Immunogenicity of the Lyme Disease Antigen OspA, Particleized by Cobalt Porphyrin-Phospholipid Liposomes

Jasmin Federizon[1], Amber Frye[2,3], Wei-Chiao Huang[1], Thomas M. Hart[2,4], Xuedan He[1], Christopher Beltran[2], Ashley L. Marcinkiewicz[2], Iain L. Mainprize[5], Melanie K. B. Wills[5], Yi-Pin Lin[2,3], and Jonathan F. Lovell[1*]

[1]Department of Biomedical Engineering, State University of New York at Buffalo, Buffalo, NY, USA 14260

[2]Division of Infectious Diseases, Wadsworth Center, New York State Department of Health, Albany, New York, USA 12208

[3]Department of Biomedical Sciences, State University of New York at Albany, Albany, NY, USA 12222

[4]Department of Biological Sciences, State University of New York at Albany, Albany, NY, USA 12222

[5]G. Magnotta Lyme Disease Research Lab, Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada N1G 2W1

ABSTRACT

Outer surface protein A (OspA) is a *Borrelia* lipoprotein and an established Lyme disease vaccine target. Admixing non-lipidated, recombinant *B. burgdorferi* OspA with liposomes containing cobalt porphyrin-phospholipid (CoPoP) resulted in rapid, particulate surface display of the conformationally intact antigen. Particleization was serum-stable and led to enhanced antigen uptake in murine macrophages *in vitro*. Mouse immunization using CoPoP liposomes elicited a Th1-biased OspA antibody response with higher IgG production compared to other vaccine adjuvants. Antibodies were reactive with intact *B. burgdorferi* spirochetes and *Borrelia* lysates, and induced complement-mediated borreliacidal activity *in vitro*. One year after initial immunization, mice maintained high levels of circulating borreliacidal antibodies capable of blocking *B. burgdorferi* transmission from infected ticks to human blood in a feeding chamber.

Keywords: Liposomes; Adjuvant; Particle vaccine; Lyme Disease; Borrelia; OspA

Highlights:

- His-tagged, recombinant *B. burgdorferi* OspA was produced and spontaneously and stably binds to liposomes containing cobalt porphyrin-phospolipid (CoPoP).
- Particleized OspA is effectively taken by macrophages *in vitro*.
- In mice, OspA is more immunogenic when admixed with CoPoP liposomes, relative to other adjuvants.
- Induced antibodies recognize spirochetes and have complement-mediated borreliacidal activity.
- One year after immunization, mice retain circulating OspA antibodies that block spirochete transmission from infected ticks to human blood in a feeding chamber.

INTRODUCTION

Lyme disease, a tick-borne disease endemic in the Northern hemisphere, is a multi-organ disorder caused by spirochetes belonging to the group, *B. burgdorferi* sensu lato complex.[1, 2] The main etiologic agent in the United States is *B. burgdorferi* sensu stricto, whereas major pathogenic genospecies in Europe also include *B. afzelii* and *B. garinii*. Without timely diagnosis and treatment, this infectious disease can lead to chronic complications in the late disseminated stage.[3] Due to climate change, regions of endemicity continue to expand, stressing the need for effective preventive measures.[4, 5] Prophylactic immunization against Lyme disease represents an attractive approach in preventing risk of contracting the disease. A leading vaccinogen is outer surface protein (Osp) A, a surface lipoprotein expressed by Lyme *borreliae* while residing in the tick gut. OspA expression facilitates spirochete colonization and persistence in the vector's gut by binding to the tick receptor of OspA (TROSPA).[6] The mechanism of action of OspA-based vaccines is based on inhibition of tick-to-human spirochete transmission, through antibody-mediated borreliacidal killing, and antibody-mediated blocking of spirochete escape from the tick midgut [7-9] Epitope mapping studies showed that some protective OspA antibodies recognize conformational epitopes in its C-terminal domain.[10-13] Hence, it is desirable to maintain the native conformation of an OspA antigen to convey protective immunity.

Historically, many advanced Lyme disease vaccine strategies have involved OspA.[14] Veterinary vaccines available in the market incorporate bacteria-derived materials that express OspA, or recombinant forms.[15-20] Valneva's VLA15, a human Lyme disease vaccine presently in clinical trials, is a multivalent OspA-based vaccine.[21-23] OspA was also the vaccinogen in the LYMErix vaccine which was withdrawn due in part to autoimmunity safety concerns.[24] Shorter linear peptide epitopes of OspA have recently been considered for vaccine development.[25] Due to the difficulty in expressing the full-length recombinant OspA in *Escherichia coli*, a truncated form of OspA was constructed by eliminating the lipidation signal sequence and the adjacent cysteine

residue that encompass the first 17 amino acid residues. Although this deletion can improve expression yield without compromising the conformational stability of the non-lipidated construct [26], it consequently lowers immunogenicity.[27, 28] Co-administration of an adjuvant is thus beneficial to enhance immunogenicity of non-lipidated OspA. Adjuvants are recognized as useful tools for improving the efficiency of vaccines, in particular for recombinant or subunit vaccines.[29, 30] Liposomes serve as versatile vaccine adjuvant, and can be used to carry a wide range of additional immunostimulatory molecules.[31] GSK's AS01 liposome adjuvant, which contains the immunopotentiators QS-21 and monophosphoryl lipid A is an adjuvant component of the Shingrix herpes zoster vaccine and RTS,S malaria vaccine [32]. Liposomes have been explored in preclinical Lyme disease vaccine research [33, 34].

Liposome metallochelation offers a strategy to surface-functionalize nanoscale scaffolds via noncovalent conjugation, using proteins with a short polyhistidine sequence (his-tag) as an anchor [34]. The present study employs a self-assembling liposomal platform containing cobalt porphyrin-phospholipid (CoPoP) that enables facile and serum-stable antigen functionalization with aqueous incubation of his-tagged antigen with the metallochelating liposome. Inaccessibility of the porphyrin moieties from the aqueous milieu has been show to render antigen attachment highly stable under physiological conditions and in the presence of large excess of competing imidazole.[35] This is compared to nickel-chelated liposomes using lipid headgroup-conjugated nickel nitrilotriacetic acid (Ni-NTA), which places the chelating metal exposed to the aqueous environment and, for example, were shown to release a substantial amount of his-tagged in 1 hr in serum at 37 °C for a his-tagged OspC Lyme disease antigen.[36] Liposomes allow for co-formulation of other immunostimulatory lipid adjuvants, such as phosphorylated hexazcyl disaccharide (PHAD), which can further boost immunogenicity. We previously showed that the approach of using liposomes incorporating CoPoP and PHAD was effective using a malaria transmission blocking antigen [37]. Lyme OspA vaccines share some similarities to malaria

transmission blocking vaccines in requiring antibodies that are active in the midgut of the vector following a blood meal [38]. In this work, we assess antigen-functionalized liposomes formed by binding his-tagged OspA to CoPoP/PHAD liposomes.

MATERIALS AND METHODS

Liposome preparation and characterization

CoPoP/PHAD liposomes composed of four parts 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Corden # LP-R4-057), two parts cholesterol (PhytoChol, Wilshire Technologies), one part PHAD (Avanti # 699800P), and one part laboratory-made CoPoP by mass were prepared as previously described [37]. In brief, liposomal components were dissolved in ethanol at 60 °C, followed by slow addition of pre-heated PBS and then nitrogen-pressurized lipid extrusion at 200 PSI using a membrane stack of decreasing size (200, 100, and 80 nm). Extruded liposomes were dialyzed in PBS at 4 °C to remove ethanol and then were characterized by dynamic light scattering using NanoBrook 90Plus PALS instrument to measure liposome size and polydispersity index. After determining CoPoP concentration, the liposome solution was diluted to adjust concentration to 320 μg/mL CoPoP and PHAD. An analogous preparation was conducted for PoP/PHAD liposomes, which lack cobalt.

