Supplementary Information for:

Structure-based Programming Lymph Node Targeting in Molecular Vaccines

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Supplementary Methods:

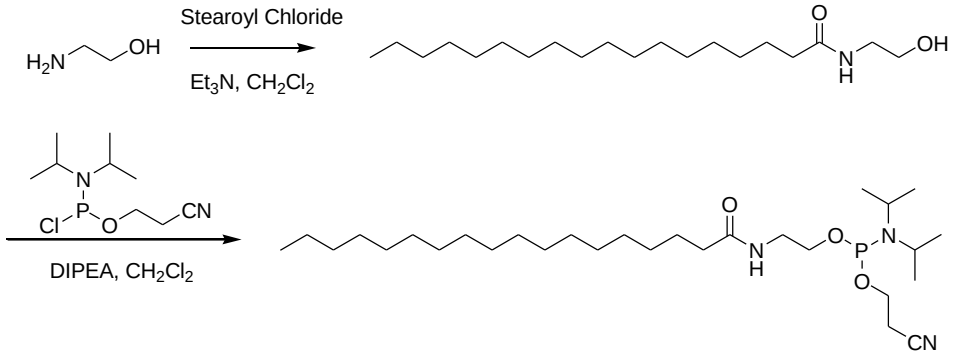
Materials. All DNA synthesis reagents including cholesteryl-TEG phosphoramadite and DMT-PEG-phosphoramadite were purchased from Glenres or Chemgenes and used according to the manufacturer's instructions. 3'-Biotin and 3'-FAM resin were purchased from Allele Biotechnology. DSPE-PEG_{2000}-Maleimide was purchased from Laysan Bio Inc. Carboxyfluorescein-labeled PEG_{2000}-DSPE was purchased from Avanti Polar lipids Inc. Carboxyfluorescein-labeled NHS-PEG_{2000} was purchased from Nanocs Inc. Incomplete Freund s adjuvant (IFA) and fatty acid-free BSA were purchased from Sigma-Aldrich. Ovalbumin protein was purchased from Worthington, SIVmac251 p27 protein was purchased from advanced bioscience laboratories. Ovalbumin peptides used for *in vitro* restimulations were purchased from Anaspec; ovalbumin protein was purchased from Worthington Biochemical Corporation. HPV-16 long peptide $E7_{43-62}$ (GQAEPDRAHYNIVTFCCKCD) was purchased from Anaspec. Cysteine- (Cys) modified peptides HPV-16 $E7_{49-57}$ (CRAHYNIVTF), SIV-$gag_{312-322}$ (CAAVKNWMTQTL) and Trp-$2_{180-188}$ (CSVYDFFVWL) were synthesized by GenScript Corp. (Piscataway, NJ) and purified by reverse phase HPLC. Murine MHC class I tetramers were obtained from Beckman Coulter (Beckman Coulter, Inc, San Diego, CA). All other reagents were from Sigma-Aldrich and used as received except where otherwise noted.

Synthesis of diacyl lipid phosphoramidite. Diacyl lipid phosphoramidite precursor for preparation of lipo-CpG was synthesized in two steps as described previously[1] (scheme S1): A solution of stearoyl chloride (6.789 g, 22.41 mmol) in $ClCH_2CH_2Cl$ (50 mL) was added dropwise to a solution of 1,3-diamino-2-dydroxypropane (1.0 g, 11.10 mmol) in the presence of $ClCH_2CH_2Cl$ (100 mL) and triethylamine (2.896 g, 22.41 mmol). The reaction mixture was stirred for 2 hours at 25 ℃ and then heated at 70 ℃ overnight.

Scheme S1. Diacyl-lipid phosphoramidite synthesis.

