Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Covalent Modification of Cell Surfaces with TLR Agonists Improves & Directs Immune Stimulation

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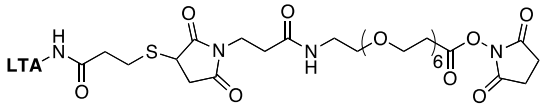
Materials and Methods

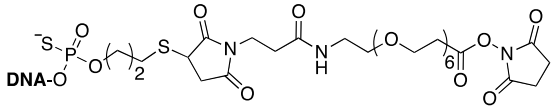
The reagents were purchased from Sigma-Aldrich and used as is unless otherwise noted. The $SM(PEG)_6$ linker was purchased from Thermo Fisher Scientific Inc. Single stranded CpG-ODN1826 (5'-TCCATGACGTTCCTGACGTT-3') with a 3'-disulfide modification and phosphorothioated backbone was purchased from Fisher Scientific Eurofins MFW Operon. Illustra NAP-5 Columns were purchased from GE Healthcare, Life Sciences. Centriprep Centrifugal Filter Devices (3K) and $ZipTip_{C18}$ for MALDI-MS were purchased from Millipore. Anti-mouse antibodies CD16/32 (93), PE anti-mouse CD86 (GL-1), -CD40 (3/23), -MHCII (M5/114.15.2), -CD80 (16-10A1), -Rat Isotype IgG2a (RTK2758), -Rat Isotype IgG2b (RTK4530), -Isotype Armenian Hamster IgG (HTK888), FITC anti-mouse CD11c (N418), APC anti-mouse IFN-γ (XMG1.2), -TNF-α (MP6-XT22), -IL-6 (MP5-20F3), -IL-10 (JES5-16E3), -IL-12 (C15.6), -Rat Isotype IgG1 (RTK2071), and -Rat Isotype IgG2b (RTK4530) were purchased from BioLegend. BD Cytofix/Cytoperm Kit for intracellular cytokine flow cytometry and GolgiPlug were purchased from BD Biosciences. JAWS II (CRL-11904) and Lewis Lung Carcinoma (CRL-1642) cell lines were purchased from ATCC. Bone marrow-derived dendritic cells (BMDCs) were harvested from 6-week-old C57Bl/6 mice (Jackson Laboratory). BMDCs were cultured in BMDC primary media: RPMI 1640 (Life Technologies), 10% heat inactivated fetal bovine serum (FBS), 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Cahalan Lab), 2 mM L-glutamine (Life Technologies), antibiotic-antimycotic (1x) (Life Technologies), and 50 μM beta-mercaptoethanol (all components were sterile filtered (0.22 μm) together before use). RAW264.7 macrophage cells (RAW-Blue) were cultured in D-MEM High Glucose media (LifeTechnologies), 10% FBS, 2 mM L-glutamine, and antibiotic-antimycotic (1x) and experiments were run in D-MEM High Glucose media (LifeTechnologies), 10% heat inactivated FBS, 2 mM L-glutamine, and antibiotic-antimycotic (1x). JAWS II dendritic cells were cultured in MEM-α media (Life Technologies), 20% fetal bovine serum (FBS), 5 ng/mL GM-CSF, 2 mM L-glutamine, and antibiotic-antimycotic (1x). LLCs were cultured in D-MEM High Glucose media (ATCC), 10% FBS, 2 mM L-glutamine, and antibiotic-antimycotic (1x). Sterile phosphate buffered saline (PBS) buffer was obtained from Life Technologies. Fluorescence-activated cell sorting (FACS) buffer contained PBS (1x), 10% FBS, and 0.1% sodium azide. ^1H NMR spectra were taken on an AVANCE600 (600 MHz) spectrometer and analyzed using iNMR software. Mass spectrum was obtained using MALDI-TOF-MS (AB SCIEX TOF/TOF 5800). Flow cytometry data was acquired using BD Accuri C6 Flow Cytometer and analyzed using the BD Accuri C6 software. RAW-Blue absorbances were measured on a Bio-Tek μQuant microplate spectrophotometer MQX200. Fast protein liquid chromatography (FPLC) traces were obtained using a Superdex G75 column, Dulbecco's PBS (DPBS) at pH 7.4, a flow rate of 0.2 mL/min, and UV/Vis detector set to 260 nm. All animal studies and mice maintenance were approved by the Institutional Animal Care and Use Committee (IACUC).

PAMP-Polymer Conjugate Synthetic Procedures.



Lipoteichoic Acid-Polymer Conjugate Synthesis (2a).

Lipoteichoic acid (LTA) was fluorescently labeled by reacting amine containing backbone of LTA (2 mg, 0.2 μmol) with rhodamine B isothiocyanate (RITC) (10.7 μL of 10 mg/mL solution in DMF, 0.2 μmol) in phosphate buffered saline (PBS) buffer (pH 7.4 with 1 mM EDTA) for 15 min at RT. Subsequently, the remaining amines were thiolated by incubating the RITC-LTA solution with N-succinimidyl-S-acetylthiopropionate (SATP)[1] (0.16 mg per 1 mg LTA) in DMF for 1 h at RT. The LTA solution was filtered using a Centriprep Centrifugal Filter Device (3K device). The thiol was deprotected by incubating the filtered LTA solution with hydroxylamine in PBS buffer (100 μL, pH 7.4 with 25 mM EDTA and 0.5 M hydroxylamine) at 4 °C overnight (~16 h). The thiolated LTA was filtered using a Centriprep Centrifugal Filter Device (3K device). The extent of RITC conjugation was quantified *via* UV-Vis to provide one RITC molecule for every five LTA molecules (1:5, RITC : LTA). Thiolation of LTA was confirmed via Ellman's Assay. Thiols per LTA molecule were determined relative to a cysteine standard curve, which resulted in a mixture where 60% of the LTA contained a thiol. (This indicated that one LTA potentially contained more than one thiol.) The thiolated LTA (150 μL) was then reacted with the SM(PEG)$_6$ linker (60 μL of 20 mg/mL solution in DMSO) for 30 min at RT. The resulting conjugate was passed through a NAP-5 Column pre-equilibrated with PBS buffer (pH 7.4). The conjugate was quantified *via* UV-Vis and confirmed using proton nuclear magnetic resonance (^{1}H NMR) and thin-layer chromatography (TLC). ^{1}H NMR spectra were taken in D_2O. The TLC was run in 65:25:4 $CHCl_3$: MeOH : H_2O.



CpG-ODN-Polymer Conjugate Synthesis (2b).

Single stranded CpG-ODN1826 (5'-TCCATGACGTTCCTGACGTT-3') was obtained with a 3'-disulfide modification. The 3'-disulfide modification was reduced to the free thiol using 3% tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in water (400 μL). The reduced CpG-ODN1826 was precipitated in EtOH (1.5 mL) and 3M NaOAc (50 μL) and placed in a -80 °C freezer for 30 min. The precipitated CpG-ODN1826 was centrifuged at 13,000 RPM and 4 °C for 20 min. The supernatant was removed and the precipitated CpG-ODN1826 was rinsed with EtOH (200 μL). The CpG-ODN1826 was placed in a speed-vacuum to remove excess solvent and dry the reduced CpG-ODN1826. The reduced CpG-ODN1826 (2.0 mM) was resuspended in Tris-EDTA (TE) buffer (1x) and passed through a NAP-5 Column, which was pre-equilibrated with TE buffer (1x). Subsequently, the SM(PEG)$_6$ linker (120 μL of 20 mg/mL solution in DMSO) was added over 2 h to the eluted DNA (0.40 mM, 100 μL). The reaction mixture was incubated at RT. The pH of the reaction was monitored to ensure a constant pH ~8. If slightly acidic, PBS buffer (40 μL) was added to the reaction mixture. Maintaining a slightly basic pH facilitated deprotonation of the thiol to increase the rate of conjugate addition. The reaction mixture was passed through a NAP-5 Column pre-equilibrated with PBS buffer (pH 7.4). The conjugate was quantified *via* UV-Vis and confirmed using MALDI-MS.

PAMP Cell Surface Modification of LLCs with LTA-Polymer (2a) and/or CpG-ODN-Polymer (2b) to Provide PAMP_LLC Constructs (3a, 3b, or 3c).

A solution of conjugate **2a** or **2b** or **2a** and **2b** (36 μM, 100 μL) in PBS buffer was incubated with Lewis Lung Carcinoma (LLC) cells ($2x10^6$ cells) for 30 min at RT. The solution was mixed thoroughly using a vortexer. The cell solution was centrifuged at 2500 RPM and RT for 10 min, and the supernatant removed. The modified cells were rinsed in PBS buffer (1 x 200 μL), then cell media (2 x 200 μL) and finally incubated with dendritic cells.

The cell surface modification was quantified using flow cytometry and fluorescent microscopy. To confirm the modification of CpG_LLCs, the 6-FAM-labeled CpG-ODN1826 anti-sense strand (10 μL, 100 μM) was incubated with the modified CpG_LLCs. The solution was incubated for 30 min at 0 °C removed from light. The cells were rinsed using PBS buffer (2 x 200 μL) and resuspended in PBS buffer (100 μL) to be analyzed.

To confirm the LTA_LLC modification, the LTA was labeled with rhodamine B as mentioned in the synthesis of **2a**.

Cell Culture and Flow Cytometry Procedures.