Protein expression and purification

The DNA sequence encoding for non-lipidated OspA (*B. burgdorferi* B31, **Supplementary Figure S1**) was synthesized into a pET21a plasmid by Genscript, which was transformed into BL21 (DE3) competent *Escherichia coli* cells. Transformed cells were grown at 37 °C in 250 mL Luria Bertani (VWR # N526) broth to an OD_{600} of 0.6 - 0.8 prior to induction with isopropyl β-D-1-thiogalactopyranoside (Corning # 46-102-RF). Bacterial growth continued at 22 °C overnight after induction. Bacterial cells were then harvested by centrifugation, re-suspended in modified binding buffer at pH 7.4, and lysed by sonication. Cell debris were pelleted by centrifugation and the

protein was purified from collected supernatant by immobilized metal affinity chromatography. The manufacturer's recommended protocol for Ni-NTA resin (G Biosciences # 786-939) was modified by including another wash buffer supplemented with 0.5 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, BioShop CHA003) that facilitates removal of endotoxin. Pure fractions determined from SDS-PAGE (Tris-Glycine buffer system) were then dialyzed to remove imidazole. Protein concentration was quantified using micro-BCA assay (Thermo Fisher Scientific # 23235). Far-UV CD spectroscopic data of non-lipidated OspA in 20 mM sodium phosphate pH 7.4 was acquired at room temperature on a bandwidth of 1 nm, scan rate of 50 nm/min, pathlength of 0.1 cm, and accumulations of three scans using Jasco J-815 CD spectrometer. Secondary structure content was calculated by deconvolution of the buffer-corrected spectral data using analysis program CDSSTR and reference set 7 provided by DichroWeb online server.

Characterization of OspA binding to CoPoP/PHAD liposomes

Non-lipidated, his-tagged OspA diluted to 80 µg/mL was incubated with CoPoP/PHAD liposomes at 4:1 mass ratio of CoPoP:protein, unless otherwise stated. Liposomes were then pelleted by high-speed centrifugation and any unbound protein in the resulting supernatant was quantified by micro-BCA assay. A non-his-tagged lysozyme (VWR # 97062-138) was used as negative control. Percent binding was calculated based on the absorbance signal of the free protein. For non-denaturing electrophoretic analysis, protein binding was evaluated using a histidine-MOPS buffer system at near neutral pH (6.8). Due to the protein's net positive charge under native conditions, polarity of the voltages applied to electrophoretic cells was reversed. Transmission electron micrographs were acquired using negative staining techniques. After deposition of 10 µL of the liposome sample on carbon-coated mesh grids (Carbon type-A, 300 mesh, copper, Ted Pella # 01821), the grids were stained with 2% uranyl acetate. Images were then captured by JEM-2010 electron microscope at 200 kV using various magnifications.

Murine vaccination and adjuvant formulations

Animal experiments were conducted in accord to University at Buffalo IACUC. Eight-week-old female CD-1 (ICR) mice received intramuscular injections containing 100 ng of non-lipidated OspA combined with indicated adjuvants on days 0 and 21. CoPoP/PHAD and PoP/PHAD liposomes were incubated with OspA at 1:4 mass ratio of protein:PHAD for 3 hr at room temperature prior to injection and diluted in PBS to achieve desired antigen dose for immunization. Vaccine formulation per one dose consists of 100 ng OspA, 0.4 μg CoPoP, 0.4 μg PHAD, 0.8 μg cholesterol, and 1.6 μg DPPC. For commercial adjuvants, AddaVax (InvivoGen # vac-adx-10) and Adju-Phos (InvivoGen, # vac-phos-250), vaccine formulations were prepared according to manufacturer's instructions. For Alhydrogel 2% aluminium gel (Accurate Chemical and Scientific Corporation # A1090BS), alum was mixed with the antigen to a final concentration of 1.5 mg/mL. Final bleed was done on day 42 unless otherwise stated. Serum was collected after centrifugation at 2,000 rcf for 15 min.

Antibody titer and immunoglobulin isotype profiling

Anti-OspA IgG titers were estimated by enzyme-linked immunosorbent assay (ELISA). A 96-well plate (Thermo Scientific Nunc # 442404) was coated with 100 ng/well OspA, blocked with 2% bovine serum albumin (BSA) in PBS containing 0.1% Tween-20 (PBS-T), and incubated with mouse serum serially diluted in 1% BSA in PBST. After incubation with horse radish peroxidase-conjugated goat anti-mouse secondary antibody IgG (Genscript # A00160), IgG1 (Invitrogen A10551), or IgG2a (Invitrogen # A10685), tetramethylbenzidine (Amresco # J644) was added. Endpoint titers were defined as the reciprocal serum dilution at absorbance (450 nm) cutoff of 0.5.

OspA fluorescent labeling

Prior to labeling with DY-490-NHS-Ester (Dyomics # 490-01), non-lipidated OspA was dialyzed at 4 °C against sodium bicarbonate solution pH 9.3 at least twice. Stock solution of the dye was added at fivefold molar excess to the protein, followed by stirring at room temperature for 2 hr. Extensive dialysis against PBS was then performed to remove free dye. Post-dialysis protein concentration was quantified using micro-BCA assay.

Serum stability of liposome-bound OspA

After incubation of liposomes with fluorescent-labeled OspA, human serum was added to a final concentration of 20% (v/v) and then incubated at 37 °C. Aliquots were taken at different time points to monitor fluorescence quenching, which directly correlates to protein binding. Fluorescence measurements were acquired at excitation and emission wavelengths of 491 and 515 nm, respectively, on a 5 nm bandwidth using TECAN Safire multi-plate reader. Recovery of fluorescence signal for CoPoP/PHAD liposomes was performed by incubating the sample in 0.1% Triton X-100, 100 µg/mL proteinase K (EMD Millipore # 539480) for 30 min at 50 °C. The percent fluorescence quenched was calculated by comparing to free DY490-conjugated OspA.

Immunoprecipitation assay

Following the recommended protocol for Protein G Magnetic Beads (New England Biolabs # S1430S), an OspA-specific monoclonal antibody LA-2 (Absolute Antibody # Ab01070-10.0) was incubated with pre-washed magnetic beads at 4 °C for at least 30 min. CoPoP/PoP/PHAD liposomes with bound non-lipidated OspA were incubated with the antibody-coated magnetic beads for 4 hr at room temperature. CoPoP and PoP were both included in the same liposomes for fluorometric analsysis. At the end of the incubation period, the beads were pelleted using a magnetic separation rack after extensive washing with PBS and then re-suspended in 0.1% Triton X-100, PBS to release any liposomal components. Fluorescence the supernatant was assessed at excitation and emission wavelengths of 420 and 670 nm, respectively, on a 5 nm bandwidth

using TECAN Safire multi-plate reader. The percent liposomes captured was calculated based on a fluorescent standard curve of the liposomes, based on its PoP component. An irrelevant rat monoclonal antibody we had on hand, specific for Pfs48/45, a malaria antigen, was used as a negative control for immunoprecipitation.

Nanoparticle uptake study

RAW264.7 murine macrophage-like cells (ATCC # TIB-71) were cultured in a 24-well plate in Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher Scientific) containing 10% fetal bovine serum, 1% penicillin/streptomycin and grown to a confluence of approximately 70-80%. Macrophage cells were incubated for 2 hr at 37 °C with the indicated liposome solution at OspA-Dy490 final concentration of 1 µg/mL. Cytochalasin B (Acros # 228090010) was supplemented to the medium at a final concentration of 10 µg/mL at least 1 hr prior to incubation with indicated sample. Following incubation, macrophage cells were re-suspended in PBS and subjected to flow cytometry using BD LSRFortessa X-20 flow cytometer. FlowJo (version 10) software was used for data analysis.

