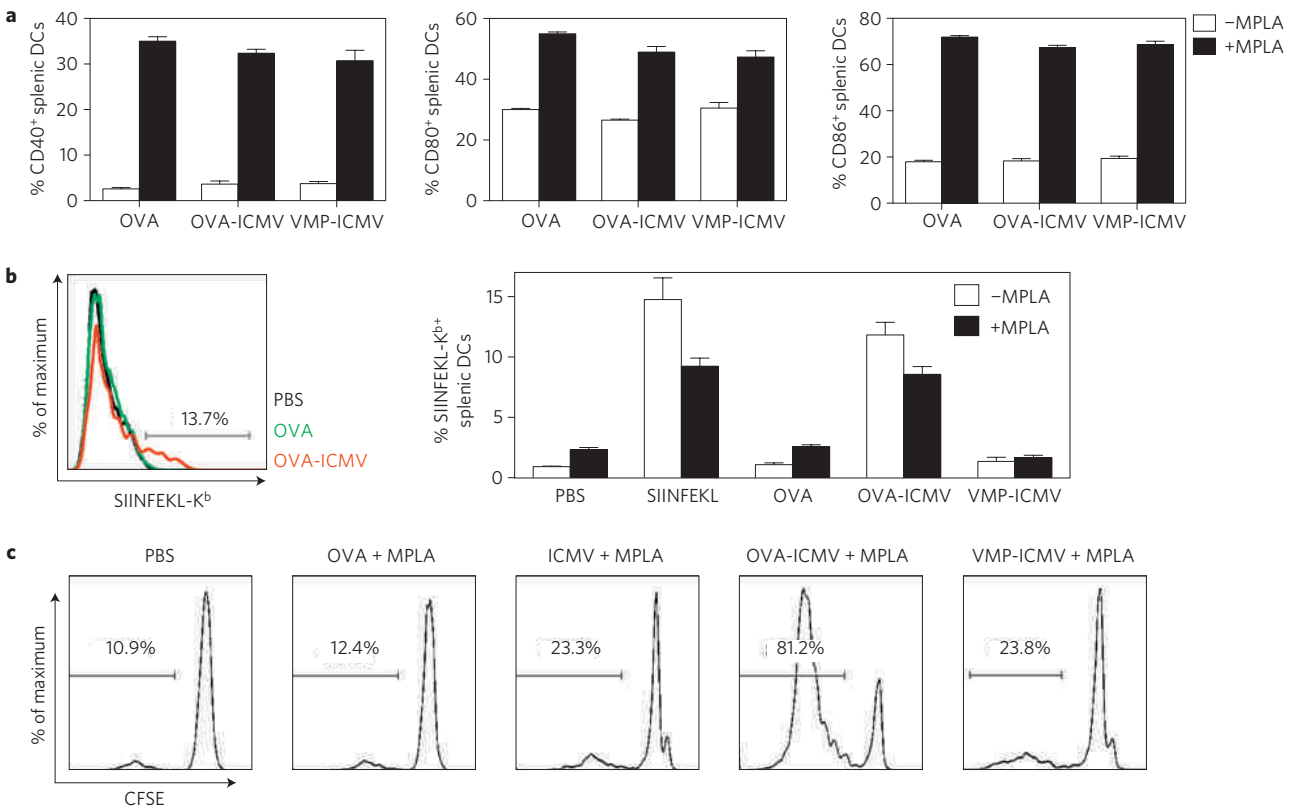

Figure 3 | *In vitro* stimulation of immune responses by ICMVs supplemented with the TLR agonist MPLA. a, Flow-cytometry analysis of expression of the cell surface costimulatory markers CD40, CD80 and CD86 on splenic DCs after 18 h incubation with 0.7 μg ml^{-1} soluble OVA, or equivalent doses of OVA loaded in ICMVs, or ICMVs loaded with an irrelevant protein (VMP), in the presence or absence of 0.1 μg ml^{-1} MPLA. **b**, Splenic DCs were incubated for 18 h with 10 μg ml^{-1} SIINFEKL peptide ($OVA_{257-264}$) and 5.0 μg ml^{-1} soluble OVA, or equivalent doses of OVA loaded in ICMVs, or VMP-loaded ICMVs in the presence or absence of 0.05 μg ml^{-1} MPLA, and the extent of cross-presentation of OVA was assessed by flow-cytometry analysis of cells stained with the 25-D1.16 mAb that recognizes SIINFEKL complexed with H-2K^b. **c**, CFSE-labelled OVA-specific naïve OT-I CD8Ts were cocultured with syngeneic splenic DCs pulsed with soluble 0.7 μg ml^{-1} OVA mixed with 0.1 μg ml^{-1} MPLA, or equivalent doses of OVA-loaded ICMVs mixed with MPLA. Empty ICMVs without antigen or ICMVs loaded with the irrelevant antigen VMP were included as negative controls. Proliferation of CD8Ts was assessed on day 3 by flow-cytometry analysis of the dilution of CFSE in the OT-I CD8Ts; shown are histograms of CFSE fluorescence. Gates on each histogram indicate the percentage of divided cells in each sample. Data represent the mean ± s.e.m. of at least three experiments with $n = 3$–4.

and cholesterol[42], we formed dehydration–rehydration vesicles (DRVs) as one of the most efficient aqueous entrapment approaches for liposomes[43], and prepared OVA-loaded PLGA particles using a double-emulsion solvent-evaporation process[44]. ICMVs exhibited superior encapsulation efficiency (~75%) compared with either DRVs or PLGA particles (two- and fourfold increases, respectively; Fig. 2b), and the amount of OVA encapsulated per total particle mass (~325 μg OVA per mg of particles) was increased in ICMVs by 1.8- and ninefold compared with DRVs or PLGA particles, respectively (Fig. 2c). Thus, ICMVs seem to be effective for encapsulating a variety of globular proteins, and, at least for the model antigen OVA, ICMVs loaded protein more efficiently than common alternative protein carriers.