Bone Marrow-Derived Dendritic Cell Harvest and Culture.
Femur bones were removed from 6-week-old C57Bl/6 mice according to Matheu, *et al.*,[2] and the bone marrow was extracted into PBS buffer. The cell suspension was made into a homogeneous solution using a pipette and subsequently filtered through a 70 μm cell strainer (Fisher Scientific). The cell solution was centrifuged at 300 RCF for 10 min at RT. The supernatant was removed, and ACK Lysing Buffer (Lonza) (3 mL) was added to the cell pellet and incubated for 2 min at RT. PBS buffer (13 mL) was then added to the cell suspension, and the cell solution was centrifuged at 300 RCF for 10 min at RT. The cell pellet was resuspended in RPMI 1640 (Fisher Scientific Hyclone), and centrifuged at 300 RCF for 10 min at RT. The cell pellet was resuspended in BMDC primary media. Harvested cells were then counted and plated at 1 million cells/mL density in 100 mm petri dishes (10 mL total media) and incubated at 37 °C in a CO_2 incubator (day 0 of cell culture). On day 3, 10 mL of BMDC primary media was added to each petri dish. Day 5 BMDCs were released using a pipette, centrifuged at 300 RCF for 10 min at RT, and replated in 24-well plates at 1.2 x 10^6 cells/mL density for cell surface marker activation and cytokine profile flow cytometry experiments.

General Procedure for Flow Cytometry for Cell Surface Marker Upregulation.
BMDCs were incubated individually with each PAMP_LLC (**3a**, **3b**, or **3c**) (9:1 BMDCs : PAMP_LLCs in 0.5 mL culture media) for 18 h at 37 °C in a CO_2 incubator. Stimulated BMDCs were released from the plate and transferred to 1 mL eppendorf tubes. The cells were centrifuged at 2500 RPM and 4 °C for 10 min and the supernatant was removed. The cell pellet was resuspended in cold FACS buffer (100 μL) and incubated with CD16/32 FcR blocking antibodies (1.0 μg/1*10^6 cells) (BioLegend) on ice for 10 min. The cell suspension was centrifuged at 2500 RPM for 10 min at 4 °C and the supernatant removed. The cell pellet was resuspended in cold FACS buffer (100 μL) and incubated with FITC-CD11c (1.0 μg/1*10^6 cells) and PE-CD86 (1.0 μg/1*10^6 cells), -CD40 (1.0 μg/1*10^6 cells), -MHCII (0.25 μg/1*10^6 cells), or -CD80 (0.5 μg/1*10^6 cells)) on ice and removed from light for 30 min. Cold FACS buffer (300 μL) was added to each sample. The cell suspension was centrifuged at 2500 RPM and 4 °C for 10 min and the supernatant was removed. The cells were then rinsed with cold FACS buffer (300 μL) one final time and the supernatant removed. The dendritic cells were resuspended in cold FACS buffer (200 μL) and kept on ice until being loaded onto the flow cytometer.

General Procedure for Intracellular Cytokine Flow Cytometry Staining.
BMDCs were incubated individually with a solution of PAMP_LLC (**3a**, **3b**, or **3c**) (9:1 BMDCs : PAMP_LLCs in 0.5 mL culture media) for 8 h at 37 °C in a CO_2 incubator. GolgiPlug (BD Biosciences), containing Brefeldin A, was added to cell culture (according to BD Biosciences Protocol) for the final 4 h of culture. After 8 h, stimulated BMDCs were released from the plate and transferred to 1 mL eppendorf tubes. The cells were centrifuged at 2500 RPM and 4 °C for 10 min and the supernatant removed. The cell pellet was resuspended in cold FACS buffer (100 μL) and incubated with CD16/32 FcR blocking antibodies (1.0 μg/1*10^6 cells) on ice for 10 min. The cell suspension was centrifuged at 2500 RPM and 4 °C for 10 min and the supernatant removed. The cell pellet was resuspended in cold FACS buffer (100 μL) and incubated with FITC-CD11c (0.25 μg/1*10^6 cells) on ice and removed from light for 30 min. Cold FACS buffer (300 μL) was added to each sample. The cell suspension was centrifuged at

2500 RPM for 10 min at 4 °C and the supernatant removed. The cells were rinsed with cold FACS buffer (300 μL) one more time and the supernatant removed. The cell pellet was resuspended in 100 μL BD Cytofix/Cytoperm solution and incubated on ice and removed from light for 20 min. The cell suspension was centrifuged at 2500 RPM and 4 °C for 10 min and the supernatant removed. BMDCs were washed in BD Perm/Wash solution (2 x 300 μL) and the supernatant removed. The cell pellet was resuspended in cold FACS buffer (100 μL) and incubated with APC-IFN-γ (1.0 μg/1*10^6 cells), -TNF-α (0.25 μg/1*10^6 cells), -IL-6 (0.25 μg/1*10^6 cells), -IL-10 (0.25 μg/1*10^6 cells), or -IL-12 (0.25 μg/1*10^6 cells)) on ice and removed from light for 30 min. Cold FACS buffer (300 μL) was added to each sample. The cell suspension was centrifuged at 2500 RPM and 4 °C for 10 min and the supernatant removed. The cells were rinsed with cold FACS buffer (300 μL) one more time. BMDCs were resuspended in cold FACS buffer (200 μL) and kept on ice until analysis *via* flow cytometer.

General Procedure for RAW264.7 Macrophage (RAW-Blue) NF-κB assay.[3]

RAW-Blue cells were plated at 10*10^4 cells/mL density (180 μL) in 96-well plates using testing media as described in Methods and Materials. RAW-Blue cells were incubated individually with 20 μL of each PAMP_LLC (**3a**, **3b**, or **3c**) (9:1 RAW-Blue : PAMP_LLCs in 200 μL total cell media volume) for 18 h at 37 °C in a CO_2 incubator. Cell media (50 μL) from the stimulated RAW-Blue cells was removed, placed into a 96-well plate, and incubated with QUANTI-Blue solution (Invivogen) (150 μL) for 1 h at 37 °C in a CO_2 incubator. The absorbance (620 nm) was measured using a Bio-Tek μQuant microplate spectrophotometer.

MALDI-MS.

CpG-ODN-Polymer Conjugate.
The reaction mixture was passed through $ZipTip_{C18}$ (Millipore) according to Millipore protocol: A $ZipTip_{C18}$ was equilibrated with 50% acetonitrile/water (2 x 10 μL) and subsequently 0.1 M triethylammonium acetate (TEAA) (3 x 10 μL). The reaction mixture was passed through the $ZipTip_{C18}$ (10 x 10 μL). The $ZipTip_{C18}$ was washed with 0.1 M TEAA buffer (3 x 10 μL) followed by nanopure water (3 x 10 μL). Conjugate **2b** was eluted using 50% acetonitrile/water (3 x 10 μL). Purified conjugate **2b** was dried using a speed-vacuum and resuspended in 0.36 M 3-hydroxypicolinic acid matrix (1:1 acetonitrile : 300 mM ammonium citrate solution in 50% acetonitrile/water) (2.0 μL). The sample was spotted on a MALDI plate and analyzed in negative ion mode.

Confocal Microscopy.

Fluorescent Cell Labeling with DiO and DiI and Confocal Microscopy Colocalization Experiments.
Stock solutions of DiI and DiO dye (1 mg/mL) were prepared in DMSO. Working solutions of DiI and DiO (100 μg/mL) were prepared by diluting the stock solutions with glucose labeling buffer (300 mM glucose and 10 mM HEPES in nanopure water).[4] The cells were plated one day prior in 6-well plates at $2*10^5$ cells/mL. Before labeling the cells, the media was removed. JAWS II and LLC cell lines were suspended in DiO or DiI solutions (0.5 mL of 100 μg/mL), respectively, and incubated at 37 °C in a CO_2 incubator for 2 h. The DiI or DiO solution was then removed. The cells were washed with glucose buffer (2 x 1 mL) and resuspended in cell media. The LLCs were labeled with **2a**, **2b**, or **2a** and **2b** as described in the synthetic procedures. The PAMP_LLCs were incubated with the dendritic cell line overnight (~ 14-18 h) at 37 °C. The cell media was removed. The cells were then resuspended in PBS buffer, and analyzed *via* confocal microscopy.

Supplemental Figures.

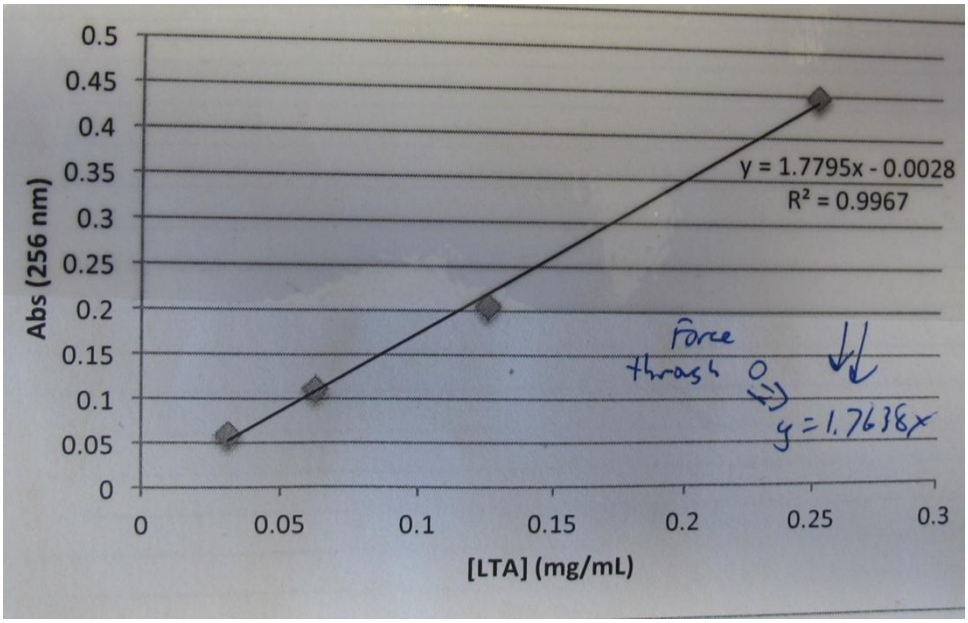


Figure S1. Ellman's assay cysteine calibration curve used to determine number of thiols per LTA molecule.