To further assess liposome uptake in macrophages, 5 x 10^4 RAW264.7 cells per were cultured in a 96-well plate. Macrophage cells were pre-incubated with the following inhibitors: Amiloride (VWR# 89152-354), Chlorpromazine (VWR# TCC2481-5G), Cytochalasin B (VWR# 200024-888), Nystatin (VWR# 97062-788), and Genistein (VWR# 89148-898), at 125, 25, 5 or 1 µg/mL for 1 hr. Cells were then incubated with PoP/PHAD liposomes (4 µg/mL of PoP) for 3 hr without removing the inhibitors. After 3 hr of incubation, cells were washed with PBS for 3 times to remove PoP/PHAD liposomes in the medium and cells were treated with 200 µl of lysis buffer (1% Triton X-100 in PBS). The fluorescence signal (420 nm excitation, 670 nm emission) of PoP was measured in microplate reader (TECAN Safire). Cell treated with PoP/PHAD liposomes without inhibitor were used as positive control, representing 100% uptake of liposomes into macrophages.

Western blot

Bacterial cultures (*B. burgdorferi* B31, ATCC #35210; *B. afzelii* BO23, ATCC #51992, *B. garinii* CIP 103362 ATCC #51383; *B. hermsii* HS1 ATCC #BAA-2821; *B. kurtenbachii* 25015 ATCC #BAA-2495) grown in BSK-H media (Sigma) containing 6% rabbit serum at 33 °C, 5% CO_2 were re-suspended in 1% SDS and boiled for at least 20 min, followed by centrifugation to remove cellular debris. After protein quantification using DC Protein Assay (Bio-Rad), 1.5 µg of cell lysate was loaded onto a 12% Tris-Glycine gel under denaturing conditions and separated proteins in the acrylamide gel were transferred to a ProTran nitrocellullose membranes (GE Healthcare LifeSciences) using a semi-dry Power Blotter XL (ThermoFisher Scientific). Immunoblot was blocked with 1% BSA in Tris-buffered saline containing 0.1% Tween20 (TBS-T) and then incubated with diluted mouse sera (1/2000) in TBS-T overnight at 4 °C. After washing, immunoblot was incubated with anti-mouse secondary antibody conjugated with horse-radish peroxidase (Jackson ImmunoResearch Laboratories Inc.) diluted 1/6667 in 1% BSA, TBS-T for 1.5 hr. Chemiluminescence was visualized using Lumina Crescendo Western HRP Substrate (Millipore Sigma).

Indirect immunofluorescence assay

For qualitative determination of specificity of IgG antibodies in mouse serum, the recommended protocol for *B. burgdorferi* (strain B31) antigen substrate slides (MBL International # BB-6112) was followed. A 1/500 dilution was carried out for both mouse serum antibody and DyLight488-conjugated goat anti-mouse IgG secondary antibody (ImmunoReagents # GtxMu-003-F488NHSX). Slides were mounted with ProLong Gold Antifade and imaged with an EVOS FL microscope using a 40× objective lens.

Splenocyte study

Murine spleen collected on day 42 post-immunization was excised and passed through a sterile Nylon cell strainer, followed by RBC lysis and re-suspension in Dulbecco's Phosphate-Buffered Saline (Thermo Fisher Scientific). After cell counting, isolated splenocytes were diluted to 2.5 x 10^5 $well^{-1}$ in RPMI 1640 Medium (ThermoFisher Scientific Gibco) containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% Eagle's medium nonessential amino acids, and 1% penicillin/streptomycin and then incubated with non-lipidated OspA (final concentration of 1 µg/mL) for 72 hr at 37 °C. Following antigen stimulation, interferon-gamma and interleukin-4 were quantified using standard ELISA based on a standard curve of the cytokine.

Borreliacidal assay

The *B. burgdorferi* strain B31-A3 used in this study is a clonal isolate of B31[39] and was cultivated at 33 °C in BSKII complete medium to mid-log phase. Two-fold serial dilution of the heat-inactivated mouse serum (56 °C, 30 min) was performed starting at 1/20 dilution. Then, 50 µL of the diluted serum and 10 µL of guinea pig serum (Sigma-Aldrich # S1639) were mixed with 40 µL of BSK II complete medium containing 5 x 10^5 cells of *B. burgdorferi* strain B31-A3 and subsequently incubated at 33 °C for 24 hr. Surviving spirochetes were quantified by direct counting of motile spirochetes under dark field microscopy. Survival percentage was determined from the proportion of serum-treated to untreated spirochetes. For quantitative comparison, 50% borreliacidal titer, which represents the dilution rate that effectively eradicated 50% of the spirochetes, was calculated using dose-response stimulation fitting in GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA, USA).

Feeding chamber assay

Artificial feeding chambers were prepared by modifying the chamber model previously reported by us and others [40-43]. The silicone rubber-saturated rayon membrane was generated as described [43] and was used to mimic the hardness and elasticity of skin. Such membrane was

attached to one side of a 2-cm length of polycarbonate tubing (hereafter called the chamber; inner diameter: 2.5 cm; outer diameter: 3.2 cm; (Amazon Inc.), as described [43]. The hair and hair extract from white-tailed deer (*Odocoileus virginianus*) were generated as described [41], used as feeding stimuli, and added into the chamber. Then, a 1.5-cm square of fiberglass mesh tape (3-mm pore) (Lowe's Inc., Mooresville, NC), a 1.5-cm plastic tile spacer (Lowe's Inc.), a nickel coin, and *I. scapularis* nymphs carrying *B. burgdorferi* strains B31-A3 (10 ticks per chamber) were added onto the chamber. The chamber was then sealed using parafilm. Prior to the experiment, human blood obtained from New York Blood Center (New York, NY) was defibrillated by mixing with citrate-phosphate-dextrose solution (final concentration as 12.28%; Sigma). Subsequently, the blood was supplemented with a cocktail of antibiotics (final concentration: 50 µg/mL rifampicin, 20 µg/mL phosphomycin and 2.5 µg/mL amphotericin; Sigma) to avoid microbial contamination. ATP (final concentration 1µM; Sigma) and glucose (final concentration as 2 mg/mL; Sigma) were also added as these reagents have been reported to enhance the efficiency of tick feeding [43]. The blood was added into six-well cell culture plate wells (VWR) and warmed to 37 °C, and the chambers with ticks were placed on these six-well cell culture plate wells. The cell culture plate wells were then placed into a 37 °C incubator with 5% of CO_2. Blood was replaced with fresh blood every 24 hours. Five days post-incubation, ticks and blood were collected. DNA was then extracted using Bio Basic EZ-10 Spin Column Genomic DNA Minipreps Kit for animal samples following the manufacturer's instructions (Bio Basic). DNA quality and quantity were assessed for each sample using a Nanodrop 1000 UV/Vis spectrophotometer (ThermoFisher) by determining the concentration of DNA and the ratio of UV adsorption at 260 nm to 280 nm. The A_{260}:A_{280} ratios were between 1.75 to 1.85, which indicates the lack of RNA or proteins. Quantitative PCR (qPCR) was then performed using an Applied Biosystems 7500 Real-Time PCR system (ThermoFisher) to determine the presence of blood in ticks and bacterial burdens in the blood and ticks. SYBR-based quantitative PCR was used to determine bacterial burdens in the ticks and blood using *Borrelia* 16s ribosomal RNA primers as previously described [44].