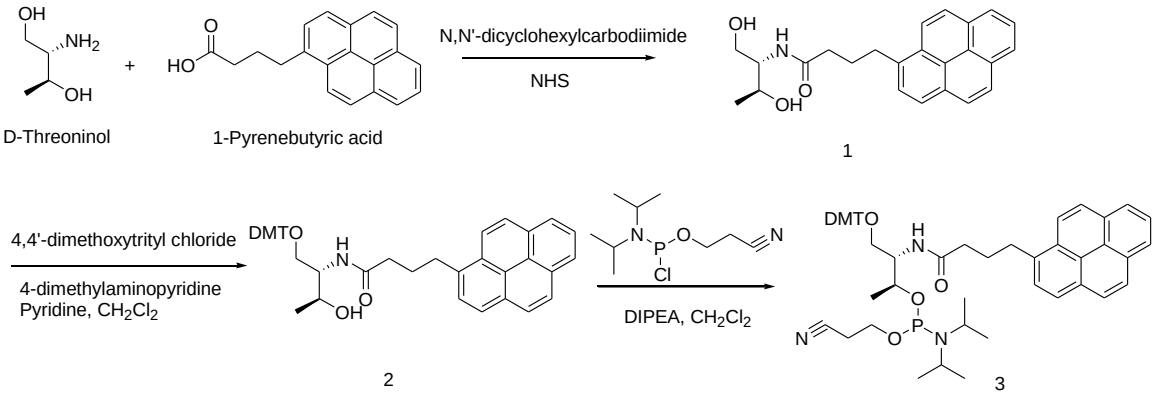
The reaction mixture was then cooled to 25 ℃, filtered, and the solid was sequentially washed with CH_2Cl_2, CH_3OH, 5% $NaHCO_3$ and diethyl ether. The product was dried under vacuum to give the intermediate product as a white solid (yield: 90%). ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 6.3 (m, 2H), 3.8 (m, 1H), 3.4-3.2 (m, 4H), 2.2 (t, 4H), 1.6 (m, 4H), 1.3-1.2 (m, 60H), 0.9 (t, 6H). The intermediate product (5.8 g, 9.31 mmol) and N,N-Diisopropylethylamine (DIPEA, 4.2 mL, 18.62 mmol) was then suspended in anhydrous CH_2Cl_2 (100 mL). The mixture was cooled on an ice bath and 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (8.6 mL, 0.47 mmol) was added dropwise under dry nitrogen. After stirring at 25 ℃ for 1 hour, the solution was heated to 60 ℃ for 90 minutes. The solution was washed with 5% $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under vacuum. The final product was isolated by precipitation from cold acetone to afford 4g (55% yield) phosphoramidite as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.4 (m, 2H), 3.9 (m, 2H), 3.8 (m, 2H), 3.6 (m, 2H), 3.0-2.9 (m, 2H), 2.6 (t, 2H), 2.2 (m, 4H), 1.6 (m, 6H), 1.3-1.2 (m, 72H), 0.9 (t, 6H). ^{31}P NMR ($CDCl_3$) 154 ppm. FTIR [film] 3649, 2926, 2854, 2361, 1653, 1540, 1365, 1265, 974, 740 cm^{-1}. HRMS [ESI] m/z calcd for $C_{48}H_{95}N_4O_4P$ $[M+Na]^+$ 845.6983, found 845.6971.

Synthesis of monoacyl phosphoramidite. Monoacyl lipid phosphoramidite was synthesized following similar procedures as described for the synthesis of diacyl phosphoramidite (scheme S2). ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 5.95 (m, 1H), 3.8 (m, 2H), 3.7 (m, 2H), 3.6 (m, 2H), 3.5 (m, 2H), 2.62 (t, 2H), 2.18 (t, 2H), 1.6 (m, 2H), 1.3-1.2 (m, 36H), 0.86 (t, 3H). ^{31}P NMR ($CDCl_3$) 149 ppm. FTIR [film] 3649, 3304, 2917, 2850, 2361, 1647, 1559, 1471, 1184, 978, 718 cm^{-1}. HRMS [ESI] m/z calcd for $C_{29}H_{58}N_3O_3P$ $[M+Na]^+$ 550.4108, found 550.4124.



Scheme S2. Monoacyl-lipid phosphoramidite synthesis.