We next determined whether interbilayer crosslinking enabled lipid vesicles to retain biodegradability while increasing protein retention in the presence of serum. OVA was loaded into PEGylated liposomes, Mg^{2+}-fused MLVs or ICMVs all with the same lipid composition, and the kinetics of protein release at 37 °C in media containing 10% fetal calf serum were quantified. Unilamellar liposomes quickly released their entire payload of entrapped OVA within ~2 days, whereas multilamellar Mg^{2+}-fused MLVs released ~50% of their entrapped cargo over the same time period (Fig. 2d). However, ICMVs showed a significantly enhanced retention of protein, releasing only ~25% of their cargo by one week, and ~90% after 30 days (Fig. 2d). Notably, ICMVs also released protein significantly more slowly than unilamellar liposomes stabilized by the inclusion of cholesterol[42] (Fig. 2d). The crosslinked vesicles also retained ~95% of their entrapped protein when stored in PBS at 4 °C for over 30 days (Fig. 2e). We also examined protein release from ICMVs in conditions modelling intracellular compartments: vesicles incubated in reducing or acidic conditions for 1 day at 37 °C retained >95% of entrapped OVA, whereas incubation with phospholipase A led to the release of >90% OVA and rapid vesicle degradation (Fig. 2e). Thus, ICMVs exhibit enhanced stability in the presence of serum compared with traditional liposomal formulations, but rapidly break down in the presence of enzymes that are present within intracellular endolysosomal compartments[31], providing a mechanism for triggered intracellular release of cargo following internalization by cells. Although some protein degradation within vesicles might be possible on administration *in vivo* before internalization by cells, critical uptake and processing of antigen will occur in the first few days after immunization in vaccine delivery[45], when such degradation processes should be minimal.

We hypothesized that the unique structure of ICMVs, with efficient retention of encapsulated protein antigens in the extracellular environment but rapid release in endosomes/lysosomes, would provide enhanced vaccine responses. To generate vaccine ICMVs, we prepared vesicles carrying the model antigen OVA (OVA-ICMVs) and mixed these vesicles with the molecular