RESULTS AND DISCUSSION

We expressed a recombinant OspA for this study. The N-terminus lipidation signal sequence was replaced with a his-tag to facilitate purification, which was carried out with Ni-NTA affinity chromatography. Electrophoretic analysis indicated a homogeneous protein preparation and confirmed the expected molecular weight of the purified protein, which is about 29 kDa (**Supplementary Figure S2**). Structural characterization using circular dichroism spectroscopy confirmed the expected foldedness of the purified protein. OspA displayed a CD spectrum with a maximum at 195 nm and a minimum at 218 nm characteristic for anti-parallel β-pleated sheets (**Supplementary Figure S2**). Calculated secondary structure content from the deconvolution of the CD spectral data is in close agreement with the reported values in the literature.[45]

Spontaneous formation of the functionalized CoPoP liposomes occurs via insertion of the his-tag into the hydrophobic bilayer and subsequent coordination of the imidazole moiety to the cobalt center.[35, 37] Binding conditions were evaluated using native electrophoresis, which allows physical separation of liposome-bound and free proteins due to the limiting pore size of the acrylamide gel. An observed optimum binding mass ratio of 1:4 of OspA:CoPoP (**Figure 1A**) was consistent with previous studies using the his-tagged malaria antigen, Pfs25.[37] Incubating 80 μg/mL OspA with an equal volume of 320 μg/mL CoPoP liposomes led to rapid binding of the antigen (**Figure 1B**). Based upon the relative band intensities, most binding occurred between 0 and 15 minutes, with some additional binding occurring with longer incubation. 3 hour incubation time was used for further studies. Based on a microBCA assay of the supernatant obtained from high speed centrifugation, specific OspA binding is estimated to be about 80% in these conditions (**Figure 1C**). Using the same method, low non-specific binding was observed for PoP/PHAD liposomes, which lacks the chelating metal. A non-his-tagged protein, lysozyme, bound neither CoPoP/PHAD nor PoP/PHAD liposomes. Based upon dynamic light scattering measurements, post-incubation liposomal size remains close to 100 nm for both CoPoP/PHAD and PoP/PHAD

liposomes, with relatively monodisperse size distribution (**Figure 1D**). Transmission electron micrographs revealed that CoPoP/PHAD liposomes retained their spherical morphology and size close to 100 nm after antigen binding (**Figure 1E**).

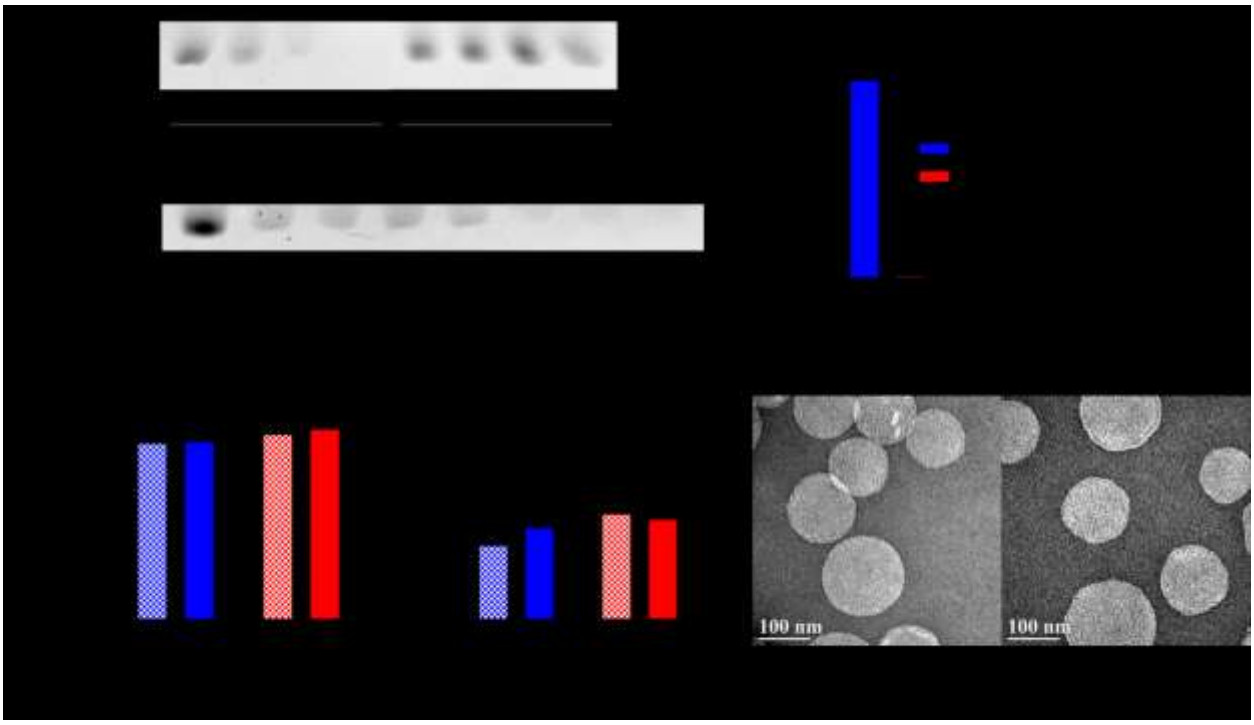

Figure 1. Spontaneous binding of his-tagged OspA to CoPoP/PHAD liposomes. A) Effect of varying mass ratios of OspA:CoPoP/PHAD liposomes evaluated by native PAGE. The visible bands that migrated in gel represent unbound protein. **B)** Kinetics of OspA binding to CoPoP/PHAD liposomes incubated at 1:4 mass ratio at room temperature. **C)** Liposome binding of his-tagged OspA or non-his-tagged lysozyme measured by microBCA assay of the supernatant following high-speed centrifugation. **D)** Hydrodynamic diameter and polydispersity index of liposomes with or without OspA incubation measured by dynamic light scattering. Error bars represent standard deviations for n=3 measurements. **F)** Negative-stained electron micrographs of CoPoP/PHAD liposomes with or without bound OspA.

Serum stability was assessed using fluorescently-labeled OspA (**Figure 2A**). Upon binding to liposomes, the fluorescent label undergoes energy transfer to the porphyrin moieties in the bilayer and the overall fluorescence becomes quenched. Incubation of OspA-bound CoPoP/PHAD liposomes with human serum at 37 °C did not significantly increase the fluorescence signal after 12 days. This reflects that OspA remains associated to the metallochelating liposomes within the

duration of the study. Liposomes lacking cobalt did not bind the antigen. Serum-stable antigen binding ensures integrity of the nanoparticles during transit to draining lymph nodes.

