Supporting information for

A universal TLR7-nanoparticle adjuvant promotes broad immune responses against heterologous strains of Influenza and SARS-CoV-2

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Materials and Methods

Reagents

L-Lactide (LLA) was purchased from TCI America (Portland, OR, USA). It was recrystallized three times in anhydrous toluene and stored at -30 °C in glove box. mPEG-PLGA (LG 50:50 (w:w), Mw 5000:10000Da) was purchased from Akina, Inc. (West Lafayette, IN, USA). TLR7 agonist (gardiquimod) was purchased from InvivoGen (San Diego, CA, USA). Alexa Fluor™ 647 NHS Ester (Succinimidyl Ester) was purchased from Thermofisher (Waltham, MA, USA). ((BDI)Zn catalysts and precursor were synthesized according to the previous report using similar procedure (ref). All other chemical reagents were purchased from Sigma-Aldrich and used as received except where otherwise noted. Recombinant SARS-CoV-2 protein (Spike, S1, RBD) and variants were purchased from Sino Biological, Inc (Beijing, China). H2-K^b SIINFEKL tetramer-PE was purchased from MBL international corporation (Woburn, MA, USA).

Antibodies

All antibodies were purchased from BD bioscience, BioLegend or ebioscience. The following antibodies were used in the mouse study: anti-CD16/CD32 (BD bioscience, Cat#: 553141, clone 2.4G2), Brilliant Violet 605 anti-mouse CD45 (BioLegend Cat#: 103140, clone 30-F11), PE/Dazzle 594 anti-mouse TCRb chain (Cat#: 109240, clone H57-597), Alexa Fluor 700 anti-mouse CD3 (BioLegend, Cat#: 100216, clone 17A2), BUV805 Rat anti-mouse CD8α (BD Biosciences, Cat#: 564920, clone 53-6.7), BUV395 Rat anti-mouse CD4 (BD Biosciences, Cat#: 565974, clone GK1.5), APC/Cy7 anti-mouse/human CD44 (BioLegend, Cat#: 103028, clone IM7), Brilliant Violet 605 anti-mouse CD62L (BioLegend, Cat#: 104438, clone MFL-14), PE/Cy7 anti-mouse CD69 (BioLegend, Cat#: 104512, clone H1.2F3), Alexa Fluor 647 anti-mouse Foxp3 (Biolegend, Cat#: 126408, clone MF-14), Brilliant Violet 711 anti-mouse CD279 (PD-1) (BioLegend, Cat#: 135231, clone 29F.1A12), Brilliant Violet 785 anti-mouse IFN-γ (Biolegend, Cat#:505838, clone XMG 1.2), FITC anti-human/mouse Granzyme B (Biolegend, Cat#:372206, clone QA16A02), PE Mouse anti-Bcl-6 (BD Biosciences, Cat#: 561522, clone K112-91).

The following antibodies were used in the human study: Alexa Fluor 700 anti-human CD45 (Biolegend, Cat#: 304024, clone HI30), PerCp-Cy5.5 anti-human CD3 (Biolegend, Cat#: 300328, clone HIT3a), PE anti-human CD19 (Biolegend, Cat#: 392506, clone 4G7), BUV 805 anti-human

CD8 (BD Biosciences, Cat#: 564912, clone SK1), Brilliant Violet 650™ anti-human CD4 (BioLegend, Cat#: 300536, clone RPA-T4), APC anti-human CD38 (Biolegend, Cat#:356606, clone HB-7), PE-Cy7 anti-human CD27 (Biolegend, Cat#:124216, clone LG.3A10).

Instrument

NMR spectra were recorded on Varian U500 (500 MHz), VXR-500 (500 MHz), or Varian U600 (600 MHz) spectrometers. Gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (Model 1260, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS multiangle laser light scattering (MALLS) detector, and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (100 Å, 103 Å, 104 Å, 105 Å, and 106 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C using DMF containing 0.1 M LiBr as the mobile phase. The molecular weights of polymers were determined from the dn/dc value assuming 100% mass recovery using ASTRA software (Version 6.1.1, Wyatt Technology). MALDI-TOF spectra were taken on a Bruker Ultra Flextreme equipped with a 337 nm nitrogen laser. The sizes and the size distributions of the NPs were determined on a ZetaPALS dynamic light scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). Flow data were acquired on LSRII or LSRII.2 flow cytometer (BD biosciences). Cells were sorted on FACSAria Fusion (BD biosciences). The absorbance of ELISA assay was measured on Microplate Reader (Bio-Rad). The whole animal in vivo imaging was performed on Spectral Instruments Imaging (Tuscon, AZ, USA).