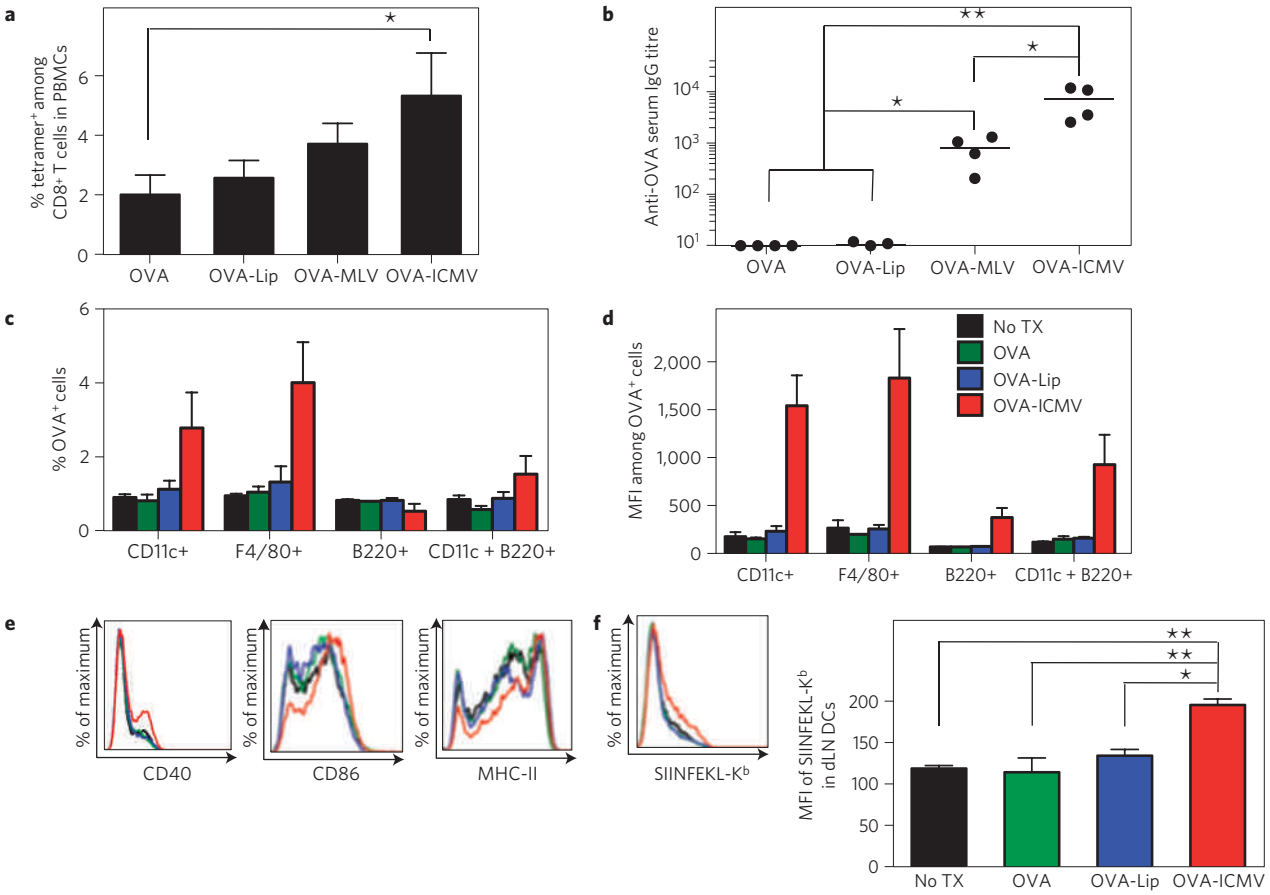

Figure 4 | *In vivo* immunization with ICMVs versus soluble antigen or antigen encapsulated in non-crosslinked vesicles. a,**b**, C57Bl/6 mice were immunized subcutaneously with a single injection of 10 μg OVA delivered in soluble, liposomal, MLV or ICMV formulations, each mixed with 0.1 μg of MPLA. **a**, The percentage of antigen-specific CD8Ts was determined by flow-cytometry analysis of PBMCs 7 days post immunization with fluorescent OVA peptide–MHC tetramers. **b**, Sera from the immunized mice were analysed by enzyme-linked immunosorbent assay 21 days post immunization for OVA-specific IgG. **c**,**d**, C57Bl/6 mice were injected with 10 μg of fluorophore-conjugated OVA mixed with 0.1 μg of MPLA as a soluble, liposomal or ICMV formulation, and the draining inguinal lymph node (dLN) cells that internalized OVA were assessed on day 2. **c**, Percentages of DCs ($CD11c^+$), macrophages ($F4/80^+$), B cells ($B220^+$) and plasmacytoid DCs ($CD11c^+B220^+$) positive for OVA uptake. **d**, The mean fluorescence intensity (MFI) of OVA^+ populations. **e**,**f**, C57Bl/6 mice were injected with 10 μg of OVA mixed with 0.1 μg of MPLA as a soluble, liposomal or ICMV formulation, and 2 d later DCs isolated from draining inguinal LNs were analysed by flow cytometry to assess DC activation and antigen cross-presentation. **e**, Overlaid histograms show costimulatory markers (CD40 and CD86) and MHC-II expression in DCs. **f**, The left panel shows overlaid histograms of inguinal LN DCs stained for SIINFEKL-K^{b+} complexes, and mean MFI levels are shown on the right panel. Data represent mean ± s.e.m. of two or three independent experiments conducted with $n = 3$–4. *, $p < 0.05$ and **, $p < 0.01$, analysed by one-way analysis of variance, followed by Tukey's HSD.