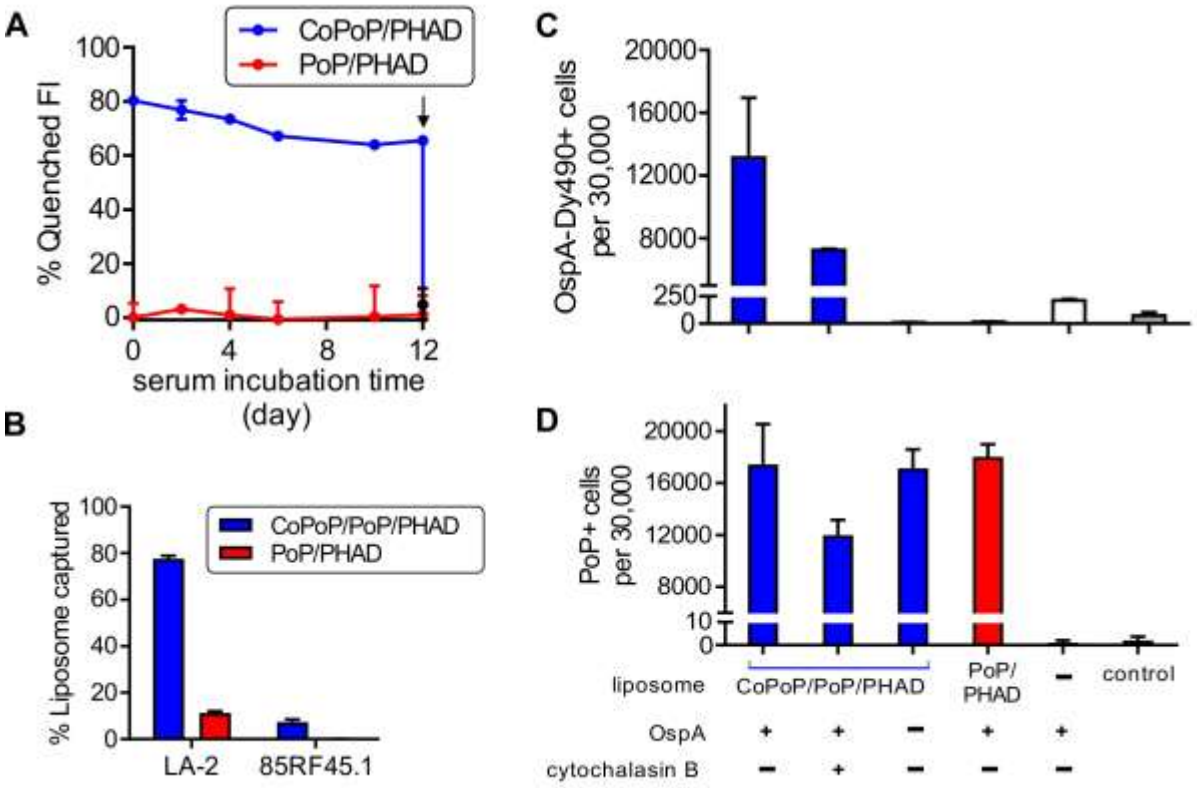

Figure 2. Serum stability, epitope availability and cellular uptake of particleized OspA. A) Stability of particleized antigen association with liposomes in 20% (v/v) human serum based on fluorescence quenching of dye-labeled OspA. The arrow shows restoration of OspA fluorescence with detergent and protease treatment. **B)** Immunoprecipitation of OspA-bound liposomes by OspA-specific monoclonal antibody LA-2. An irrelevant antibody specific for a malaria antigen served as a negative control. CoPoP liposomes included additional PoP for analysis, since CoPoP has weak fluorescence. Uptake of fluorescently labeled OspA (**C**) or liposomes themselves (**D**) in RAW 264.7 murine macrophage cells following 2 hr incubation with indicated samples at 37 °C. Cytochalasin B, a phagocytosis inhibitor, was added to medium 1 hr prior to incubation. Error bars represent standard deviations for n=3 experiments.

Previous studies using analogous nanoparticle systems demonstrated variation of the immunogenicity and protective efficacy with the point of attachment to the nanoparticle scaffold.[46, 47] This highlights the importance of proper antigenic epitope presentation on the particle surface. In this study, the his-tag was appended at the N-terminus opposite to the locality

of protective epitopes to ensure epitope accessibility on the liposomal surface and avoid possible occlusion of the important C-terminal epitopes. This configuration putatively mimics the lipoprotein integration and antigen orientation in the outer membrane of Lyme *borreliae*. Assessment of the epitope availability using a whole-liposome immunoprecipitation method confirmed surface exposure and epitope intactness of the LA-2 epitope on the liposome-bound OspA (**Figure 2B**). LA-2 is a protective monoclonal antibody against OspA that binds the C-terminus domain of OspA.[10, 48] In this experiment, the LA-2 antibody could immunoprecipitate CoPoP liposomes functionalized with OspA, based on the detection of additional PoP added to the liposomes. Liposomes lacking cobalt were not immunoprecipitated. The recognition of the particleized OspA by LA-2 suggests that structural integrity of the antigen is maintained after attaching to liposomes. As a negative control, a rat monoclonal antibody specific for an irrelevant malaria antigen was ineffective at immunoprecipitating the OspA-functinoalized liposomes, although this control antibody was from a different species and not isotype-matched.

One method for liposomes to enhance antigen delivery is based on enhanced uptake of liposomes by antigen presenting cells. Nanoparticle internalization studies were assessed with flow cytometry, with the gating strategy used shown in **Supporting Figure S3.** Murine macrophage cells showed high antigen uptake only with the surface-functionalized liposome (**Figure 2C**). Minimal uptake was observed with the free non-adjuvanted or non-associated (*i.e.* PoP/PHAD) forms of the antigen. These results corroborate studies demonstrating enhanced antigen internalization in the nanoparticulate form. In all cases, the liposomes themselves were uptaken in macrophages, regardless of antigen attachment (**Figure 2D**). Cellular uptake of the liposome-bound antigen may proceed in part via phagocytosis, which was somewhat diminished in the presence of cytochalasin B, which inhibits actin polymerization.[49] Additional studies were carried out to examine the uptake mechanism of PoP liposomes in the presence of selective uptake inhibitors at concentrations from 1-125 μg/mL (**Supporting Figure S4**). Besides

cytochalasin B, chlorpromazine, an endocytosis inhibitor that inhibits clathrin-mediated endocytosis, also could effectively inhibit liposome uptake. Amilrode, genistein, and nystatin, which inhibit pinocytosis or micropinocytosis through mechanisms including caveolae inhibition were generally inefficient. Taken together, these data suggest that liposomes and any associated antigens are uptaken by macrophages (and possibly other immune cells) by endocytosis and phagocytosis.

Next, the immunogenicity of OspA with various adjuvants was assessed in outbred CD-1 mice with prime-boost intramuscular vaccination with 100 ng OspA. CoPoP/PHAD liposomes elicited OspA-specific IgG antibody titer higher than other adjuvants assessed (**Figure 3A**). Otherwise identical liposomes that included PHAD but lacked cobalt in the PoP macrocycle, and thus did not induce OspA particle formation, exhibited lower antibody production. Although more work is needed to elucidate the mechanism of the CoPoP/PHAD efficacy, enhancing antigen delivery to antigen presenting cells, as implied by the *in vitro* data (**Figure 2C**), likely contributes. Other adjuvants produced a lower average anti-OspA IgG titer relative to CoPoP liposomes, and higher inter-subject variability was observed. Such variance in the antibody response could reflect that the selected dose (100 ng) was insufficient to produce consistently high antibody response.

Immunofluorescence labeling of *B. burgdorferi* B31 spirochetes (**Figure 3B**) demonstrated recognition by OspA-specific antibodies induced by immunization with functionalized CoPoP/PHAD liposomes. Fluorescence micrographs further reveal low functional recognition of the anti-OspA antibodies induced from immunization with alum and PoP/PHAD liposomes. As expected, no labeling was observed for the pre-immune serum, which served as the negative control.

Immunoblot analysis using antisera from CoPoP/PHAD immunization showed that OspA-specific antibodies recognized several different strains of Lyme *borreliae* albeit to different extents (**Figure 3C**). Different band intensities reflect antigenic heterogeneity of OspA across different

genospecies. The relapsing fever agent *B. hermsii*, which lacks the *ospA* gene, has no visible band. Minimal non-specific bands were observed for an *E. coli* lysate expressing recombinant his-tagged OspA. The slightly lower molecular weight observed for the recombinant OspA band may be due to the substitution of the lipidation signal sequence with a polyhistidine segment.