Animals

8-12 weeks old female C57BL/6J mice were purchased from the Jackson Laboratory. All the animals were cared in Stanford Animal Facility under federal, state and NIH guidelines. The study protocol was reviewed and approved by the University Administrative Panel on Laboratory Animal Care.

Human samples

Whole tonsils from consented individuals undergoing surgery for obstructive sleep apnea, hypertrophy or recurrent tonsillitis were collected in accordance with the Stanford University Institutional Review Board (IRB). Ethics approval was granted by the Stanford University IRB (protocols 30837 and 47690). Written informed consent was obtained from adult participants and from the legal guardians of children aged 0–17 years; written informed assent was also obtained from children aged 7 years and older. Whole tonsils were collected in saline after surgery and then immersed in an antimicrobial bath of Ham's F12 medium (Gibco) containing Normocin (InvivoGen), penicillin and streptomycin for 1 h at 4 °C for decontamination of the tissue. Tonsils were then briefly rinsed with PBS and processed as needed for culturing[1].

Preparation of gardiquimod-PLA (TLR7-PLA) polymer conjugate

In a glovebox, gardiquimod (3.1 mg, 0.01 mmol) was dissolved in anhydrous THF (300 μL) and mixed with a THF solution (500 μL) containing (BDI-EI)ZnN(TMS)$_2$ [(BDI) is 2-((2,6-diethylphenyl) amido)-4-((2,6-diisopropylphenyl)-imino)-2-pentene] (6.5 mg, 0.01 mmol). The mixture was stirred for 15 min. LA (14.4 mg, 10 eq) was dissolved in THF (500 μL) and added to the stirred (BDI-EI)ZnN(TMS)$_2$ and the mixture. The reaction proceeded in the glovebox overnight. After LA was completely consumed, the reaction was stopped by quenching the polymerization solution with cold methanol solution (30 μL). The polymer was precipitated with ether (10 mL), collected by centrifugation, and dried by vacuum.

Particle size measurement by dynamic light scattering (DLS)

The hydrodynamic size was measured with 90Plus Particle Size Analyzer (Brookhaven) by dispersing the NPs in DI water at concentration of 0.5 mg/mL. Measurements were taken at a 90° scattering angle.

Release kinetic study of gardiquimod (TLR7 agonist) from NPs

The collected NPs were resuspended in FBS/PBS buffer (0.5 mL, 1:1, v/v) in a dialysis tube with a cut of 1kD. Immerge the dialysis tube in a tube contain PBS (4.5 mL). Collect 0.5 mL sample off the tube at each time point while supplement with 0.5 mL PBS at the same time at 0, 1, 2, 3, 4, 5 days. The gardiquimod concentration was measured by UV spectrometer at $\lambda = 321$nm. The 100 %

release control was achieved by co-incubating above NP with 0.1 M NaOH at 37° C for 6 h and measured with UV spectrometer.

Synthesis of AF647 modified gardiquimod and gardiquimod-PLA (TLR7-PLA)

For the synthesis of AF647 labeled gardiquimod-PLA, AF647-NHS (0.5 mg, 0.4 μmol) was dissolved in ddH_2O (10 μL) and then mixed with gardiquimod-PLA (2.0 mg, 0.5 μmol, 1.2 equiv) in DMF (40 μL), stirred overnight. The reaction was purified by dialysis with a dialysis kit with a cut of 3.5 kD and lyophilized to get the powder.