adjuvant monophosphoryl lipid A (MPLA). MPLA is a Food and Drug Administration-approved agonist for Toll-like receptor (TLR) 4 expressed by dendritic cells, B cells and innate immune cells, and potently amplifies vaccine responses[1,46]. Antigen-loaded ICMVs mixed with MPLA promoted upregulation of costimulatory molecules on splenic and bone-marrow dendritic cells (DCs) *in vitro*, compared with DCs pulsed with ICMVs without MPLA (Fig. 3a and Supplementary Fig. S7). DCs pulsed with ICMVs cross-presented peptides derived from OVA with greatly enhanced efficiency compared with those pulsed with soluble OVA (with or without added MPLA), as determined by staining DCs with the 25-D1.16 mAb that recognizes the SIINFEKL peptide ($OVA_{257–264}$) complexed with MHC class I H-$2K^b$ molecules ($p < 0.001$, compared with soluble OVA or ICMVs loaded with irrelevant antigen (vivax malaria protein, VMP), Fig. 3b). Splenic DCs incubated with OVA-ICMVs+MPLA triggered robust proliferation of OVA-specific naïve OT-1 CD8Ts *in vitro*, as assessed by a 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE) dilution assay. In contrast, weak T-cell responses were detected when DCs were pulsed with equivalent doses of soluble OVA and MPLA, empty ICMVs or VMP-ICMVs, indicating the specificity of the T-cell responses elicited by ICMVs (Fig. 3c). These results suggest that addition of MPLA enables equivalent DC activation by ICMVs or soluble OVA, but ICMVs trigger enhanced cross-presentation of the antigen, as expected for particulate antigen delivery.

To determine the influence of vesicle structure on the immune response *in vivo*, we vaccinated C57Bl/6 mice with equivalent doses of OVA, MPLA and lipids (10 μg, 0.1 μg and 142 μg, respectively) in the form of PEGylated unilamellar liposomes, MLVs or ICMVs. Seven days after immunization, we assessed the strength of the endogenous CD8T response by analysing the frequency of OVA peptide–MHC $tetramer^+$ (antigen-specific) CD8Ts among peripheral blood mononuclear cells (PBMCs) by flow cytometry, and found a trend toward increasing T-cell responses in the order soluble OVA < liposomes < Mg^{2+}-fused MLVs < ICMVs (Fig. 4a). At 3 weeks post-immunization, Mg^{2+}-fused MLVs elicited ~100 times greater OVA-specific IgG titres in the sera of the animals compared with soluble OVA or unilamellar liposomes. However, ICMV immunization generated a substantially stronger humoral response, ~1,000 times and ~10 times greater than the