Synthesis of pyrene phosphoramidite. Pyrene phosphoramidite was synthesized as shown in scheme S3. Synthesis of compound 1: In a 300 mL round-bottom flask, D-threoninol (0.95 g, 9.1 mmol), 1-Pyrenebutyric acid (2.88 g, 10.0 mmol), dicyclohexylcarbodiimide (DCC, 2.06 g, 10.0 mmol) and N-Hydroxysuccinimide (NHS, 1.15 g, 10 mmol) were dissolved in 50 mL DMF. The reaction mixture was stirred at room temperature for 24 hours. The insoluble N,N'-dicyclohexylurea was filtered, and DMF was removed with a rotary vacuum evaporator to obtain an oily crude product. Compound 1 was purified by flash chromatography (yield: 85%). 1H NMR (300 MHz, $CDCl_3$, ppm): δ 8.1-7.7 (m, 9H), 6.2 (d, 1H), 4.2-3.8 (m, 4H), 3.0 (m, 2H), 2.3-2.2 (m, 4H), 1.2 (d, 3H).



Scheme S3. Pyrene phosphoramidite synthesis.

Synthesis of compound 2: Compound 1 (2.93 g, 7.2 mmol) and 4-dimethylaminopyridine (0.043 g, 0.36 mmol) were dissolved in 40 mL dry pyridine in a 100 mL round-bottom flask under dry nitrogen. The solution was cooled on an ice bath. 4,4'-dimethoxytrityl chloride (2.93 g, 8.64 mmol) was dissolved in 10 mL dry CH_2Cl_2 in a 50 mL flask and slowly added to the above pyridine solution under dry nitrogen. The reaction was slowly warmed to 25 ℃ and stirred for 24 hours. The solvent was removed under vacuum, and compound 2 was isolated by chromatography (50:50:3 ethyl acetate:hexane:triethylamine) (yield: 75%). ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 8.3-7.5 (m, 22H), 6.1 (d, 1H), 4.2-3.9 (m, 2H), 3.7 (d, 6H), 3.4-3.3 (m, 4H), 2.4-2.2 (m, 4H), 1.2 (d, 3H).

Synthesis of compound 3: Compound 2 (1 g, 1.48 mmol) was dissolved in CH_2Cl_2 and cooled on an ice bath. Then, DIPEA (0.57g, 4.44 mmol) and 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (0.42 g, 1.78 mmol) were added dropwise over 30 min under dry nitrogen. The reaction mixture was stirred on ice for 3 hours. The solvent was evaporated, and compound 3 was purified by chromatography (50:50:3 ethyl acetate:hexane:triethylamine) (Yield: 70%). ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 8.3-6.6 (m, 21H), 5.82 (d, 1H), 4.4-4.2 (m, 2H), 3.8 (s, 3H), 3.7 (d, 6H), 3.6-3.1 (m, 8H), 2.5 (m, 1H), 2.4-2.2 (m, 5H), 1.3-0.9 (m, 20H). ^{31}P NMR ($CDCl_3$, ppm) 149. FTIR [film] 3432, 3051, 2967, 1734, 1653, 1507, 1364, 1250, 1179, 1034, 977, 829, 736 cm^{-1}. HRMS [ESI] m/z calcd for $C_{54}H_{60}N_3O_6P$ $[M+Na]^+$ 900.4112, found 900.4019.

DNA synthesis and lipophilic conjugation. All DNA sequences were synthesized using an ABI 394 synthesizer on a 1.0 micromole scale. All lipophilic phosphoramidites were conjugated as a final 'base' on the 5' end of oligos. Lipophilic phosphoramidites were dissolved in dichloromethane and coupled to oligos using the so-called syringe synthesis technique[2]. Briefly, lipid phosphoramidites (200 µL) were mixed with activator (0.2 mM 5-Ethylthio Tetrazole in 200 µL Acetonitrile), and the mixture were pushed back and forth through the column using 2 syringes for 10 min. Alternatively, lipophilic phosphoramidite could also be coupled using the DNA synthesizer (15 min coupling time). After the synthesis, DNA was cleaved from the solid support, deprotected, and purified by reverse phase HPLC using a C4 column (BioBasic-4, 200 mm x 4.6 mm, Thermo Scientific), 100 mM triethylamine-acetic acid buffer (TEAA, pH 7.5)-acetonitrile (0-30 min, 10-100%) as an eluent. Lipophilic ODNs typically eluted at 20 min while unconjugated oligos eluted at 8 min. ODNs were characterized by MALDI-TOF mass

spectrometry (Supplementary Table 1). Immunostimulatory CpG oligos employed were a type B sequence known as 1826[3]:

Typical sequence of Lipo-G_n-CpG:
5'diacyllipid-*G_n*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*-3'