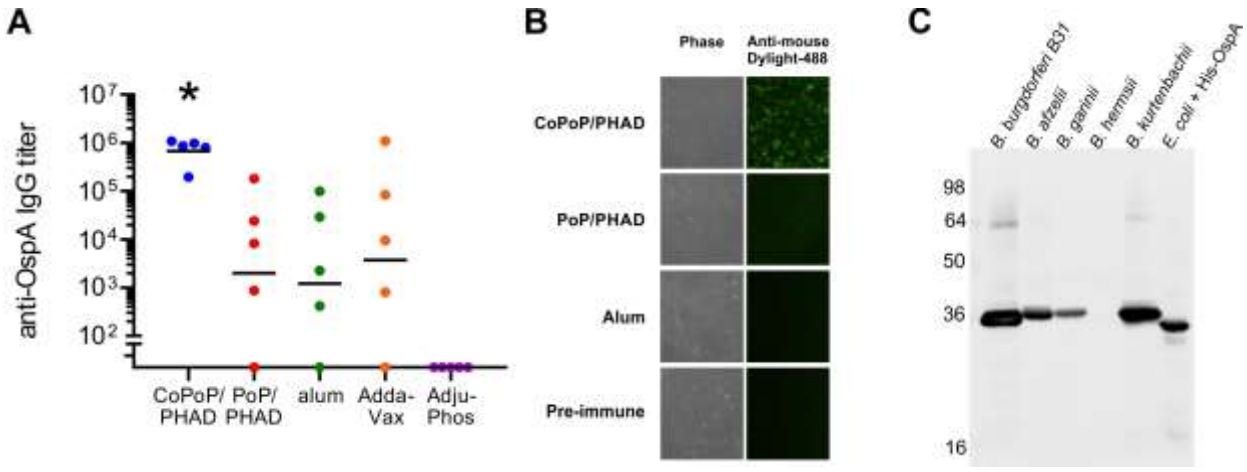

Figure 3. Immunogenicity of OspA adjuvanted with CoPoP liposomes. 100 ng OspA, admixed with indicated adjuvants, was injected intramuscularly on day 0 and day 21 and serum was collection on day 42. **A)** Anti-OspA IgG titers induced by CoPoP/PHAD liposomes compared to other commercial adjuvants. Horizontal lines represent geometric mean. Asterisk shows that the anti-OspA IgG titer was significantly higher in the CoPoP/PHAD adjuvant compared to all others (one-way ANOVA followed by post-hoc Tukey's test; $P<0.05$). **B)** An indirect immunofluorescence assay of *B. burgdorferi* B31 using goat anti-Mouse IgG DyLight-488 secondary antibody conjugate. **C)** Immunoblot assay using whole cell lysates of different *Borrelia* species. CoPoP/PHAD post-immune mouse serum was used. The molecular weight size, in kDa is indicated.

CoPoP/PHAD liposomes produced higher levels of OspA-specific IgG2a antibodies than IgG1 (**Figure 4A**). This suggests the immune response was based towards a Th1 response. Alum, on the other hand, induced higher ratio levels of the IgG1 isotype. Predominance of IgG2a isotype is significant as this isotype exhibits higher bactericidal activity and greater capacity to activate complement than IgG1 isotype. The Th1-biased immune response observed for CoPoP/PHAD liposomes correlates with the higher stimulation of interferon-gamma than interleukin-4 when splenocytes from immunized mice were stimulated with OspA (**Figure 4B**).

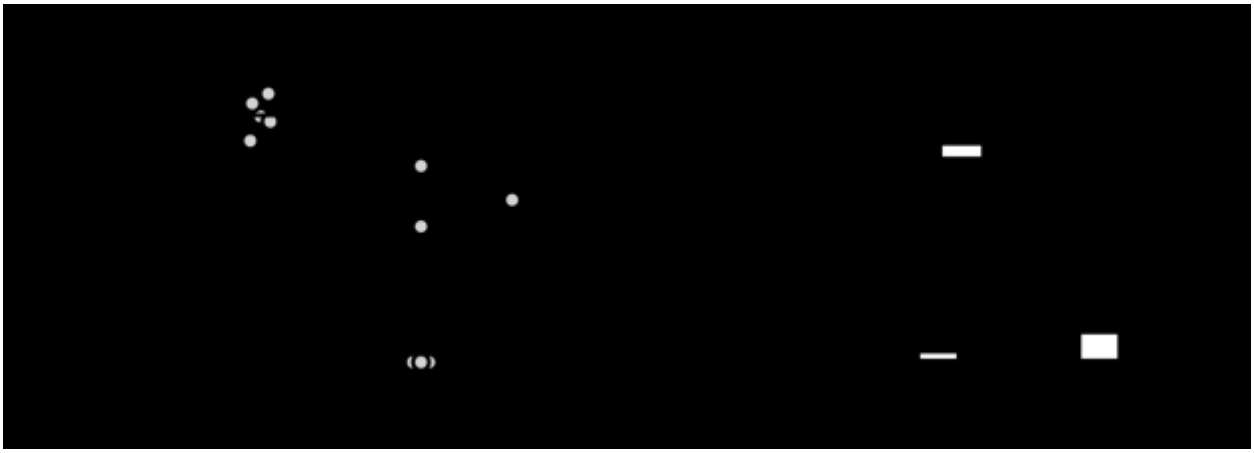

Figure 4. Th1-biased immune response induced by OspA with CoPoP/PHAD liposomes. **A)** IgG isotype profiling for post-immune sera (day 42) using ELISA. Horizontal lines show geometric mean. **B)** Splenocyte stimulation study to detect interferon-gamma and interleukin-4 secretion after 72-hr stimulation with OspA. Splenocytes were isolated from murine spleen collected on day 42 post-immunization. Error bars represent standard deviations from n=3 triplicate stimulation experiments.

To demonstrate whether OspA-specific antibodies could eliminate spirochetes *in vitro*, a complement-mediated bactericidal assay was performed and the borreliacidal titers were then compared. Antibodies generated from CoPoP/PHAD and OspA immunization exhibited higher bactericidal activity compared to OspA adjuvanted with Alum or PoP/PHAD liposomes over a broad range of serum dilutions (**Figure 5A**). The calculated 50% borreliacidal titer for CoPoP/PHAD liposomes was significantly higher than the other adjuvants, including Alum and identical liposomes lacking cobalt (**Figure 5B**). CoPoP/PHAD liposomes themselves, without addition of OspA, did not induce any complement killing.

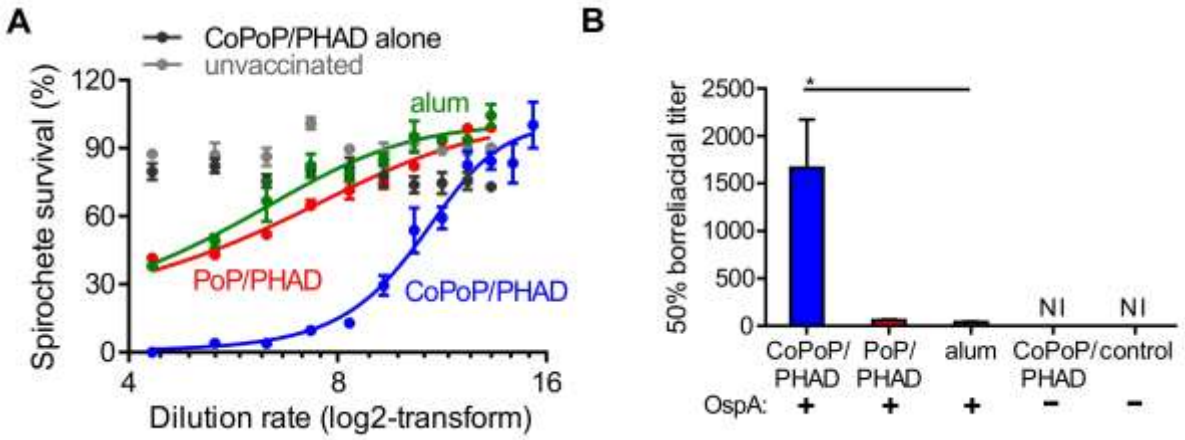

Figure 5. Borreliacidal antibodies induced by murine immunization with OspA admixed with CoPoP/PHAD liposomes. (A) Serum bactericidal antibody assay performed using guinea pig complement incubated with varying concentrations of mouse IgG collected on day 42 after priming on day 0 and boosting on day 21 with 100 ng OspA. Survival percentage was derived from normalization of the number of spirochetes after overnight serum treatment to that immediately after incubation. Surviving *B. burgdorferi* B31-A3 were counted using dark-field microscopy. (B) Average 50% borreliacidal activity (serum dilution rate that effectively eliminated 50% of the bacteria) from three different mice sera. Error bars represent standard error of the mean. "NI"; no inhibition. Statistical significance ($P < 0.05$, indicated by asterisk) of differences between bactericidal titers is assessed by Kruskal-Wallis test with Dunn's post-hoc analysis.