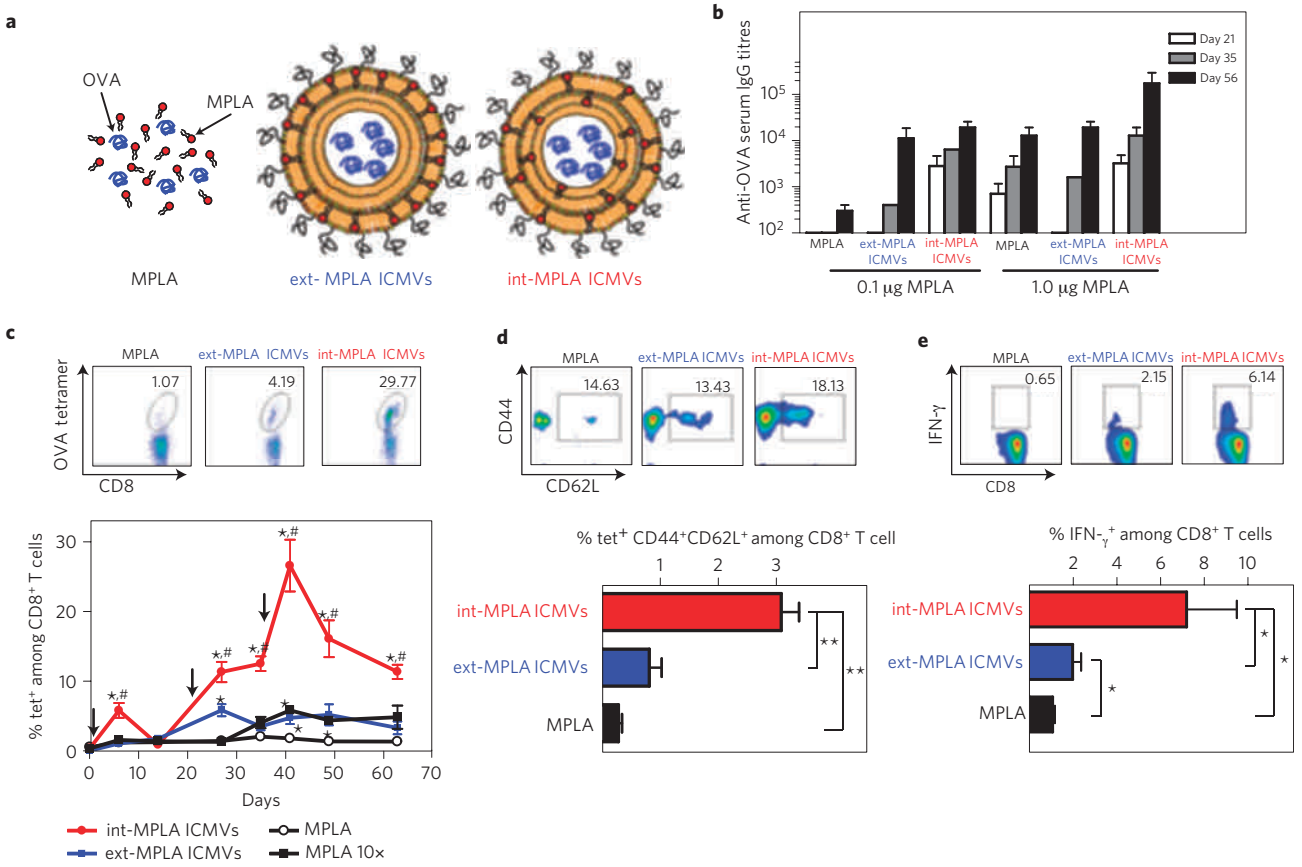

Figure 5 | ICMVs carrying antigen in the aqueous core and MPLA embedded in the vesicle walls elicit potent antibody and CD8T responses. **a**, Schematic illustration of the vaccine groups: soluble OVA mixed with MPLA (MPLA), OVA-loaded ICMVs with MPLA only on the external surface (ext-MPLA ICMVs) or OVA-loaded ICMVs with MPLA throughout the lipid multilayers (int-MPLA ICMVs). **b**–**e**, C57Bl/6 mice were immunized on days 0, 21 and 35 at the tail base subcutaneously with 10 μg OVA and either 0.1 μg or 1.0 μg of MPLA formulated as either MPLA, ext-MPLA ICMVs or int-MPLA ICMVs. **b**, Enzyme-linked immunosorbent assay analysis of total OVA-specific IgG in sera. **c**, Frequency of OVA-specific T cells in peripheral blood assessed over time through flow-cytometry analysis of tetramer$^+$CD8$^+$ T cells for vaccinations with 10 μg OVA and 0.1 μg MPLA. Response to vaccinations with soluble OVA + 1 μg MPLA (MPLA 10×) is also shown for comparison. Shown are representative flow-cytometry scatter plots from individual mice at d41 and mean tetramer$^+$ values from groups of mice versus time. **d**, Analysis of T-cell effector/effector memory/central memory phenotypes in peripheral blood by CD44/CD62L staining on tetramer$^+$ cells from peripheral blood on d41. Shown are representative cytometry plots from individual mice and mean percentages of tet$^+$CD44$^+$CD62L$^+$ cells among CD8Ts at d41. **e**, The functionality of antigen-specific CD8Ts was assayed on d49 with intracellular IFN-γ staining after *ex vivo* restimulation of PBMCs with OVA peptide *in vitro*. Representative flow-cytometry histograms of IFN-γ^+CD8$^+$ T cells from individual mice and mean results from groups are shown. Data represent the mean ± s.e.m. of two independent experiments conducted with $n = 3$. **c**, *, $p < 0.05$ compared with sol OVA + MPLA; #, $p < 0.05$ compared with ext-MPLA ICMVs. **d,e**, *, $p < 0.05$; **, $p < 0.01$, analysed by two-way analysis of variance, followed by Tukey's HSD.