Lipo-GpC:
5'diacyllipid-*T*C*C*A*T*G*A*G*C*T*T*C*C*T*G*A*G*C*T*T*-3'

Supplementary Table 1: Sequences and MALDI-TOF MS Characterization of Synthetic ODNs

ODNs Sequence		Mass	
		Calculated	Found
C18-CpG-F	5'single chain-*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*-Fam-3'	7348.9	7344.9
Lipo-CpG	5'diacyllipid-*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*-3'	7060.1	7060.0
Lipo-G_2-CpG	5'diacyllipid-*G*G*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*-3'	7749.2	7754.0
Lipo-G_4-CpG	5'diacyllipid-*G*G*G*G*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*-3'	8439.2	8435.2
Lipo-G_6-CpG	5'diacyllipid-*G*G*G*G*G*G*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*-3'	9130.3	9134.2

Synthesis of fluorescein-PEG amphiphiles. PE lipids (1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine, DLPE; 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, DMPE; 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, DPPE; 1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine, DSPE, Avanti polar lipids Inc.) were dissolved in 500 μL $CHCl_3$ and 500 μL DMF, 3 eq of triethylamine and 1.2 eq of fluorescein-PEG_{2000}-NHS (Creative PEG Works Inc.) was added and the reaction mixture were agitated overnight. The amphiphilic fluorescein PEG amphiphiles were purified by reverse phase HPLC using a C4 column (BioBasic-4, 200 mm x 4.6 mm, Thermo Scientific), 100 mM triethylamine-acetic acid buffer (TEAA, pH 7.5)-methanol (0-30 min, 10-100%) as an eluent. The final products were dissolved in H_2O and quantified by UV-Vis spectroscopy (fluorescein, *extinction coefficient* 70,000 $M^{-1}cm^{-1}$ at 490 nm, pH 9) and characterized by MALDI-TOF mass spectrometry. Fluorescein labeled diacyl lipo-PEGs with different PEG length were synthesized by repetitively coupling hexa-ethylene glycol phosphoramidite via solid phase DNA synthesis.

Synthesis of peptide amphiphiles. N-terminal cysteine-modified peptides (HPV-16 $E7_{49-57}$ (CRAHYNIVTF), SIV-$gag_{312-322}$ (CAAVKNWMTQTL), Trp-$2_{180-188}$ (CSVYDFFVWL) and HPV-16 $E7_{43-62}$ (GQAEPDRAHYNIVTFCCKCD)) were dissolved in DMF and mixed with 2 equivalents maleimide-PEG_{2000}-DSPE (Laysan Bio, Inc.), and the mixture was agitated at 25℃ for 24 hours. Bioconjugations were judged to be essentially complete by HPLC analysis. Peptide amphiphiles were characterized by MALDI-TOF mass spectrometry. The peptide conjugates were then diluted in 10x ddH_2O and lyophilized into powder, redissolved in H_2O and stored at -80 ^oC.

Amphiphile characterization. Micelle sizes were determined by dynamic light scattering (DLS) using a 90Plus/ZetaPals particle size and ξ-potential analyzer (Brookhaven Instruments). Circular dichroism (CD) spectra of G-quartet-forming lipo-G_n-CpG molecules were measured using 5 μM CpG ODNs in pH 7.4 PBS with 20 mM KCl and 10 mM $MgCl_2$ on an Aviv Model 202 Circular Dichroism spectrometer at 20 ℃. Scans from 220 to 320 nm were performed with 100 nm/min scanning speed, 1 nm bandwidth. For each spectrum, an average of three scans was taken; spectral contribution from the buffer was subtracted.

Size-exclusion chromatography. Size-exclusion chromatography was carried out on a Shimadzu HPLC system equipped with a SEC-Bio-sil column (5 µm silica particle size, Bio-rad, repacked in a 200x4.6mm column). Samples were eluted using 1xPBS + 20 mM KCl at a flow rate of 0.5 mL per minute. In a typical experiment, 80 µL of 5 µM lipo-G_nT_{10-n}CpG-Fam in 1xPBS + 20 mM KCl were added 20 µL FBS (Greiner Bio-one), samples were briefly vortexed and incubated at 37°C for 2 hours then diluted in 500 µL 1xPBS with 20 mM KCl, sample were then analyzed by SEC, FBS was monitored using absorptions at 280 nm, while ODNs were monitored at 480 nm (Fam peak).