For the synthesis of AF647 labeled gardiquimod, AF647-NHS (0.5 mg, 0.4 μmol) was dissolved in ddH_2O (10 μL) and then mixed with gardiquimod (132 μg, 0.42 μmol) in ddH_2O (10 μL), stirred overnight. The reaction was purified by flashing on a C18 column (Teledyne ISCO, 30 g, Lincoln, NE, USA) with NH_4HCO_3 (5mM in ddH_2O) and acetonitrile (25% ~ 80%, v/v) and then lyophilized to get the product.

Whole-mouse imaging, lymph node accumulation, and cell uptake study

Mice were immunized with Alum adsorbed ovalbumin (OVA) (100 μg) mixed with either TLR7-Alum or TLR7-NPs (equivalent dose of gardiquimod in these two formulations). Gardiquimod in the TLR7-Alum or TLR7-NPs was labeled by AF647 and the signals of AF647 were measured by longitudinal whole-animal in vivo Lago spectral imaging system from day 0 to day 3. Overplayed images were collected on a Largo X imaging system and analyzed with Aura imaging software (Spectral Instruments Imaging, Tucson, AZ, USA). Total radiance at the s.c. injection site was normalized to the initial fluorescence signal at day 0 taken 30 min after injection. Inguinal draining LNs were excised at various time points and whole-tissue fluorescence was measured by a Lago spectral imaging system at Ex640 nm and Em690 nm wavelengths. Values represent the integrated fluorescence intensity. After imaging, the excised LNs were gently busted with a 3mL syringe plunger thumb rest and digested with 1mg/mL collagenase type 4 for 20-25 minutes at 37°C. Reactions were stopped with 2mM EDTA and single cell suspension were prepared by passing through 40μm cell strainers. The cells were washed, blocked with Fc-blocker (anti-mouse CD16/CD32 monoclonal antibody on ice for 10 min), stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain and surface markers, and analyzed on BD LSRII flow cytometer.

Immunization study

Immunogenicity experiments with recombinant hemagglutinin (HA) and TLR7 agonist used immunization formulations of 10 μg of alum-bound antigen mixed with 20 μg of TLR7 agonist either in TLR7-Alum or TLR7-NPs in 100 μl of PBS. Immunogenicity experiments with recombinant full-length SARS-Cov-2 spike protein and TLR7 agonist used immunization formulations of 5 μg of alum-bound antigen mixed with 20 μg of TLR7 agonist either in TLR7-Alum or TLR7-NPs in 100 μl of PBS. Mice were s.c. immunized at the tail base at week 0 and boosted at week 3. Sera were collected by face bleeding two weeks post either priming or boosting for ELISA measurements.

ELISA analysis of antibody titers

For ELISAs to measure anti-HA titers or anti-SARS-CoV-2 titers, high protein binding plates (Costar) were directly coated with antigen of interest (2 μg ml^{-1}, 50 μL) overnight. The plates were blocked with the blocking buffer for 1 h, washed, and cultured with diluted mouse sera (1:500 or 1:2500) for 2h. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies to IgG was used to detect bound antibodies. Plates were developed with TMB substrate solution (Thermo Scientific), quenched with sulfuric acid and read at 450 nm by Microplate Reader (Bio-Rad). In all ELISA assays, unvaccinated mouse serum was used as negative control.

Phenotypic and functional analysis of T cells

Female C57BL/6 mice (8-12 weeks) were immunized with Alum-bound NP-OVA (50 μg) and TLR7-Alum or TLR7-NPs (equivalent gardiquimod dose: 20 μg) in 100μL PBS at s.c. injection of tail base. For phenotypic analysis, the Inguinal draining LNs (dLNs) were excised at day 4, day 7 and day 14 to prepare single cell suspension. The cells were washed, blocked with Fc-blocker (anti-mouse CD16/CD32 monoclonal antibody on ice for 10 min), and stained with PE-labeled H2-K^b- SIINFEKL tetramer (MBL international corporation) at room temperature for 1 h. Cells were then washed twice and stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain and surface markers (CD45, CD3, TCRb, CD4, CD8, CD44, CD62L, CD69), and analyzed on BD LSRII flow cytometer. For functional analysis, cells from dLNs were plated in 24-well plates in complete T-cell media with eBioscience™ Cell Stimulation Cocktail (plus protein transport

inhibitors) for 5 h. After stimulation, the cells were collected, washed, blocked with Fc-blocker and then stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain and surface markers (CD45, CD3, TCRb, CD4, CD8, PD-1) and then fixed using eBioscience™ Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's instructions. Cells were then washed, permeabilized, stained for IFN-γ, Foxp3, BCL6, and Granzyme B, and analyzed on a BD LSRII flow cytometer.