soluble OVA ($p < 0.01$) and non-crosslinked MLV immunizations ($p < 0.05$), respectively (Fig. 4b). Thus, the stabilized structure of ICMVs promoted both T-cell and antibody responses. Enhanced T-cell and antibody responses to immunization with ICMVs compared with other formulations could be attributed to improved antigen delivery to antigen-presenting cells, enhanced activation of DCs, enhanced antigen cross-presentation (as seen *in vitro*) or a combination of these factors. To distinguish between these possibilities, mice were immunized with fluorophore-conjugated OVA mixed with MPLA as a soluble, liposomal or ICMV formulation, and the draining inguinal lymph node cells that internalized OVA were assessed on day 2. OVA delivered by ICMVs was readily detected in total DCs, macrophages and plasmacytoid (CD11c$^+$B220$^+$) DCs in the draining lymph nodes (dLNs), whereas soluble and liposomal formulations showed fluorescence barely above background ($p < 0.01$, Fig. 4c,d). Repeating this analysis with unlabelled OVA, we found that administration of OVA-ICMVs with MPLA triggered a minor enhancement of costimulatory marker and MHC-II expression among DCs in dLNs compared with soluble or liposomal OVA + MPLA (Fig. 4e and Supplementary Fig. S8). However, using the 25-D1.16 antibody to detect OVA peptide presentation, we readily detected OVA peptide–MHC complexes on DCs in the dLNs following ICMV immunization, whereas soluble OVA or liposomal OVA injections did not give staining above the expected background cross-reactivity of 25-D1.16 with self-peptide MHC complexes[47] (Fig. 4f). All together, these results suggest that improved retention of entrapped antigen in the crosslinked multilamellar structures of ICMVs leads to enhanced antigen delivery to antigen-presenting cells, followed by enhanced cross-presentation.

The multilamellar structure of ICMVs offers the opportunity to sequester not only protein antigen (in the aqueous core) but also lipophilic molecules (in the vesicle walls). We thus tested whether embedding MPLA throughout the walls of the ICMVs would impact the immune response *in vivo*, by enabling better retention of MPLA together with antigen in the vesicles. The TLR agonist was incorporated throughout the vesicle layers by codissolving MPLA with the other lipids in the first step of the

synthesis (int-MPLA ICMVs), and we compared these vesicles with ICMVs carrying the same amount of MPLA incorporated only on the vesicle surfaces through a post-insertion approach (ext-MPLA ICMVs, Fig. 5a, Supplementary Methods). Mice were immunized subcutaneously with OVA (10 μg) and MPLA (0.1 μg or 1.0 μg) in ICMVs or soluble form, and boosted on d21 and d35 with the same formulations. As shown in Fig. 5b, immunizations using the low dose of MPLA led to a barely detectable antibody response against soluble OVA even following two boosts, whereas both int-MPLA and ext-MPLA ICMVs elicited strong anti-OVA serum IgG titres by d56. An IgG response to soluble OVA could be obtained using ten times more MPLA, but int-MPLA ICMVs with 1.0 μg MPLA elicited higher titres than soluble protein (∼13 times greater on d56). On the other hand, we did not observe any significant level of antibodies directed against lipid components of ICMVs throughout these immunization studies (data not shown).