Protection conferred by OspA-based transmission blocking vaccines heavily relies on the levels of circulating antibodies in the host blood, which enters the tick gut at the start of a blood meal. Therefore, a durabile antibody response is desirable for sustained vaccine efficacy. We assessed long-term durability of circulating antibodies in mice following immunization with 100 ng OspA with CoPoP/PHAD liposomes on day 0 and day 21. Anti-OspA IgG titers calculated at different time points remained fairly similar throughout the year-long period, even up to one year after initial vaccination (**Figure 6A**). This demonstrates a highly durable antibody response The antibodies obtained at one year post initial immunization induced similar levels complement-mediated bacterial killing as those collected at 6-week post initial immunization (**Figure 6B, 6C**). These results suggest that the CoPoP/PHAD adjuvant could potentially require less frequent booster injections to retain protective antibody levels. However, it is difficult to predict the durability of the immune response in other species, based on observations from mice.

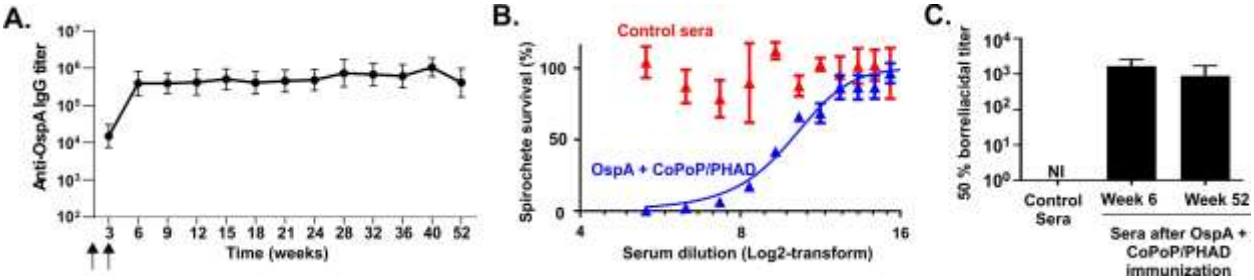

Figure 6. Longevity of anti-OspA response following immunization. CD-1 mice were immunized on day 0 and 21 with 100 ng OspA with CoPoP/PHAD liposomes **A)** Anti-OspA IgG titer following immunization. Data points and error bars represent geometric mean and 95% confidence interval. Arrows show days of immunization. **B)** Serum bactericidal antibody assay on week 52 sera, performed using guinea pig complement incubated with varying post-immune serum dilutions. **C)** Average 50% borreliacidal activity from three different mice sera. Error bars represent standard error of the mean. No statistical difference of the 50% borreliacidal titer in the serum collected at 6 week or one year post initial immunization. "NI"; no inhibition.

To verify whether the sera collected at one-year post immunization could block spirochete transmission from ticks, a feeding chamber assay was used (**Figure 7A**). Human blood was mixed dilute sera from CoPoP/PHAD-OspA immunized mice and infected *I. scapularis* nymphs were then allowed to feed with each of these sera via our previously reported feeding chamber model.[43] The bacterial burdens in ticks feeding on blood treated with sera from CoPoP/PHAD-OspA-immunized mice were reduced compared to that in ticks feeding on blood incubated with normal mouse sera (**Figure 7B**).

An OspA-based Lyme disease vaccine should inhibit the transmission of *Borrelia* from the ticks to the human host, following a blood meal with the induced OspA antibodies. This could be assessed in the feeding chamber (**Figure 7C**). 5 days after infected ticks were placed in the feeding chamber, spirochetes could be detected in the human blood pool containing diluted normal mouse sera. This shows that the bacteria migrated from the tick midgut, spread to the salivary glands, and into the human blood pool. However, bacteria were undetectable in the human blood mixed with post-immune sera from CoPoP/PHAD-OspA-immunized mice (one year after immunization). This demonstrates that the sera from CoPoP/PHAD-OspA-immunized mice

was active in the tick midgut to prevent tick-borne transmission of *B borgdorferi* for extended periods following immunization with 100 ng antigen doses.

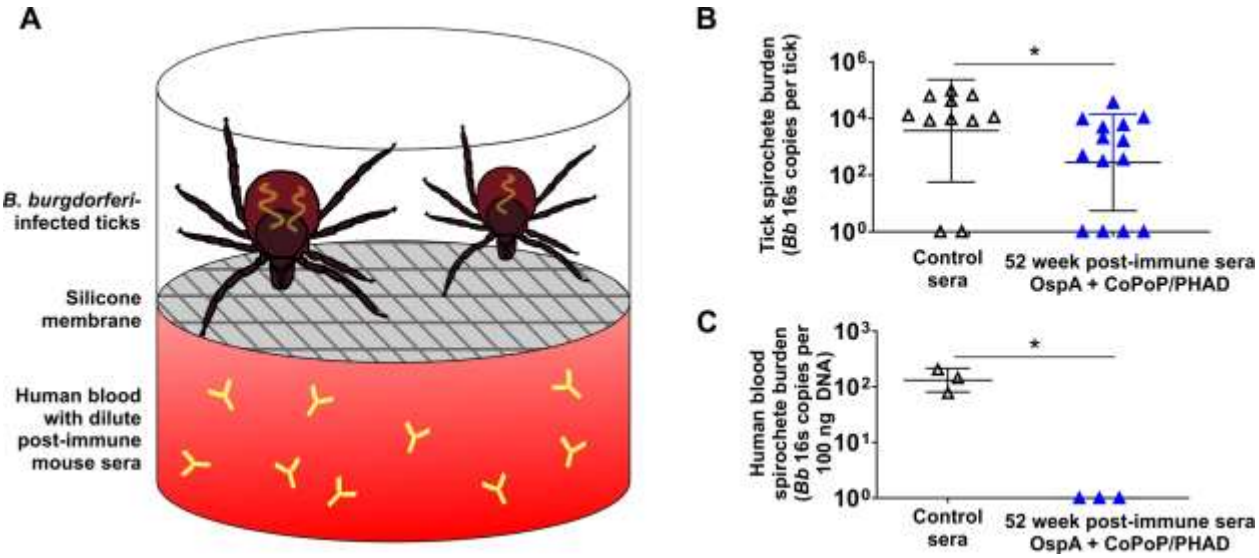

Figure 7. Assessment of 52 week post-immune mouse sera using infected ticks in a human blood feeding chamber. A) *I. scapularis* nymphal ticks carrying *B. burgdorferi* (*Bb*) strain B31-A3 were placed in feeding chambers with human blood containing diluted mouse serum (1:424 dilution) from mice immunized 52 weeks earlier with OspA and CoPoP/PHAD liposomes or normal mouse sera. After 5 days, nymphs were pulled from the membrane and spirochete burden was determined in the ticks (**B**) or in the human blood (**C**) using qPCR. Geometric mean ± geometric S.D. is shown. For ticks, *Bb* burden was determined in individual ticks (11 and 14 ticks for dilute normal or post-immune mouse sera, respectively). For human blood, *Bb* burden was determined on day 5 of feeding, in three feeding chambers. Asterisk indicates a statistically significant difference in spirochete burden ($P<0.05$, unpaired t-test).

This study did not assess potential toxicity of immunization with OspA and CoPoP/PHAD liposomes. Previously, we reported that high doses of CoPoP itself did not induce any weight loss, overt histological changes in major organs, or abnormal blood profiles.[37] Additional toxicity studies are warranted with CoPoP/PHAD liposomes and OspA. Furthermore, *B. burgdorferi* OspA itself has been linked with human autoimmunity based on a short epitope predicted to bind to human HLA-DR4 [50]. Others have shown that this short *B. burgdorferi* OspA epitope can be replaced with the analogous sequence from *B. afzelii* to address this potential problem [51]. It could be useful to assess this substituted OspA antigen with CoPoP/PHAD liposomes.