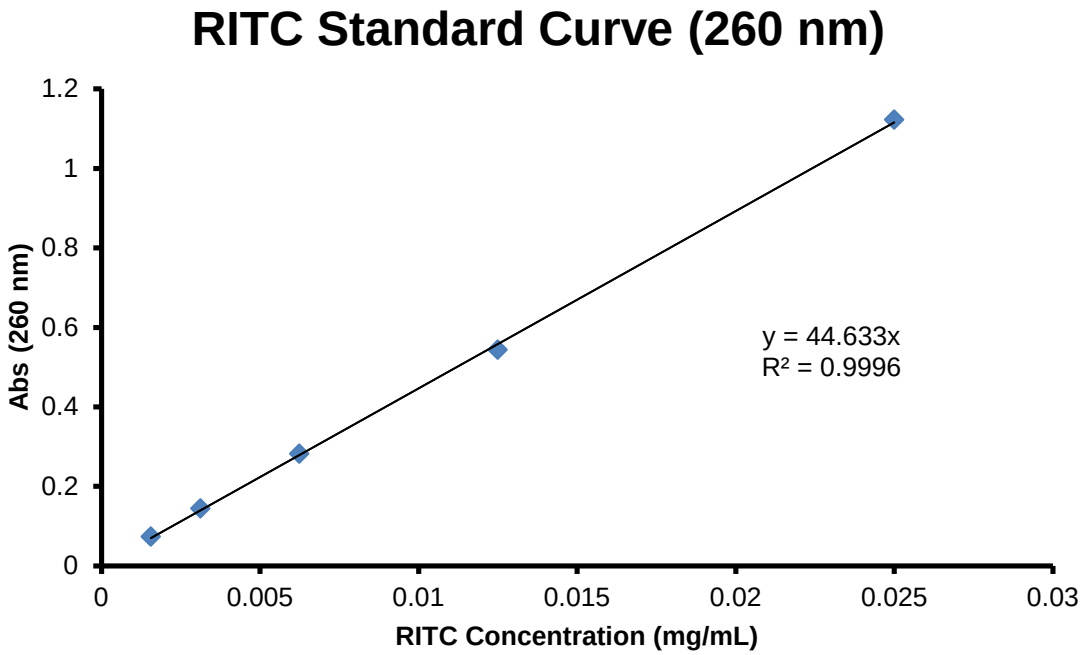


Figure S2. RITC standard curve at 260 nm used to quantify the number of RITC molecules conjugated to LTA.

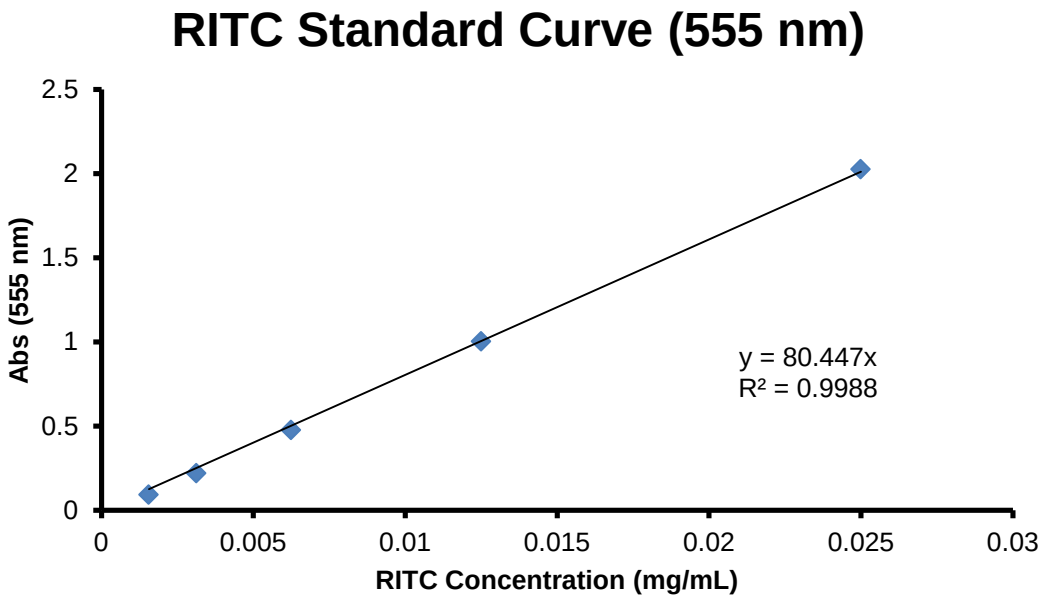


Figure S3. RITC standard curve at 555 nm used to quantify the number of RITC molecules conjugated to LTA.

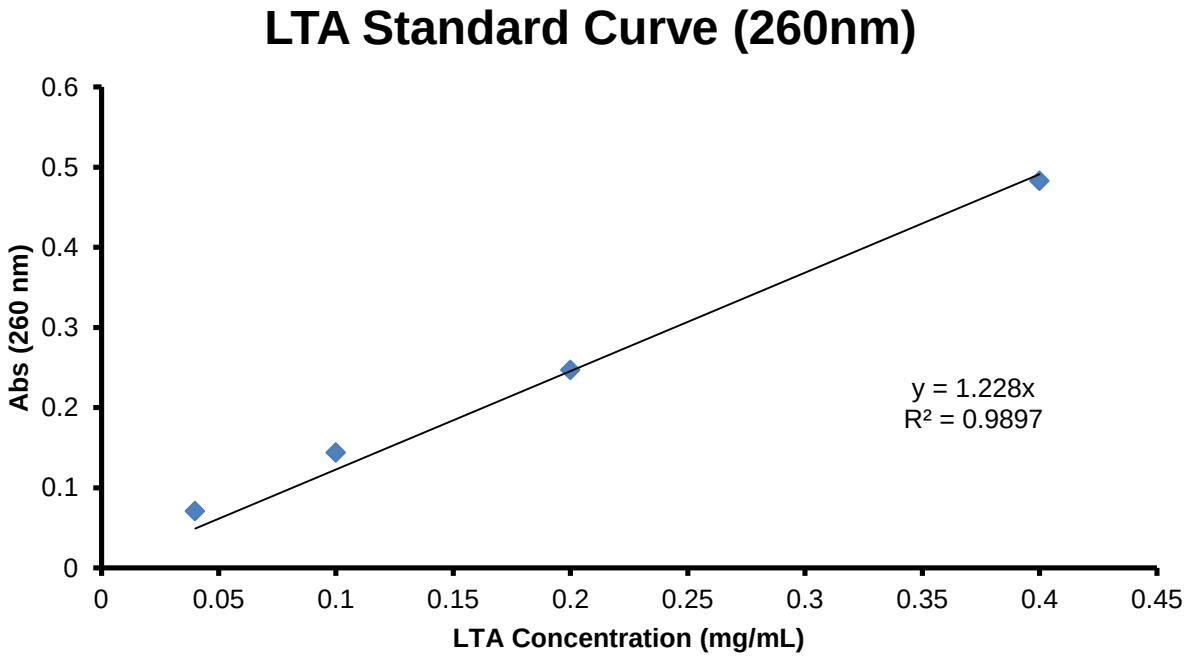


Figure S4. LTA standard curve at 260 nm used to quantify the number of RITC molecules conjugated to LTA.

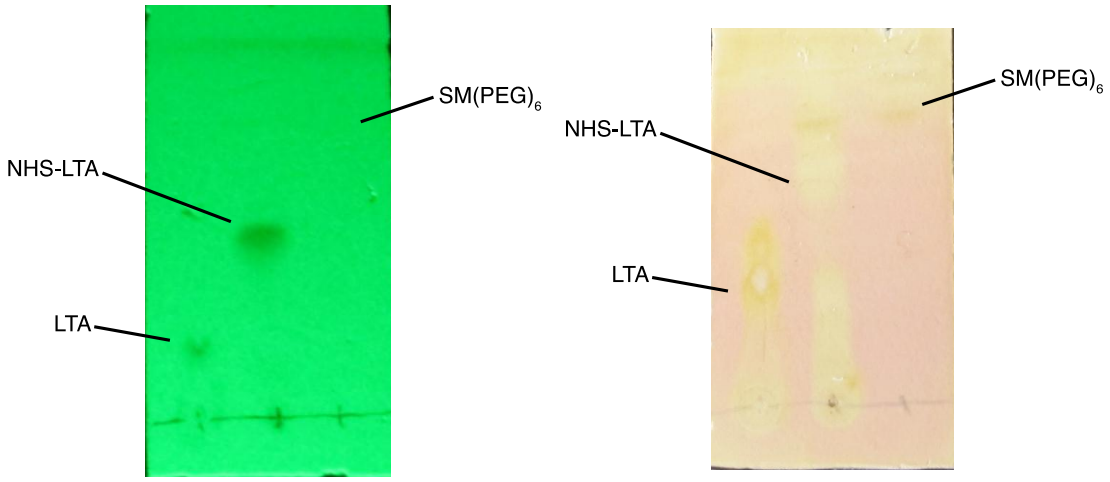


Figure S5. Thin-layer chromatography (in 65:25:4 $CHCl_3$: MeOH : H_2O)[5] confirming the synthesis of NHS-LTA (**2a**): LTA (left lane), NHS-LTA (middle lane), $SM(PEG)_6$ linker (right lane) under UV light (254 nm). TLC plate under UV 254 nm (left plate) and stained with $KMnO_4$ (right plate).