Bio-layer interferometry (BLI) measurements. The binding affinity of amph-CpG to albumin was measured by a BLItz Bio-Layer Interferometry system (BLI, Fortébio Inc.) at 25°C. Bio-Layer Interferometry measures the change in the interference pattern of light as ligand in solution binds an immobilized target on a biosensor probe, yielding an apparent K_d. Briefly, streptavidin BLItz Dip and Read™ biosensor probes (Fortébio Inc.) were loaded with 100 µM biotinylated BSA (Sigma-Aldrich) in PBS buffer or mock loaded with non-biotinylated IgG, washed in PBS buffer twice and baseline readings were taken for 30 seconds in PBS. Association phase readings for amph-CpG were performed for 300 seconds at 100 nM, 250 nM, 500 nM, and 1 µM concentrations in PBS followed by dissociation phase in the same buffer for 300 seconds. The dissociation constant was obtained by global curve fitting of the responses to yield a k_{on} value of $1.48x10^4$ $M^{-1}s^{-1}$ ($\pm 4.0x10^2$ $M^{-1}s^{-1}$), a k_{off} value of $1.82x10^{-3}$ s^{-1} ($\pm 4.4x10^{-5}$ s^{-1}), and thus an apparent K_d value of 125 ± 5.0nM; background binding (apparent affinities) of lipo-CpG interacting with mock-loaded probes were less than 2% of binding to albumin-loaded probes.

Protein pull-down assay and SDS-PAGE. FBS was passed through a G-25 column to deplete endogenous biotin. Biotin-labeled CpG (CpG-B, 2.5 µM final concentration) and lipo-CpG (lipo-CpG-B, 2.5 µM final concentration) were incubated with FBS (100x dilution) at 37°C for 1 hour, after which 50 µL prewashed Streptavidin magnetic beads (Pierce) were added, the samples were agitated at room temperature for 15 min and magnetic beads were collected with a magnetic stand. After washing the beads with 500 µL PBS, the samples were mixed with 100 µL SDS-PAGE reducing sample buffer and heated at 95 °C in a heating block for 5 minutes. Beads were removed magnetically and the supernatant was analyzed by SDS-PAGE.

Albumin immobilized Agarose and affinity chromatography. 20 mg fatty acid-free BSA was dissolved in 3 mL PBS and then directly added to 250 mg NHS-activated Agarose resin. The reaction was kept at 4 °C overnight and unbound BSA was filtered. After quenching the reaction with Tris-HCl buffer (pH 8), the resin was washed extensively using PBS. Affinity chromatography was performed by mixing CpG-F or lipo-CpG-F (0.6 µM final concentration) with 300 µL BSA-agarose beads (50 mg dry beads/mL). The mixtures were equilibrated at 37 °C for 1 hour, after which the beads were separated using spin columns and fluorescent images were recorded by a gel-imager (Bio-rad).

Albumin-CpG conjugate. Thiol-terminated CpG was prepared by solid-phase coupling of fluorescein-labeled CpG with C6 S-S CE phosphoramidite (Thiol-Modifier *C6 S-S*, ChemGenes) at the 5' end of the oligo. Mouse serum albumin (MSA, 10 mg in 200 µL PBS) was co-dissolved with 0.79 mg N-(ß-Maleimidopropyloxy)succinimide ester (BMPS, Aldrich) in 20 µL DMSO

and the mixture was agitated at 25 ℃ for 2 hours. Extra BMPS was removed by diluting in PBS solution and passing the mixture through a PD MidiTrap G-25 desalting column (GE Healthcare). 246 μg of 5'-disulfide-modified fluorescein-CpG (pre-activated by 20 μL 100 mM 3,3′,3″-Phosphanetriyltripropionic acid, TCEP) was added to the modified-MSA solution. The mixture was allowed to react overnight at 25 ℃ and unconjugated CpG was removed by dialysis (50K MWCO); free CpG removal was confirmed by size-exclusion chromatography.