Phenotypic analysis of GC B cells, follicular T cells and plasma blasts in lymph nodes

For flow cytometry analysis of GC B cells, follicular T cells and plasma blasts, cells from the draining lymph nodes were stains with Ghost Dye™ Violet 510 (Tonbo Bioscience). Cell were then washed and blocked with Fc receptor antibody (clone: 2.4G2, BD Bioscience) prior to staining with fluorochrome conjugated antibodies: B220 (clone: RA3-6B2, BD Bioscience), CD19 (clone1D3: BD Biosciences), CD38 (clone: 90, BD Biosciences), CD95 (clone: Jo2, BD Biosciences), CXCR4 (clone: L276F12, BioLegend), CD86 (clone: GL1, prepared in our lab), CD138 (clone: 281-2, BD Biosciences), CD44 (clone: IM7, BioLegend), IgM (goat polyclonal, Jackson ImmunoResearch), IgG1 (clone: A85-1, BD Bioscience), CD3 (clone: 17A2, BioLegend), CD4 (clone: GK1.5, BioLegend), CXCR5 (clone: L138D7, BioLegend), PD1 (clone: 29F.1A12, BioLegend). After staining, cells were washed and fixed with 1.5% PFA. Stained cells were analyzed on an LSRII flow cytometer. Data were analyzed with FlowJo 10 software. Gating strategy: GCBC were gated on live single $CD3^-$ $CD19^+$ $CD95^+$ $CD38^-$ cells, TFH were gated on live single $CD19^-$ $CD3^+$ $CD4^+$ $CXCR5^+$ $PD1^{hi}$, plasma blasts were gated on live single $CD138^+$ $CD44^+$ cells.

Immunofluorescence

Cryostat sections (7 μm) made from OCT (TissueTek) embedded draining lymph nodes were fixed with 2% PFA for 20 minutes, and then washed and blocked in blocking buffer (PBS with 1% BSA, 0.3% Triton-100, Fc receptor antibody (clone: 2.4G2, BD Biosicence) and 5% rat serum and mouse serum). Sections were then stained in blocking buffer with biotin labeled anti mouse IgD (clone: 11-26c, eBioscience) and PE labeled anti mouse BCL6 (clone: K112-91, BD Biosciences) antibodies, and subsequently stained with Al488 conjugated Streptavidin (Thermo Fisher

Scientific). Fluorescent images were captured using a 4x objectives on a fluorescence microscope (Keyence).

Influenza viral challenge

Mice were s.c. immunized with different formulations of 10 µg of Alum-adsorbed H1(NC99) mixed with 20 µg of TLR7 agonist either in TLR7-Alum or TLR7-NPs in 100 µl of PBS. at the tail base at week 0 and boosted at week 3. At week 7, the immunized mice were anesthetized and intratracheally infected with the heterologous influenza A/PR/8/34 H1N1 (Charles River, Cat# 10100374, Lot# 4XP201023) in 20ul sterile PBS (1/40000 dilution from 10^7 PFU/mL virus stock). Body weight and survival of mice were monitored for 12 days after the challenge. In this study, the virus titers in the stock were determined with plaque assay. Briefly, 1:200 diluted virus stock was the start point for plaque assay, followed by a series of 3-fold dilution with DMEM plus 0.2% BSA. The virus dilution was used to inoculate 90% confluent monolayer MDCK cells for 1hr at 35°C. Equal volumes of 2.4% Avicel diluted in 2X DMEM were added to the inoculum to yield a 1.2% Avicel, 1X DMEM final concentration. Plaque assays were incubated for 36 hours, followed by immunostaining protocol at room temperature. Cells were fixed with 4% paraformaldehyde for 30min, permeabilized with buffer containing 0.5% Triton X-100 and 20mM glycine PBS for 30min and stained with 1:1000 dilution of anti-influenza A NP clones A1 and A3 primary antibody (Millipore) followed by adding a 1:2000 dilution of anti-mouse IgG secondary antibody (KPL). Plates were washed (0.05% Tween-20/PBS) 3 times after each step. Colorimetric staining was completed per manufacturer's instruction using ImpactDAB kit (Vector Labs).