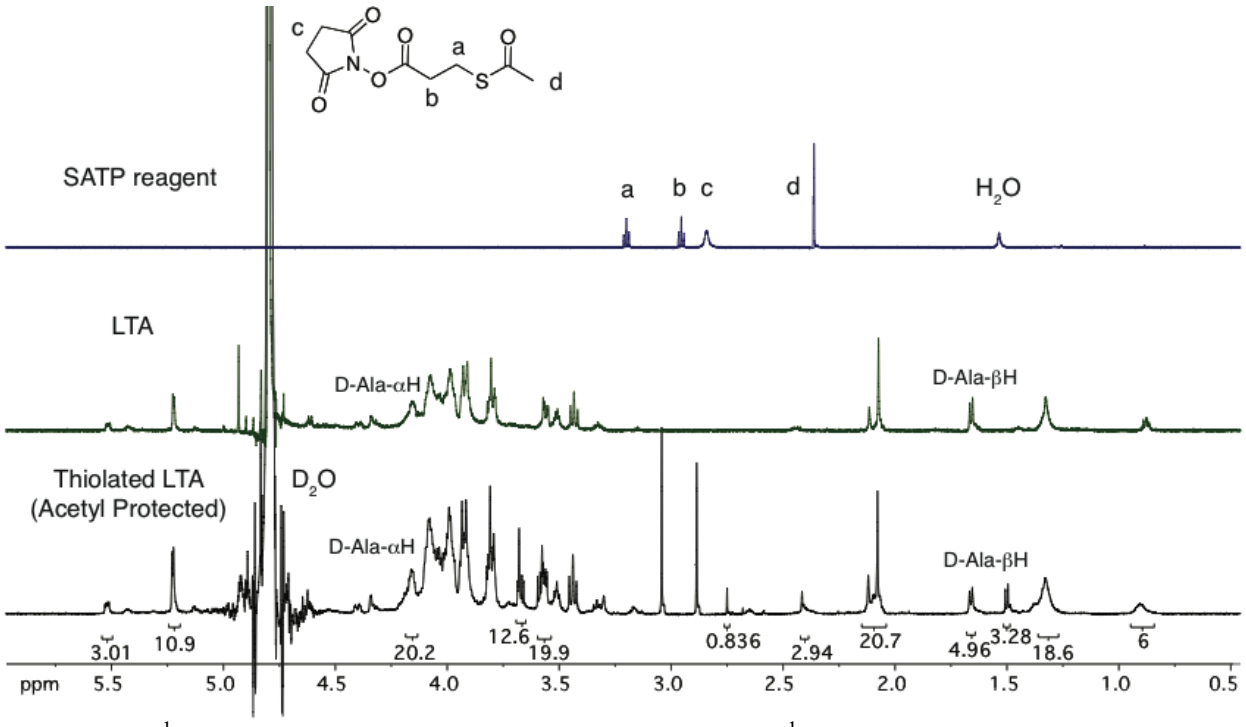


Figure S6. ^{1}H NMR spectra of SATP reagent (blue-top),[1] LTA (green-middle), and acetyl protected sulfhydryl LTA (black-bottom). All samples were taken in D_2O.[6] (Refer to Ref. 6 for LTA structure. ^{1}H NMR of **2a** was attempted. However, the spectrum was unclear as many of the peaks overlapped and thus was inconclusive.)

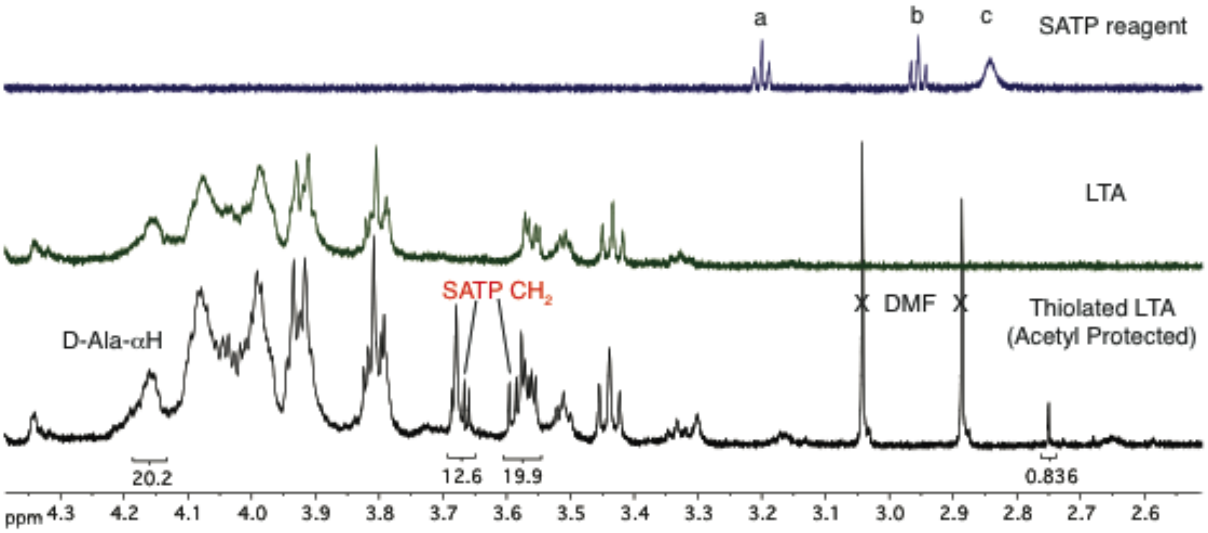


Figure S7. [1]H NMR spectra of SATP reagent (blue-top),[1] LTA (green-middle), and acetyl protected sulfhydryl LTA (black-bottom). All samples were taken in D_2O. D-Ala-αH was observed at 4.16 ppm. SATP methylene protons were observed at 3.59 and 3.68 ppm.