Animals and cells. Animals were cared for in the USDA-inspected MIT Animal Facility under federal, state, local and NIH guidelines for animal care. Female C57BL/6 mice (6-8 weeks) and female TLR2 knockout mice (B6.129-Tlr2^{tm1Kir}/J, 6 weeks) were obtained from the Jackson Laboratory. B16F10 cells were obtained from American Type Culture Collection (ATCC), TLR reporter cell lines were purchased from invivogen (San Diego, California), TC-1 cells were kindly provided by Dr. T. C. Wu at John Hopkins University. Cells were cultured in complete medium (MEM, 10% fetal bovine serum (Greiner Bio-one), 100 U/mL penicillin G sodium and 100 μg/mL streptomycin (Pen/Strep), MEM sodium pyruvate (1 mM), NaH_2CO_3, MEM vitamins, MEM non-essential amino acids (all from Invitrogen), and 20 μM β-mercaptoethanol (β-ME)).

***In vitro* uptake assay.** Bone marrow-derived dendritic cells were generated by culturing bone marrow cells flushed from femurs of C57BL/6J mice (Jackson) in DC media: DMEM supplemented with 10% FBS, pen/strep, sodium pyruvate and 20 ng/mL GM-CSF (Peprotech). Media was replaced on day 3; non-adherent and loosely adherent immature dendritic cells (DCs) were collected on day 6 and phenotyped by determining expression of CD11c, CD86 and CD40 (routinely 60-80% CD11c$^+$). Rhodamine labeled CpG or amph-CpG (1 μM) was incubated with murine bone marrow-derived dendritic cells (BMDCs) at 37 ℃ for 4 hours with LysoTracker® (Life Technologies) and imaged using a Zeiss LSM 510 confocal microscope (Oberkochen, Germany). In some experiments, Rhodamine labeled CpG or amph-CpG (1 μM) was incubated for 30 minutes, 2 hours, 6 hours, and 24 hours with the murine dendritic cell line DC2.4. Cells were stained with DAPI and uptake was quantified using the mean fluorescence intensity (MFI) of viable (DAPI-) cells by FACS.

***In vitro* TLR reporter assay.** Amph-CpG was incubated for 24 hours with the InvivoGen HEK-Blue™ murine TLR2 or RAW-Blue™ mouse macrophage reporter cell lines, both secreted embryonic alkaline phosphatase (SEAP) reporter system. SEAP levels were quantified by incubating supernatant with QuantiBlue™ substrate for 1h and reading absorption at 620 nm.

***In vitro* cross-presentation assay.** CD11c$^+$ BMDCs were selected using magnetic bead-based positive selection (Miltenyi Biotec) according to the manufacture's method. These cells were plated at 15,000 cells per well in a 96-well U-bottom plate and incubated overnight with indicated concentrations of OVA and maturation stimuli (or DC media). DCs were washed 3 times with PBS and 30,000 CFSE-labeled OT-I CD8$^+$ T cells were then added to each well in 200 μL T cell media (RPMI1640 supplemented with 10% FBS, pen/strep, β-mercaptoethanol, and sodium pyruvate). Cells were collected after 2 days of proliferation, and stained and gated for DAPI- (viable) CD8$^+$ T cells using Flowjo v.7.6.5 (Treestar). Extent of proliferation was quantified by determining the % of cells that had undergone division by determining % of viable

$CD8^+$ T cells that had diluted CFSE using T cells alone as a control for the no division/dilution peak. Bars represent medians and whiskers represent range (*n*=2).

***In vitro* cell membrane insertion assay**. Freshly-isolated splenocytes from C57Bl/6 mice ($50x10^6$ cells/mL) were incubated with fluorescent amph-PEGs with different PEG lengths (final concentration 1.67 μM) in the presence of 100 μM albumin at 37°C for 1 hr. Amph-PEG in cell pellets and supernatants was quantified by fluorescence spectroscopy after mixing with Glo lysis buffer (Promega).

Lymph node imaging. After injection of fluorescein-labeled probes, animals were sacrificed and inguinal and axillary LNs were excised and imaged at different time points using an IVIS Spectrum Imaging System (Caliper life Sciences, Hopkinton, MA). An image set (Ex: 465, Em: 520, f 4, 10 Sec) was collected. Living Image software Version 4.2 (Caliper Life Sciences) was used to acquire and quantitate the fluorescence imaging data sets. A Region of Interest tool was used to measure the radiant efficiency from each lymph node, unless stated otherwise, data are presented mean ±s.e.m.