CONCLUSION

CoPoP liposomes induced serum-stable binding of recombinant, his-tagged OspA while preserving antigen conformation. The uptake in macrophages and immunogenicity of particleized OspA was enhanced compared to other vaccine adjuvants. Vaccination with OspA admixed with CoPoP/PHAD liposomes generated antibodies that recognized *B. burgdorferi* and had strong borreliacidal activity. A durable, functional antibody response was observed that blocked *B. burgdorferi* transmission from infected ticks to human blood in a feeding chamber. Taken together we conclude that CoPoP liposomes warrant further investigation for use with Lyme disease immunization strategies.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

WH and JFL are named co-inventors on one or more University at Buffalo patent applications describing CoPoP technology and hold equity in POP Biotechnologies, a university startup company licensing the technology.

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Supporting Information

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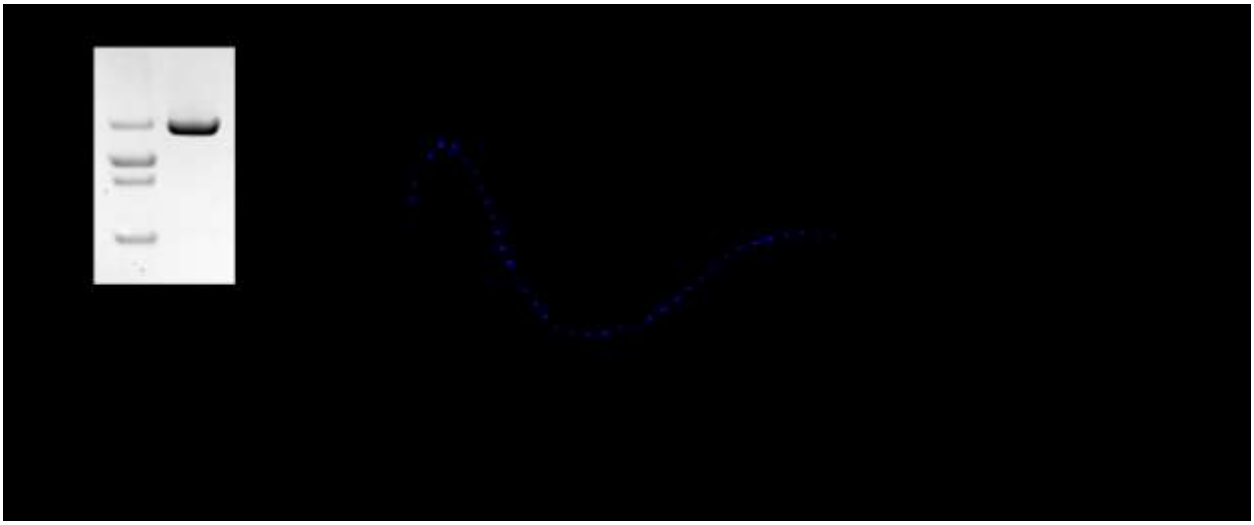
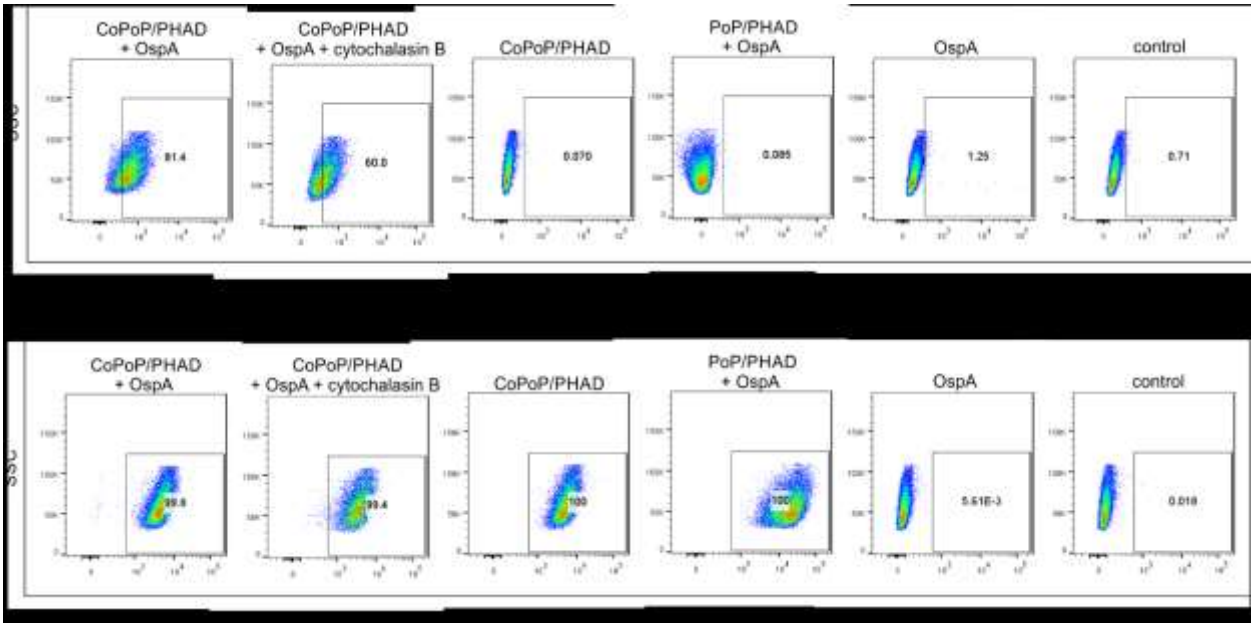
Supplementary Figure 1. Amino acid sequence of the recombinant non-lipidated construct of OspA (*B. burgdorferi* B31). The first 17 residues (not shown) which contain the lipidation signal sequence, were deleted and substituted with the his-tag.



Supplementary Figure 2. Characterization of purified recombinant non-lipidated OspA. (A) Degree of purity assessed by SDS-PAGE of 2 µg OspA (MW 29 kDa). (B) Far-UV circular dichroism spectrum of OspA in 20 mM sodium phosphate pH 7.4 solution (solid line) and calculated best fit spectrum (blue dotted line) with NRMSD of about 0.05. (C) Secondary structural content in percentage deconvoluted from buffer-corrected spectral data using CDSSTR provided by Dichroweb server.



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731 **Supplementary Figure 3.** Gating Strategy for macrophage uptake of DY-490-OspA and
732 liposomes. Cells were first gated based on the forward and side scatter. Images are
733 representative for three different experiments.
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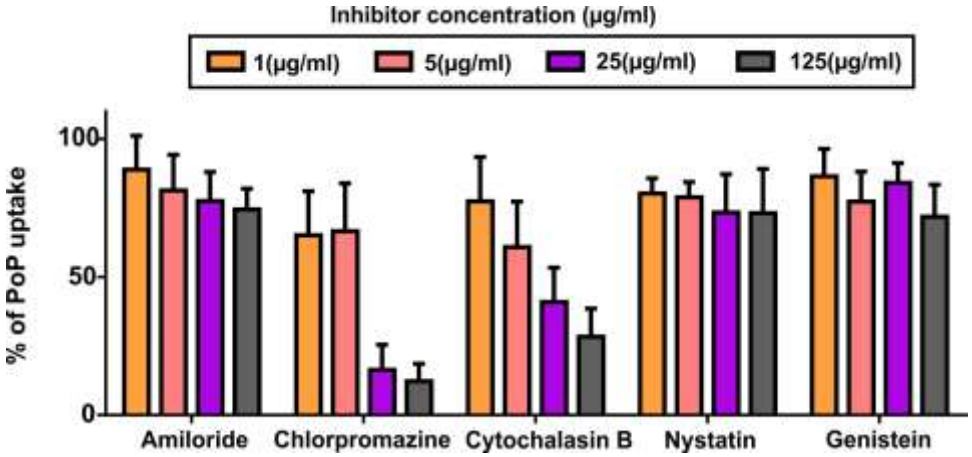

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737 **Supplementary Figure 4.** Uptake of PoP liposomes in murine macrophages pretreated with
738 indicated uptake inhibitors. PoP/PHAD liposomes were incubated with cells for 3 hr and uptake
739 was assessed, relative to untreated cells by PoP detection in the cells lysed with 1 % Triton-X100.
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