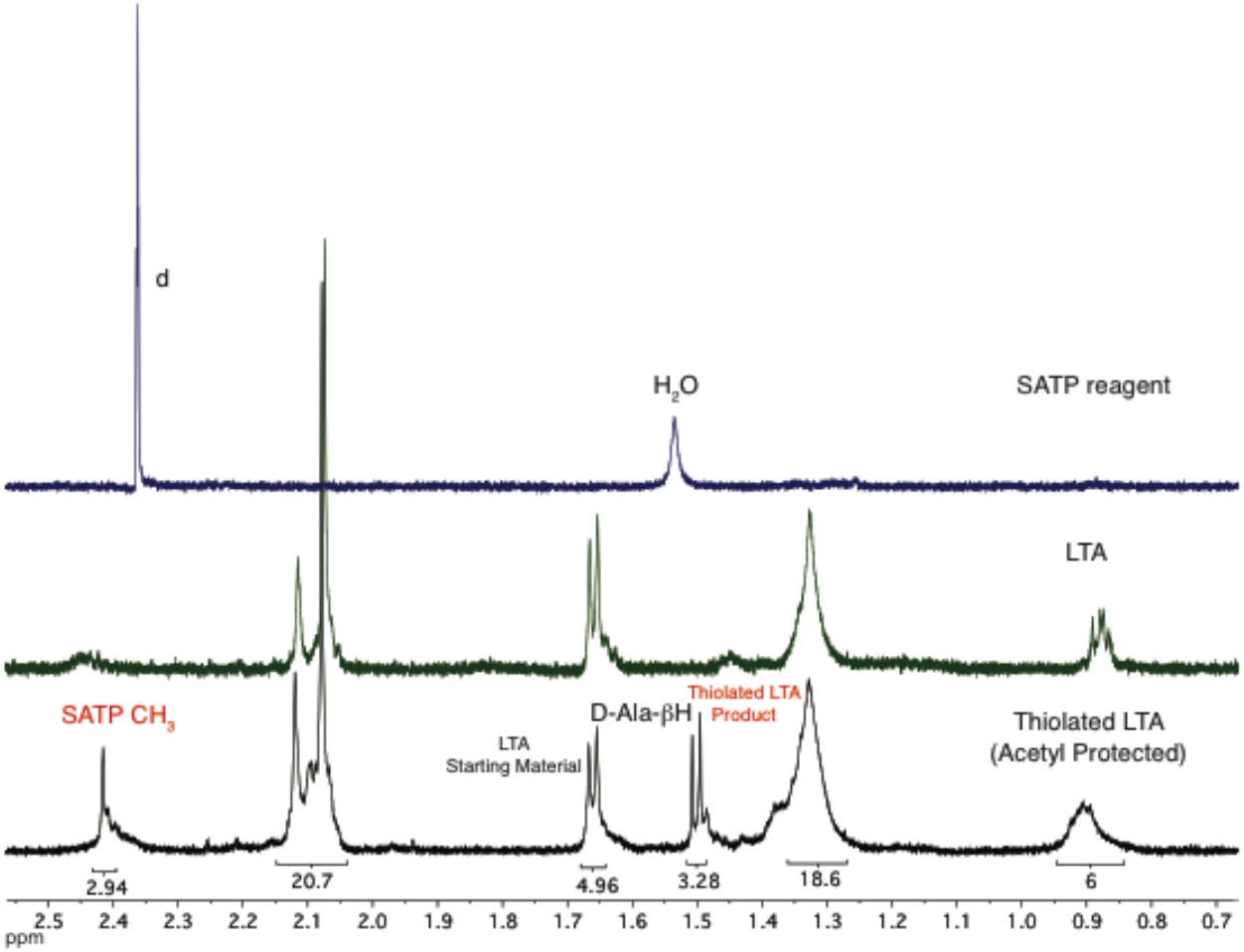


Figure S8. ^{1}H NMR spectra of SATP reagent (blue-top),[1] LTA (green-middle), and acetyl protected sulfhydryl LTA (black-bottom). All samples were taken in D_2O. D-Ala-βH was observed at 1.50 (protected sulfhydryl LTA) and 1.66 ppm (starting material LTA). SATP methyl protons were observed at 2.42 ppm.

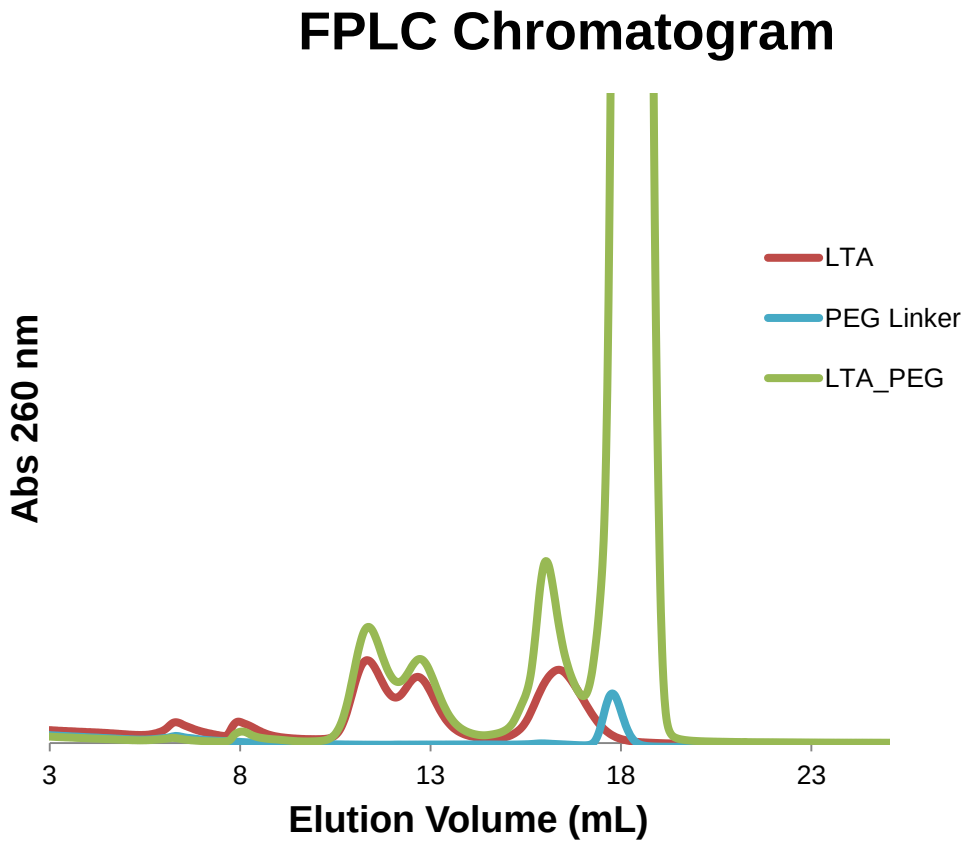


Figure S9. FPLC trace of LTA (red), SM(PEG)$_6$ linker (blue), and **2a** (green) (samples run in DPBS). LTA conjugation to SM(PEG)$_6$ linker does not result in a significant shift in the FPLC trace, so **2a** cannot be definitively confirmed using FPLC.

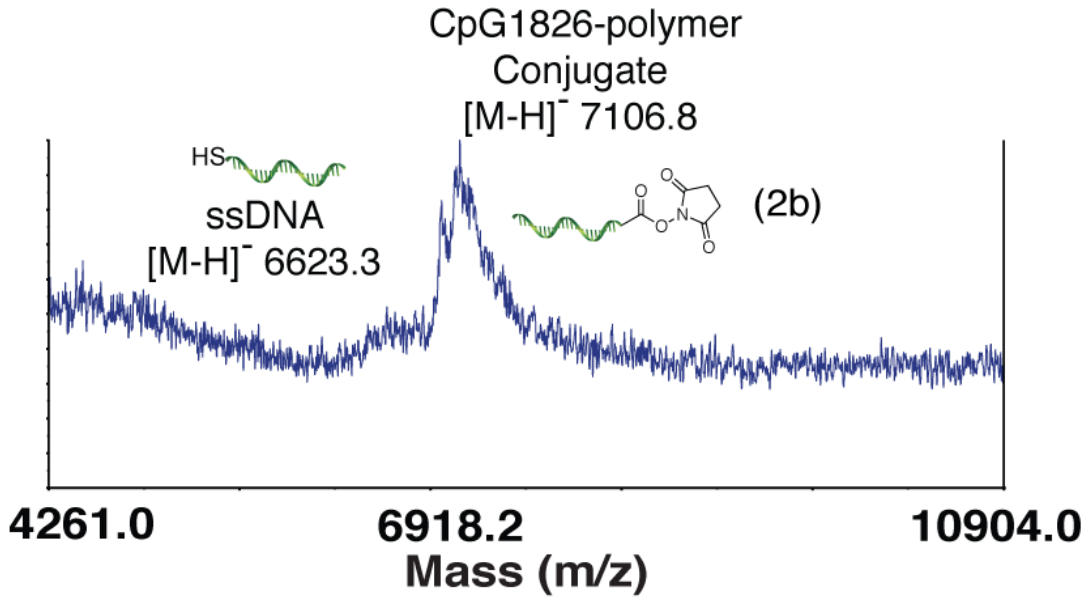


Figure S10. MALDI-MS confirming the synthesis of NHS-CpG-ODN (**2b**) at m/z = 7106.8.

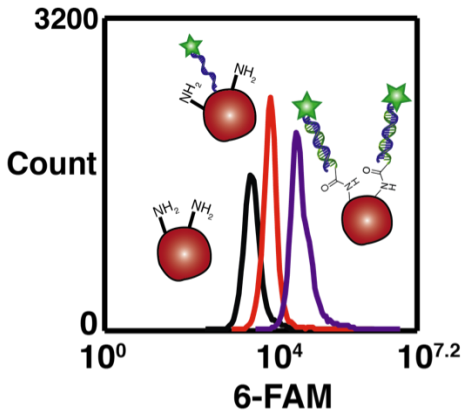


Figure S11. Flow cytometry confirmation of 6-FAM-labeled CpG-ODN cell surface modification of LLCs to provide **3b**: unmodified LLCs (black), non-specific sticking of 6-FAM CpG-ODN1826 anti-sense strand (red), and 6-FAM-labeled CpG-ODN_LLCs (purple).

‡‡ After 2-5 hours, we observed disappearance of the modification from the surface of the LLCs. Endocytosis is the most likely mechanism (data not shown).

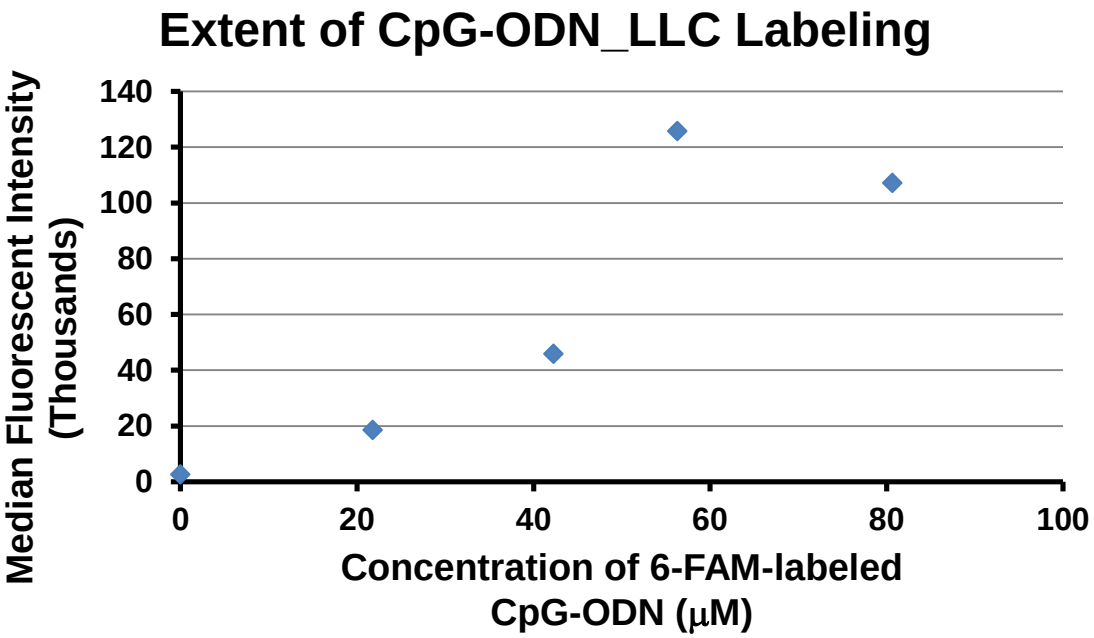


Figure S12. Flow cytometry quantification of 6-FAM-labeled CpG-ODN_LLCs. LLCs were incubated with varying concentrations of 6-FAM-labeled CpG-ODN1826 conjugate **2b** for 30 min at RT.