Immunizations. C57Bl/6 mice (6-8 weeks) were vaccinated by a homologous prime-boost regimen; animals were primed on day 0 and boosted on day 14 (unless stated otherwise) with 10 μg OVA or SIV-gag protein and 1.24 nmol CpG (in soluble or amphiphile forms) suspended in PBS with 20 mM K^+ and 10 mM Mg^{2+}. The volume of all vaccine injections was 100 μl. In experiments in which IFA was used, CpG/OVA in 50 μL PBS with 20 mM K^+ and 10 mM Mg^{2+} was combined with an equal volume of IFA and emulsified by vortexing vigorously until a thick emulsion formed. For amph-peptides, mice were primed with peptide-PEG_{2000}-DSPE conjugate (10 μg peptide) mixed with 1.24 nmol amph-CpGs suspended in PBS with 20 mM K^+ and 10 mM Mg^+; animals were boosted with same doses of peptides and CpGs. All injections were performed s.c. at the base of the tail.

Flow cytometry. All Antibodies were purchased from BD pharmingen or ebioscience. The following primary antibodies were used: anti-CD16/CD32 (BD bioscience, Cat#: 553142, clone: 2.4G2), anti-CD8-APC (ebioscience, Cat#: 17-0081-83, clone: 53-6.7), anti-IFN-γ-PE (BD bioscience, Cat#: 554412, clone: XMG1.2) and anti-TNF-α-FITC (BD bioscience, Cat#: 554418, clone: MP6-XT22), anti-CD11c-PE (ebioscience, Cat#: 12-01184-02, clone: N418), anti-F4/80-APC (ebioscience, Cat#: 17-4801-82, clone: BM8), anti-B220-PE (BD bioscience, Cat#: 553089, clone: RA3-6B2), anti-CD3-APC antibody (ebioscience, Cat#: 17-0031-81, clone: 145-2C11), anti-CD207-eFluor 660 (ebioscience, Cat#: 50-2073-80, clone: RMUL.2). Flow data were acquired on a FACSCanto flow cytometer (BD biosciences) and analyzed using Flowjo software (Tree Star Inc. Ashland, OR).

Tetramer staining. Blood was collected and red blood cells were depleted by ACK lysing buffer. Cells were then blocked with Fc-blocker (anti-mouse CD16/CD32 monoclonal antibody) and stained with phycoerythrin-labeled tetramers (Beckman Coulter) and anti-CD8-APC (ebioscience, Cat#: 17-0081-83, clone: RMUL.2) for 30 minutes at room temperature. Cells were washed twice, resuspended in FACS buffer (5 μg/mL DAPI), and analyzed on a BD FACSCanto flow cytometer. Analysis typically gated on live, $CD8^+$, Tetramer positive live cells.

Intracellular cytokine staining. Cells were plated in 96-well round-bottomed plates and pulsed with optimal peptides (OVA, SIINFEKL; HPV E7, RAHYNIVTF; SIV-$gag_{312-322}$, AAVKNWMTQTL; or $Trp\text{-}2_{180-188}$, SVYDFFVWL) for 2 hr at 37℃ in T-cell media (RPMI 1640, 10%FBS, 50 μM β-mecaptoethanol, 100 U/mL Penn/Strep, 1x Gibco® MEM Non-Essential Amino Acids Solution (Life technologies), 1 mM Sodium pyruvate, 1 mM HEPES), followed by the addition of brefeldin A for 4 hours. Cells were stained with anti-CD8-APC (ebioscience, Cat#: 17-0081-83, clone: RMUL.2) and then fixed using Cytofix (BD biosciences) according to the manufacturer's instructions. Cells were then washed and permeabilized. Intracellular staining for anti-IFN-γ-PE (BD bioscience, Cat#: 554412, clone: XMG1.2) and anti-TNF-α-FITC (BD bioscience, Cat#: 554418, clone: MP6-XT22) was performed according to the manufacturer's instructions and cells were analyzed on a BD FACSCanto flow cytometer.