Mouse Lung tissue isolation and processing

Lung isolation was performed as described[2, 3] with some modifications. Briefly, euthanized mice were perfused by via the right cardiac ventricle with PBS. Harvested lungs were dissected into gentleMACSTM C tubes (Miltenyi) containing 4mL of a mixture of collagenase (25 µg/mL liberase^{TM}, Roche) and DNaseI (10 µg/mL, Sigma) in PBS containing 2% FBS. Then lungs were dissociated with pre-set program m_lung_02_01 using a gentleMACS^{TM} octo dissociator (Miltenyi) and incubated for 30 min at 37°C. Next digested lungs were homogenized with the gentleMACS program m_lung_02_01 and 10mM EDTA were added. Suspensions were passed through a 100µm cell strainer. Red blood cells were lysed with Ack lysing buffer (Gibco). Cells were

resuspended in 4mL 36% Percoll (GE Healthcare) and washed once for further experiments.

Human tonsil organoid stimulation

The tonsil cells were cryopreserved as previously described[1]. For culture of cryopreserved cells, aliquots were thawed into complete medium, enumerated, and resuspended to 6×10^6 cells per mL. Cells were plated, 100 μL per well, into permeable (0.4-μm pore size) membranes (24-well size PTFE or polycarbonate membranes in standard 12-well plates with single-well receiver trays; Corning or Millipore), with the lower chamber consisting of complete medium (1 mL) supplemented with 0.5 μg/mL of recombinant human B cell-activating factor (BAFF; BioLegend) every three or four days. Full-length SARS-CoV-2 spike protein (2.5 μg per well) with or without TLR7-NPs (5 μg per well) were added directly to the cell-containing portion of the culture setup. Cultures were incubated at 37 °C, 5% CO_2 with humidity. Cells from organoids were harvest after 14-day culture and analyzed by flow cytometry. Supernatants from the lower chamber were harvest for measuring SARS-CoV-2 spike-specific antibodies by ELISA.

BD Rhapsody single-cell targeted RNA sequencing and data analysis

Tonsil organoid cells cultured with different days and stimulants were FACS-sorted, library prepared and sequenced in one batch. They were first stained with oligonucleotide-conjugated Sample Tags (the BD Human Single-Cell Multiplexing Kit, Cat. #633781) and the FACS antibodies (live/dead Aqua Zombie stain, BioLegend: CD45 clone HI30, CD19 clone SJ25C1, CD3 clone SK7) following the manufacturer's protocol. Live B cells (CD45+CD19+CD3−) were FACS-sorted from the barcoded samples and pooled. Barcoded samples were then washed and spun down at 350xg for 10 minutes and pooled. Pooled sample was then stained concurrently with a panel of 32 oligonucleotide-conjugated antibodies for the AbSeq. Stain in BD stain buffer for 30 minutes on ice. Samples were then spun down at 350xg for 10 minutes and washed three times. Pellet was resuspended in Rhapsody Sample Buffer for capture. Cell capture and library preparation were completed using the BD Rhapsody Targeted mRNA and AbSeq Reagent Kit (Cat. # 633774). Briefly, cells were captured with beads in microwell plate, followed by cell lysis, bead retrieval, cDNA synthesis, template switching and Klenow extension, and library preparation in the Stanford Human Immune Monitoring Center (HIMC) following the BD Rhapsody protocol.

Libraries were prepared for sample tags, targeted mRNA using the customized Immune Response panel, and AbSeq. Sequencing was completed on NovaSeq (Illumina, San Diego, CA) at Novogene USA Inc. (US Davis, CA).