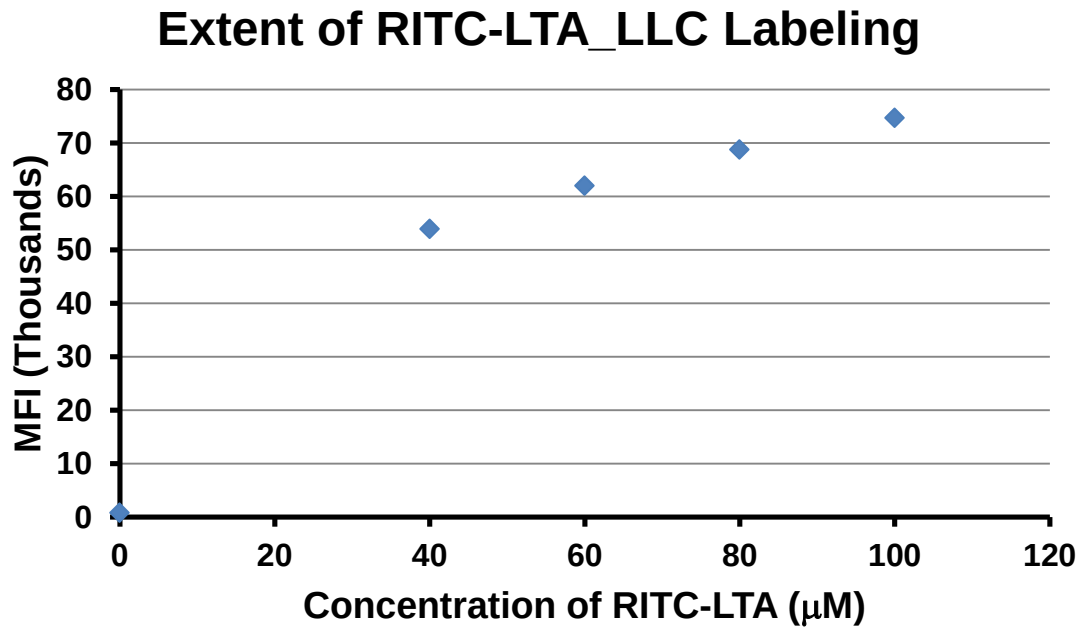


Figure S13. Flow cytometry quantification of RITC LTA_LLCs. LLCs were incubated with varying concentrations of RITC LTA conjugate **2a** for 30 min at RT.

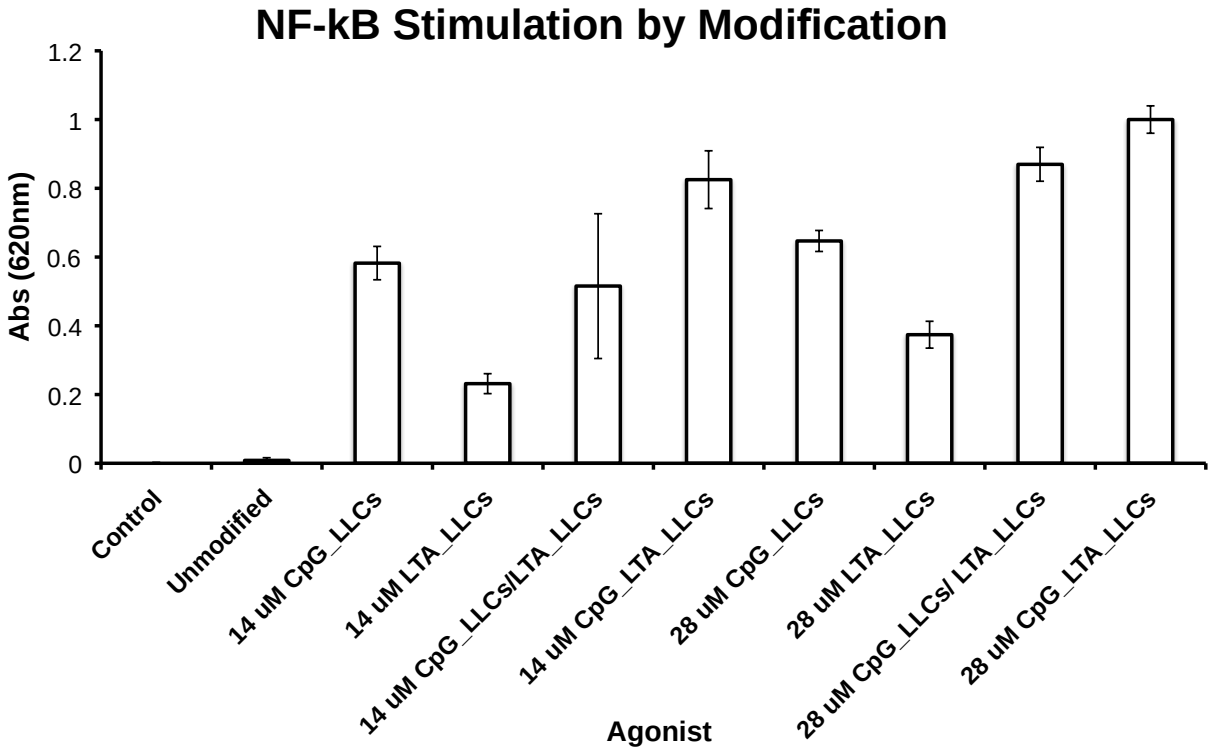


Figure S14. RAW264.7 macrophage NF-κB stimulation *via* alkaline phosphatase secretion. Data displays absorbance (620 nm) caused by macrophage cell incubation with PAMP_LLC constructs for 19 h at 37 °C, which correlates to NF-κB stimulation. LLCs were modified with half of the concentration of PAMP-polymer conjugate (14 μM) compared to the full concentration (28 μM) to determine that the activation from CpG_LTA_LLCs is more than the additive stimulation from just CpG_LLCs and LTA_LLCs. More important, the CpG_LTA_LLCs displayed the greatest stimulation over using a single PAMP. Data obtained over six independent experiments, where $p < 0.025$ for CpG_LLCs relative to CpG_LTA_LLCs, $p < 0.05$ for LTA_LLCs relative to CpG_LTA_LLCs, and $p < 0.01$ for the unmodified LLCs relative to all PAMP_LLCs and for the CpG_LTA_LLCs relative to all other samples.