Splenomegaly measurements. C57Bl/6 mice (3 per group) were injected with 1.24 nmol, 2.48 nmol and 2.48 nmol CpGs at day 0, day 2 and day 4, respectively; on day 6, animals were sacrificed and spleens were collected, weighed, and photographed. Splenomegaly was normalized to individual animals' body weights.

Proinflamatory cytokines assays. C57Bl/6 mice (3 per group) were administered a single dose (6.2 nmol) of CpGs s.c. at the tail base. Serum samples were collected at 2 hours and 24 hours post injection, and cytokines present were quantified using a Milliplex MAP mouse cytokine kit (EMD Millipore Corporation, Billerica, MA).

In vivo cytotoxicity assays. Groups of C57Bl/6 mice were immunized as described above with soluble or amphiphile peptide vaccines on days 0 and 14. On day 21, vaccinated animals were injected i.v. with $10x10^6$ donor splenocytes from naïve C57Bl/6 mice, half of which were pulsed with 10 μM SIINFEKL peptide for 30 min (peptide-pulsed targets) and half of which were left peptide-free (control cells). Prior to injection, the peptide-pulsed target cells were labeled with 1 μM Carboxyfluorescein succinimidyl ester (CFSE), while peptide-free control cells were labeled with 0.1 μM CFSE; CFSE labeling was performed for 10 min at 37℃ in serum-free medium and cells were then extensively washed prior to transfer into immunized recipients. Eighteen hours after transfer, immunized recipients were sacrificed and splenocytes from each recipient mouse were analyzed by flow cytometry to quantify the ratio of peptide-pulsed vs. non-pulsed donor cells present. Specific killing was defined as percentage of specific lysis=[1-non-transferred control ratio/experimental ratio]x100.

immunohistochemistry staining. Immunofluorescent staining was performed on 10 μm frozen sections of LN biopsy specimens. To reduce fading of fluorescein, sections were mounted in Vectashield mounting medium (Vector Laboratories, Inc. Brulingame, CA) and were viewed in a Zeiss LSM 510 confocal microscope (Oberkochen, Germany). Staining of lymph nodes sections as done directly with a PE-labeled anti-CD11c (ebioscience, Cat#: 12-01184-02, clone: N418) and APC-labeled anti-F4/80 (ebioscience, Cat#: 17-4801-82, clone: BM8), or PE-labeled anti-B220 (BD bioscience, Cat#: 553089, clone: RA3-6B2) and APC-labeled anti-CD3 antibody (ebioscience, Cat#: 17-0031-81, clone: 145-2C11) (pre-blocked with anti-CD16/32).

Tumor inoculation and tumor therapy experiments. C57Bl/6 mice (6-8 weeks) were anaesthetized and inoculated subcutaneously on the right hind flank with $3x10^5$ TC-1 cells (a

tumor cell line derived from primary lung epithelial cells of C57BL/6 mice and transformed with human papillomavirus 16 (HPV-16) E6/E7) or B16F10 Cells (mouse melanoma tumor cell line express Trp2). Tumors were allowed to establish for six days (for TC-1) or five days (for B16F10) before the treatment. TC-1 tumor bearing mice were randomized into groups and were vaccinated on day 6 (10 μg peptide, 1.24 nmol CpG), day 13 (20 μg peptide, 1.24 nmol CpG) and day 19 (20 μg peptide, 1.24 nmol CpG). B16F10 tumor bearing mice were treated on day 5 (10 μg peptide, 1.24 nmol CpG), day 11 (20 μg peptide, 1.24 nmol CpG) and day 17 (20 μg peptide, 1.24 nmol CpG). Tumor sizes were measured every 1–2 days by electronic calipers and calculated as the product of 2 orthogonal diameters ($D1 \times D2$).

Statistical analysis. Based on pilot immunization and tumor treatment studies, we used group sizes of 4 animals/group for immunogenicity measurements and at least 8 animals/group for tumor therapy experiments to obtain 80% power at the 95% confidence level to detect 30% differences in T-cell expansion or functionality. All plots show mean values and error bars represent the SEM. Comparisons of mean values of two groups were performed using unpaired Student's t tests. One-way analysis of variance (ANOVA), followed by a Bonferroni post-test was used to compare >2 groups. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ unless otherwise indicated. Statistical analysis was performed using GraphPad Prism software (San Diego, CA).

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