The Rhapsody raw data was first preprocessed by the Seven Bridges Genomics online platform (San Francisco, CA) using the BD Rhapsody Targeted Analysis Pipeline to align genes and calculate molecular counts with molecular index correction. After the preprocessing, the single cell Rhapsody count tables composing of 493 genes and 32 surface markers from the different timepoints, and stimulants were imported in Seurat package of R[4] for the following processing. The gene counts were log-normalized by a factor of 10,000, while the abseq counts were central log ratio normalized. The PCA with 30 dimensions were used to dimensionally reduce the gene and abseq combined count data. The graphical-based clustering and the manual annotation were applied to identify B cell subsets. To identify the genes that are significantly differentially expressed between the two treatments, we used the Wilcoson ranksum tests[5] with the Benjamini-Hochberg adjusted p-values below 0.05 and absolute values of the log2 of fold-change between the average expression of the raw molecular counts above 0.25. The 'Biological Processes' gene ontology terms of the significantly differentially expressed genes were calculated using DAVID [6, 7] and the GO terms were shown with the false discovery rate less than 0.05.

Statistics

Statistics were analyzed using GraphPad Prism software. All graphs represent mean and standard deviations unless otherwise noted. Comparisons of more than two groups were performed using a one-way ANOVA with a Tukey post hoc test to determine statistical significance. For comparisons of experiments with only two groups, two-tailed unpaired Student's t-tests were used.

Reference

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Fig. S1

LA/Gardiquimod (M/I)	Expected M_n (kD)	Obtained M_n (kD)	Obtained M_w (kD)	M_w/M_n
25	3.9	2.7	2.8	1.05
100	14.7	15.4	18.3	1.19

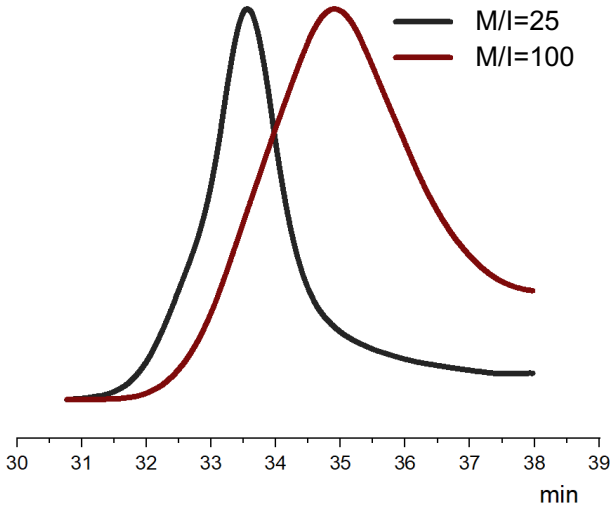
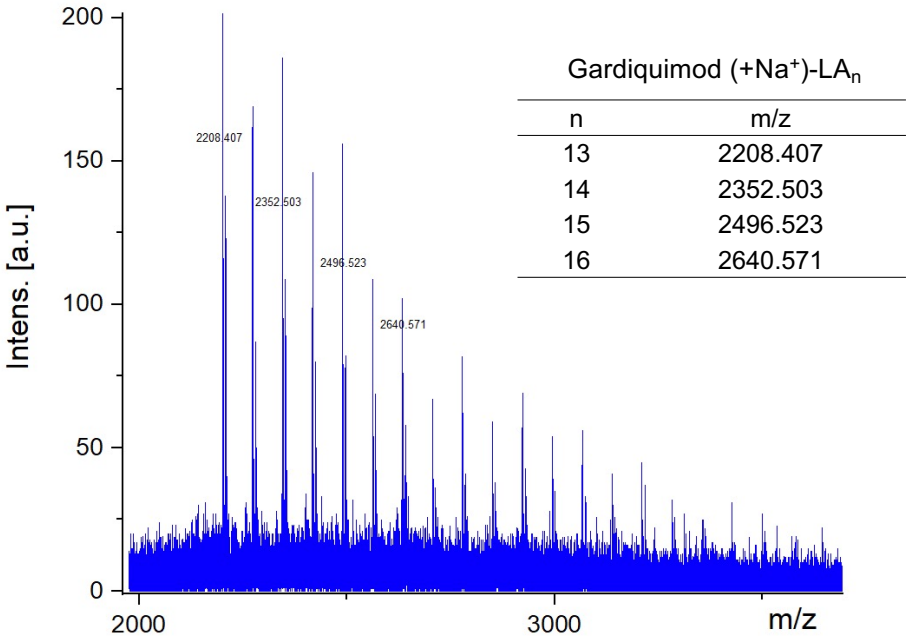


Figure S1: GPC analysis of Gardiquimod-LA_n, equipped with dRI detector.