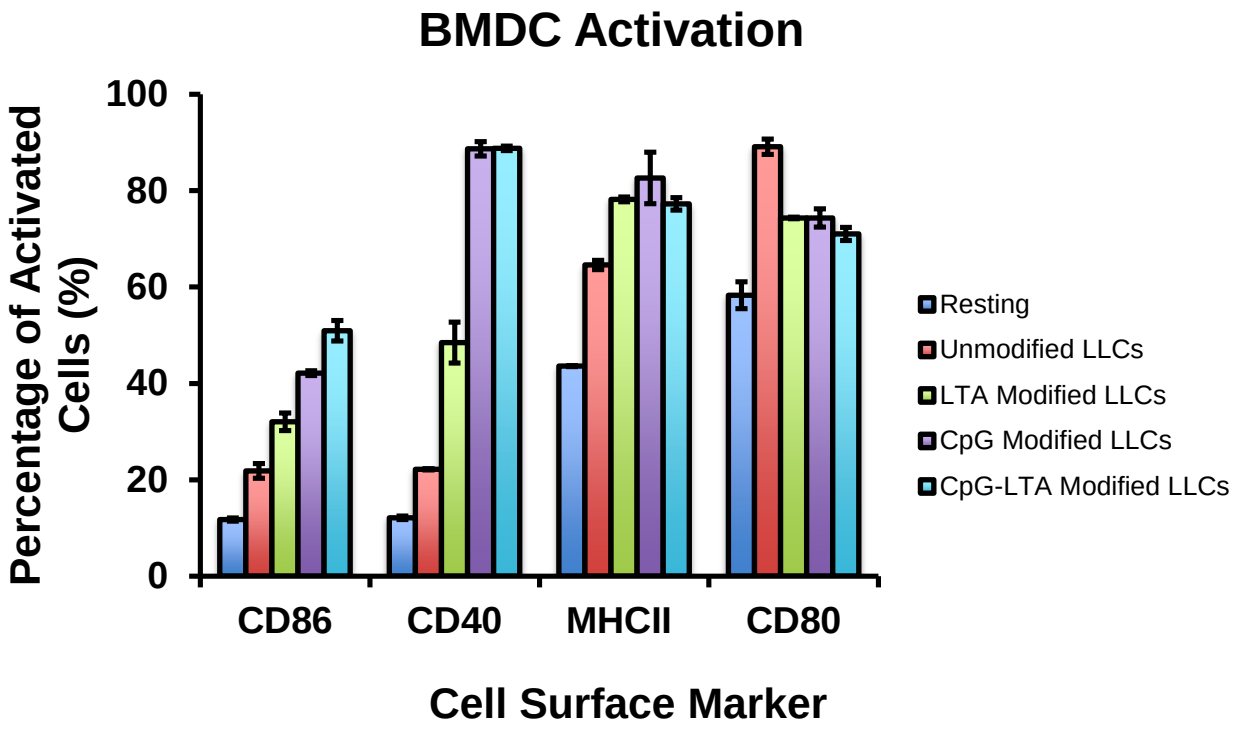


Figure S15. Flow cytometry analysis of BMDC cell surface marker upregulation, including CD80, using PAMP_LLCs: control (dark blue), unmodified LLCs (red), LTA_LLCs (green), CpG_LLCs (purple), CpG_LTA_LLCs (light blue). P values represent each PAMP_LLC in comparison to the resting state where $p < 0.01$ for CD86, CD40, and MHC II, $p < 0.025$ for CD80 over three independent experiments, $p < 0.05$ for CD86 comparing unmodified LLCs to LTA_LLCs, $p < 0.01$ comparing unmodified LLCs to CpG_LLCs and CpG_LTA_LLCs, $p < 0.025$ comparing CpG_LLCs to CpG_LTA_LLCs, and $p < 0.01$ for CD40 comparing unmodified LLCs to all PAMP_LLCs.

‡ For flow cytometry experiments, **2a**, **2b**, and **2c** did not contain fluorescent tags, so that the conjugates would not interfere with quantifying the fluorescently tagged antibodies.

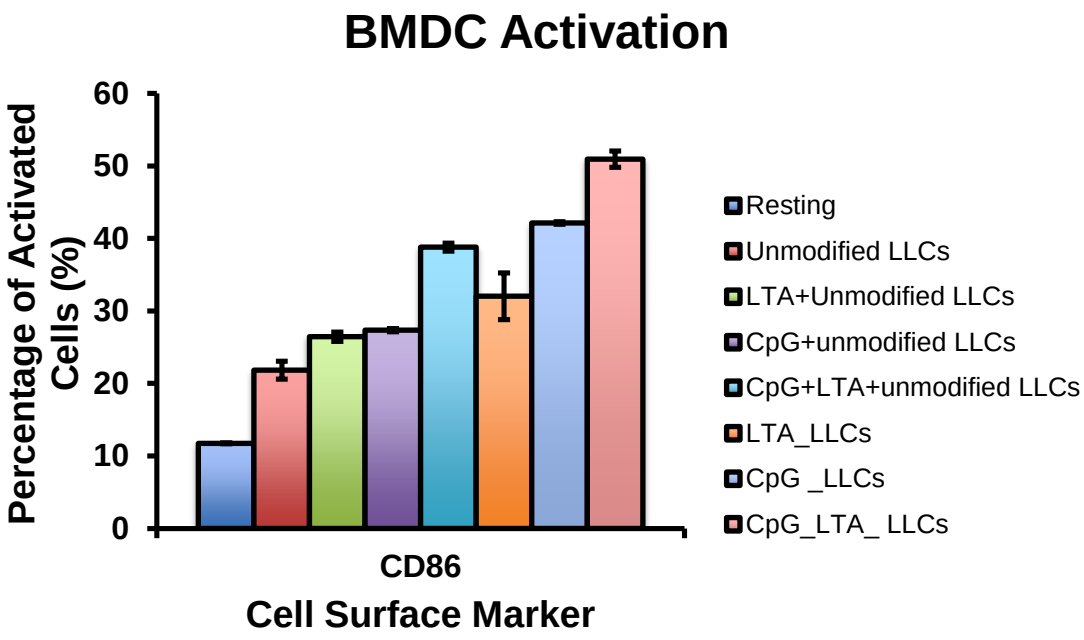


Figure S16. Flow cytometry analysis of BMDC CD86 upregulation, including PAMPs free in solution (positive controls): control (dark blue), unmodified LLCs (red), LTA (1 μg/mL) and unmodified LLCs (green), CpG-ODN (5 μg/mL) and unmodified LLCs (purple), and CpG-ODN (5 μg/mL)/LTA (1 μg/mL)/unmodified LLCs (light blue), LTA_LLCs (orange), CpG_LLCs (light purple), CpG_LTA_LLCs (pink).

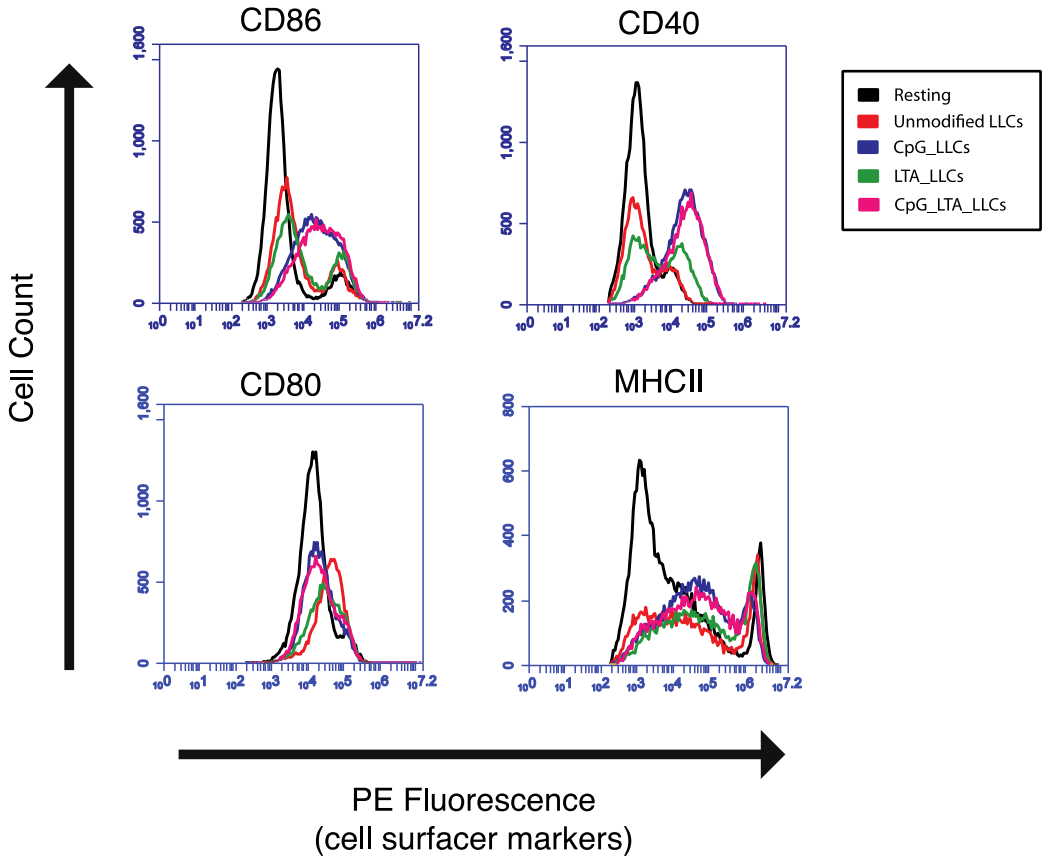
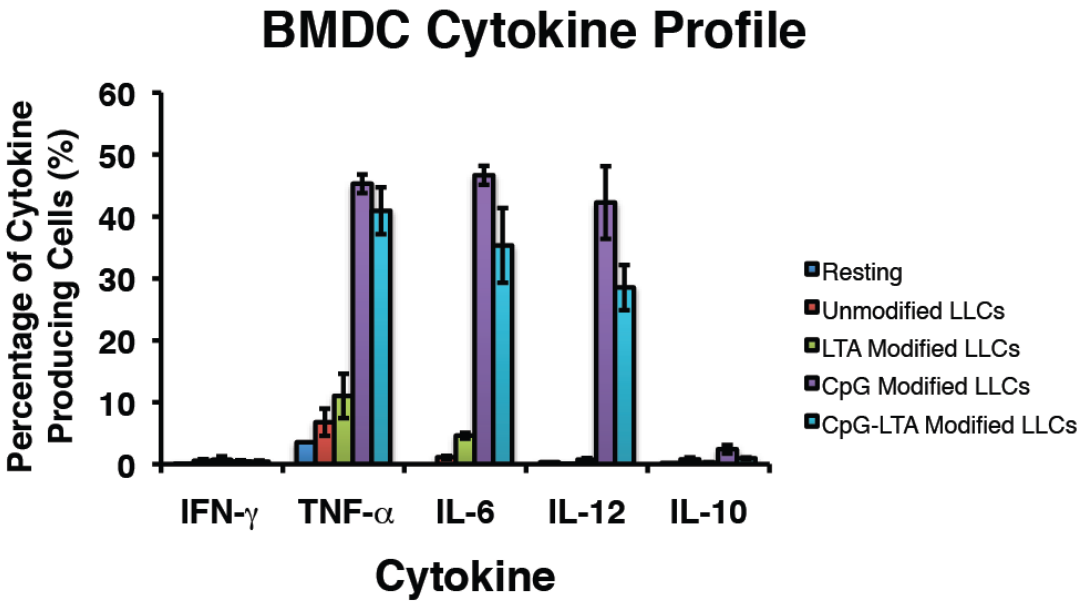


Figure S17. Activation of BMDCs using PAMP_LLCs. Expression of CD86, CD40, CD80, and MHCII was analyzed *via* flow cytometry: resting (black), unmodified LLCs (red), CpG_LLCs (blue), LTA_LLCs (green), and CpG_LTA_LLCs (pink).