Embedding MPLA in the multilayers of ICMVs had a more striking effect on the CD8T response to vaccination. Soluble OVA mixed with 0.1 μg MPLA elicited barely detectable antigen-specific T-cell expansion as assessed by tetramer staining on PBMCs; ext-MPLA ICMV delivery led to a 2.5-fold increased tetramer$^+$ T-cell population by d41 (Fig. 5c, $p < 0.05$). Adding ten times more MPLA enabled soluble OVA immunizations to eventually reach T-cell responses equivalent to ext-MPLA ICMVs following boosting. By contrast, immunization with int-MPLA ICMVs elicited dramatically stronger CD8T responses that continued to expand following each boost, achieving a peak 28% tetramer$^+$ T cells in the CD8$^+$ T-cell population by d41 (five times greater than ext-MPLA ICMVs ($p < 0.05$) and 14 times greater than soluble OVA + MPLA ($p < 0.01$) Fig. 5c). Notably, int-MPLA ICMVs elicited overall a significantly higher frequency of tetramer$^+$CD44$^+$CD62L$^+$ cells ($p < 0.01$, Fig. 5d), a phenotype for central memory T cells known to confer long-lived protection against pathogens and tumours[48]. Antigen-specific T cells elicited by int-MPLA ICMVs persisted even one month after the final boosting, with ∼11% tetramer$^+$ T cells among CD8Ts (three and eight times greater than ext-MPLA ICMVs and soluble OVA + MPLA, respectively, $p < 0.05$ for both, Fig. 5c). To test the functionality of T cells expanded by these immunizations, we assessed the ability of CD8Ts from peripheral blood to produce Interferon γ (IFN-γ) on restimulation *ex vivo* on d49. Mice immunized with int-MPLA ICMVs had much higher levels of IFN-γ-competent T cells than mice receiving ext-MPLA ICMVs or soluble OVA immunizations ($p < 0.05$, Fig. 5e). To our knowledge, in terms of the degree of antigen-specific T-cell expansion, persistence of memory cells and IFN-γ functionality, this is one of the strongest endogenous T-cell responses ever reported for a protein vaccine, comparable to strong live vectors such as recombinant viruses[5,6]. Notably, this is achieved through 'homologous' boosting, repeated immunization with the same particle formulation, a strategy that cannot be used with many live vectors owing to immune responses raised against the pathogen-based delivery vector itself[7].

These studies demonstrate the synthesis of a new class of submicrometre-particle reagents based on crosslinked multilamellar lipid vesicles, which combine a number of attractive features for biomedical applications: the particle synthesis does not require exposure of protein cargos to organic solvents, the lipid basis of the particles makes them inherently biodegradable to metabolizable by-products, the phospholipid shell enables modular entrapment of both lipophilic and hydrophilic cargos, proteins are encapsulated at very high levels per mass of particles and protein release from the particles can be sustained over very long durations. These results suggest that ICMVs may be a very effective vehicle for delivering biomacromolecules, and in particular for vaccine applications. The ability to achieve such strong combined T-cell and antibody responses using a synthetic-particle vaccine could open up new possibilities for vaccination in the setting of infectious disease and cancer.

Materials and methods

Synthesis of ICMVs. 1.26 μmol of lipids in chloroform (typical lipid composition DOPC:DOPG:MPB = 4:1:5 molar ratio, all lipids from Avanti Polar Lipids, Alabaster, AL) were dispensed to glass vials, and the organic solvents were evaporated under vacuum overnight to prepare dried thin lipid films. The lipid films were rehydrated in 10 mM bis–tris propane at pH 7.0 with cargo proteins for 1 h with rigorous vortexing every 10 min, and then 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, Farmingdale, NY). The liposomes formed in this first step were induced to undergo fusion by addition of divalent cations such as Mg^{2+} and Ca^{2+} at a final concentration of 10 mM. The resulting MLVs were incubated with 1.5 mM DTT (maleimide:DTT molar ratio of 2:1) for 1 h at 37 °C to conjugate opposing bilayers of maleimide-functionalized lipids and form crosslinked ICMVs; the resulting vesicles were recovered by centrifugation at 14,000 × g for 4 min, and washed twice with deionized water. For PEGylation, the particles were incubated 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 °C. The resulting particles were centrifuged and washed 3× with deionized water. The final products were either stored in PBS at 4 °C or lyophilized in the presence of 3% sucrose as a cryoprotectant and stored at −20 °C. For some assays, simple liposomes or Mg-fused MLVs were harvested before crosslinking with ultracentrifugation at 115,000 *g* using an Optima ultracentrifuge for 6 h (Beckman Coulter).