Fig. S2



Gardiquimod (+Na^+)-LA_n

n	m/z
13	2208.407
14	2352.503
15	2496.523
16	2640.571

Figure S2: MALDI-TOF MS analysis of Gardiquimod-LA_n. The obtained m/z is identical to the calculated m/z of Gardiquimod (+Na^+)-LA_n (336.3+144.08*n). Matrix： HCCA

Fig. S3

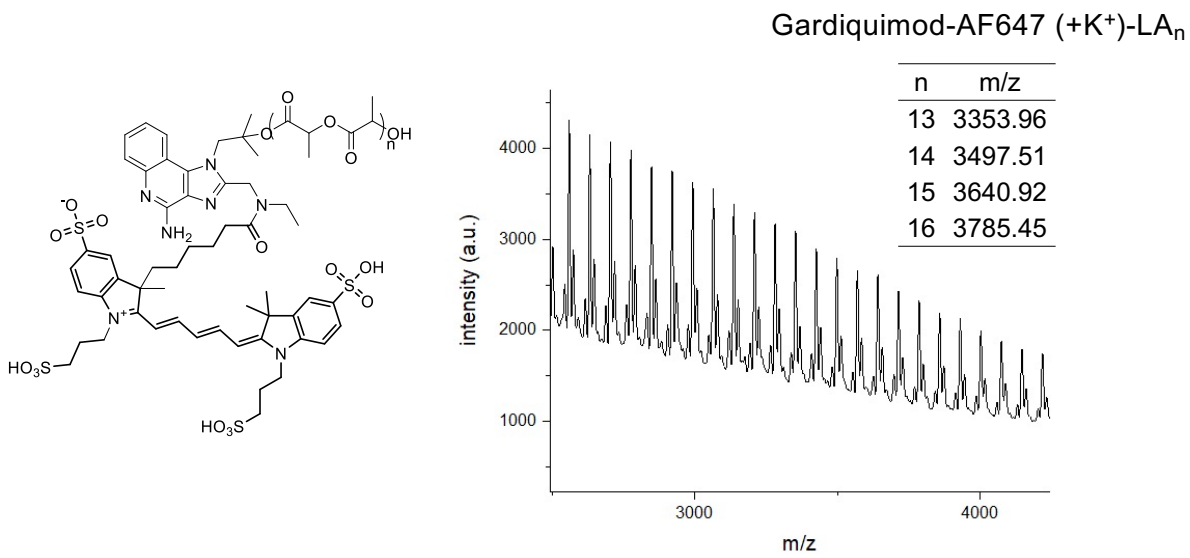


Figure S3: MALDI-TOF MS analysis of Gardiquimod-AF647-LA_n. The obtained m/z is identical to the calculated m/z of Gardiquimod-AF647 ($+K^+$)-LA_n (1192.36+144.04*n). Matrix： DMB