Figures S18. Intracellular cytokine flow cytometry analysis, including IL-10, using PAMP_LLCs: control (dark blue), unmodified LLCs (red), LTA_LLCs (green), CpG_LLCs (purple), CpG_LTA_LLCs (light blue). P values represent that for resting state relative to CpG_LLC and CpG_LTA_LLCs where $p < 0.01$ for TNF-α, IL-6, and IL-12 over three independent experiments, and $p < 0.1$ for IL-6 and IL-2 comparing CpG_LTA_LLCs to CpG_LLCs.

‡‡‡‡‡ Cytokines can polarize an immune response in order to elicit a specific response against a targeted pathogen. Pro-inflammatory cytokines help recruit APCs toward an infected site to eliminate a pathogen. In contrast, anti-inflammatory cytokines inhibit the recruitment of APCs and suppress an immune response, which is necessary for autoimmune diseases. A balance of the different types of immune responses is required to effectively combat foreign pathogens.

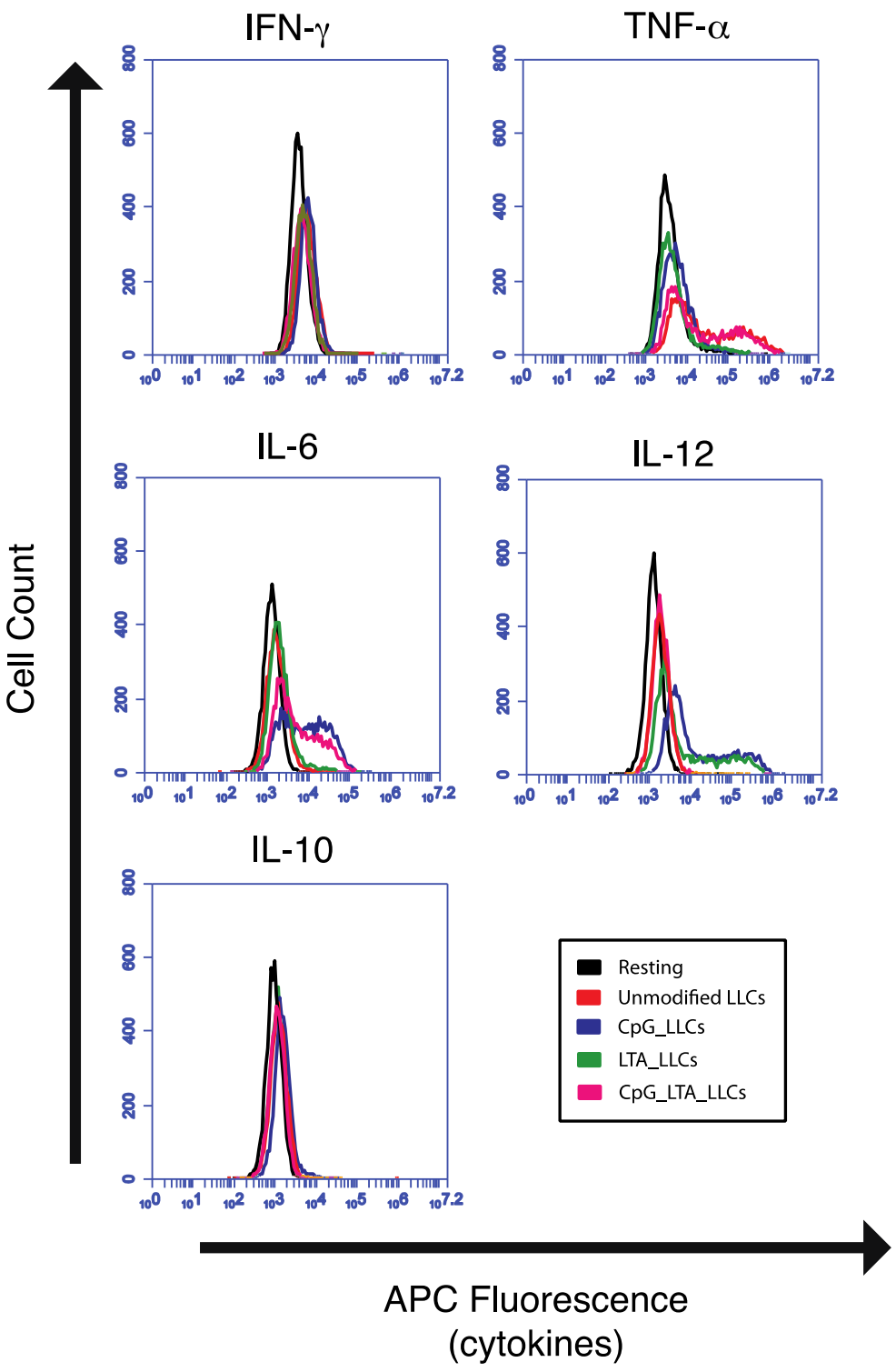


Figure S19. Activation of BMDCs using PAMP_LLCs. IFN-γ, TNF-α, IL-6, IL-12, and IL-10 cytokine production was analyzed *via* flow cytometry: resting (black), unmodified LLCs (red), CpG_LLCs (blue), LTA_LLCs (green), and CpG_LTA_LLCs (pink).

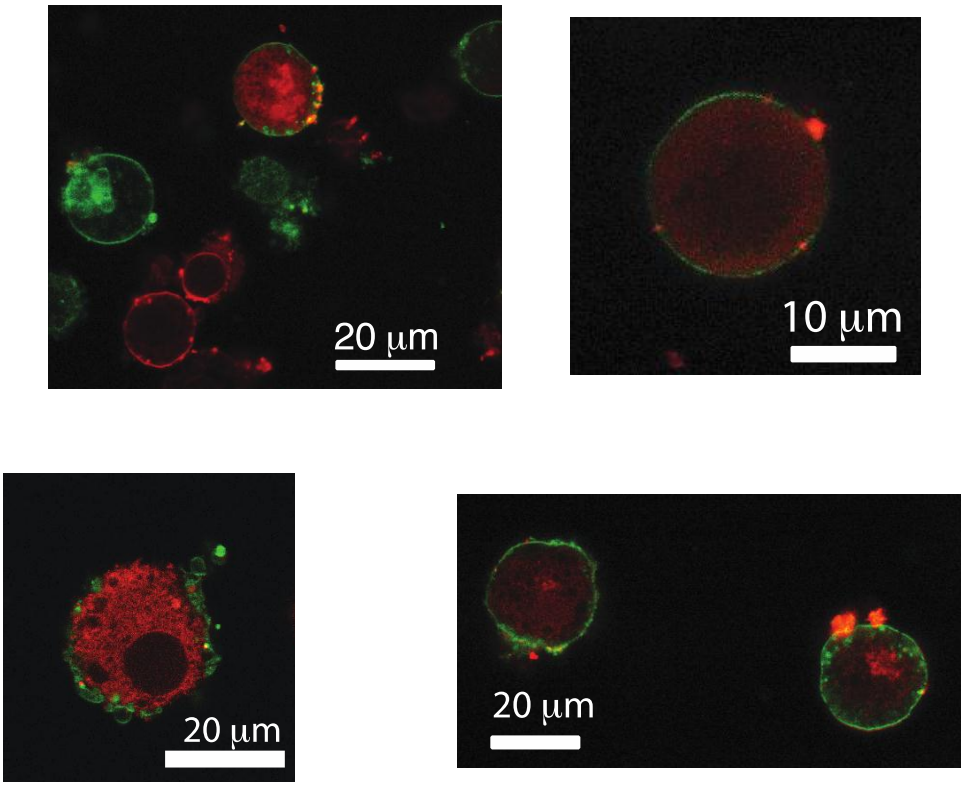


Figure S20. Confocal microscopy image of DiI-labeled CpG_LLCs (red) macrophagocytosed by DiO-labeled dendritic cells (green).

‡‡‡ DiO is a lipophilic, membrane bound green fluorophore. DiI is a lipophilic, membrane bound red fluorophore. These lipophilic dyes are commonly used to track multiple cells. There are hypotheses that these dyes can exchange/diffuse between cells, since they embed into the cell membrane *via* lipophilic interactions. However, dialkylcarbocyanine dyes have been used for many years in the field of immunology for cell labeling and tracking experiments.[7,8] Also, no DiI-labeled CpG_LLCs were observed with DiO-labeled cell interiors and no DiI was observed on the outer cell membrane of the DCs. These results strongly suggest that the dendritic cells engulfed the CpG_LLCs and that the images were not a result of the interchange of the lipophilic dyes.

‡‡‡‡ DC stimulation using CpG-ODN recruits TLR9 from the ER through the Golgi to endosomal compartments *via* membrane fusion. TLR9 is processed and cleaved after passing through the Golgi to provide functional TLR9 in the endosome as opposed to full-length (unprocessed, non-functional) TLR9, which does not become activated by ligands.[9,10]

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