***In vitro* protein loading and drug release.** For encapsulation studies, ovalbumin (OVA, Worthington, Lakewood, NJ), SIV-gag (Advanced Bioscience Laboratories, Kensington, MD) and FLT-3L (Peprotech, Rocky Hill, NJ) were labelled with Alexa-Fluor 555 (Invitrogen, Carlsbad, CA) for direct fluorometric quantification of the amount of protein entrapped. OVA was also encapsulated in DRVs and PLGA nanoparticles as described previously[43,44]. In some experiments, ICMVs were loaded with a recombinant VMP as an irrelevant antigen control[49] (provided by A. Yadava, Walter Reed Army Institute of Research). Capped-thiol OVA was prepared by incubating 1 mg of OVA with 1.5 mM tris(2-carboxyethyl)phosphine for 1 h at RT, followed by incubation with 1.5 mM ethyl maleimide (Pierce, Rockford, IL) at 37 °C for 1 h. The extent of thiol protection was >95% as assessed with Ellman's assay[50]. Release of OVA labelled with Alexa-Fluor 555 from lipid vesicles was quantified in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal calf serum at 37 °C using dialysis membranes with a molecular mass cutoff of 100 kDa. At regular intervals, the releasing media were removed for quantification of fluorescence, and an equal volume of fresh medium was replaced for continued monitoring of drug release. Residual OVA remaining at the end of the time-course was determined by lipid extraction of vesicles with 1% Triton X-100 treatment and measuring released protein by fluorescence spectrophotometry. OVA release assays were also carried out in Hank's buffered saline solution supplemented with 500 ng ml^{-1} of phospholipase A (Sigma, St Louis, MO). To examine the stability of encapsulated cargo molecules, monoclonal rat IgG encapsulated in ICMVs was retrieved with 1% Triton X-100 treatment and analysed with SDS-PAGE under non-reducing conditions with silver staining (Pierce).

Vaccination study with ICMVs. Groups of C57Bl/6 mice (Jackson Laboratories) were immunized subcutaneously in the tail base with indicated doses of OVA (with or without TLR agonists, MPLA). Frequencies of OVA-specific CD8Ts and their phenotypes elicited by immunization were determined by flow-cytometry analysis of PBMCs at selected time points following staining with 4,6-diamidino-2-phenylindole (to discriminate live/dead cells), anti-CD8 α, anti-CD44, anti-CD62L and SIINFEKL/H-2K^b peptide–MHC tetramers (Becton Dickinson). To assess the functionality of primed CD8Ts, PBMCs were stimulated *ex vivo* with 1 μM OVA-peptide SIINFEKL for 6 h with GolgiPlug (Becton Dickinson), fixed, permeabilized, stained with anti- IFN-γ and CD8 α, and analysed by flow cytometry. Anti-OVA IgG titres, defined as the dilution of sera at which the 450 nm OD reading is 0.5, were determined by enzyme-linked immunosorbent assay analysis of sera from immunized mice. Animals were cared for following National Institutes of Health, state and local guidelines.

Statistical analysis. Statistical analysis was carried out with Jmp 5.1 (SAS Institute, Cary, NC). Data sets were analysed using one- or two-way analysis of variance, followed by Tukey's HSD test for multiple comparisons. *p*-values less than 0.05 were considered statistically significant. All values are reported as mean ± s.e.m.

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Author contributions

J.J.M. and D.J.I. designed the experiments. J.J.M. carried out the experiments; H.S. assisted in the *in vivo* characterization and immunization studies. A.B., H.K., J.T.G., J.R. and W.C. contributed cryoelectron microscope imaging. M.T.S. and S.H.U. contributed experimental suggestions. H.L., B.H., M.S. and S.L. provided technical support. J.J.M. and D.J.I. analysed the data and wrote the paper.

Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper on www.nature.com/naturematerials. Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions. Correspondence and requests for materials should be addressed to D.J.I.