Fig. S4

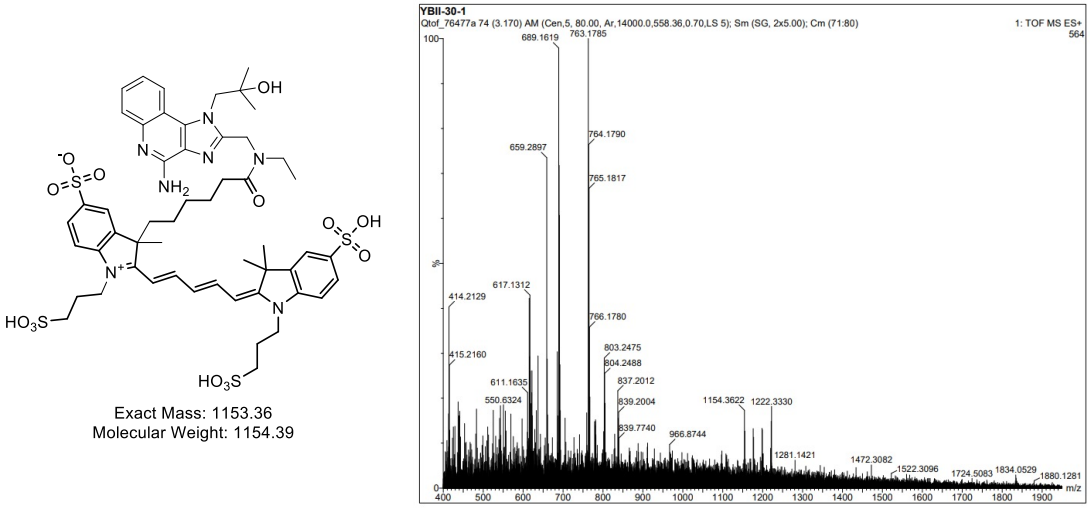


Figure S4: ESI-MS analysis of Gardiquimod-AF647. observed 1154.36 M+ H^+

Fig. S5

A

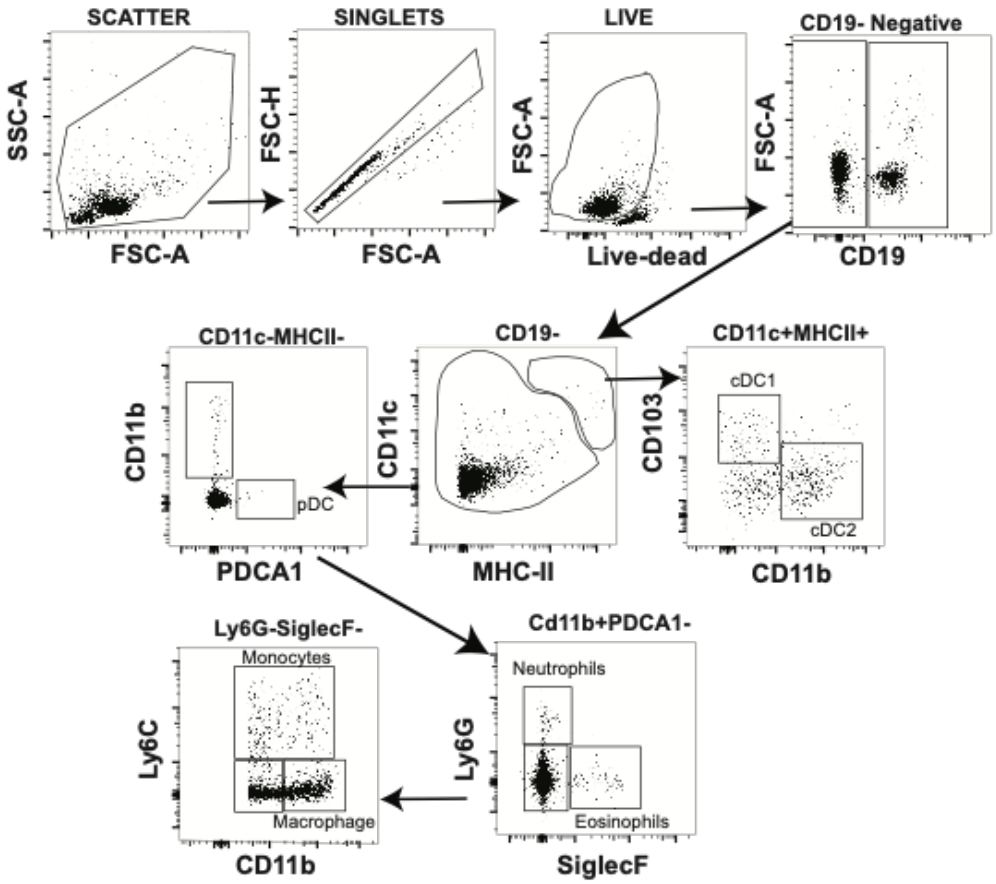


Figure S5: Gating scheme for flow cytometry analysis of DCs and cells of monocytic lineage.

Fig. S6

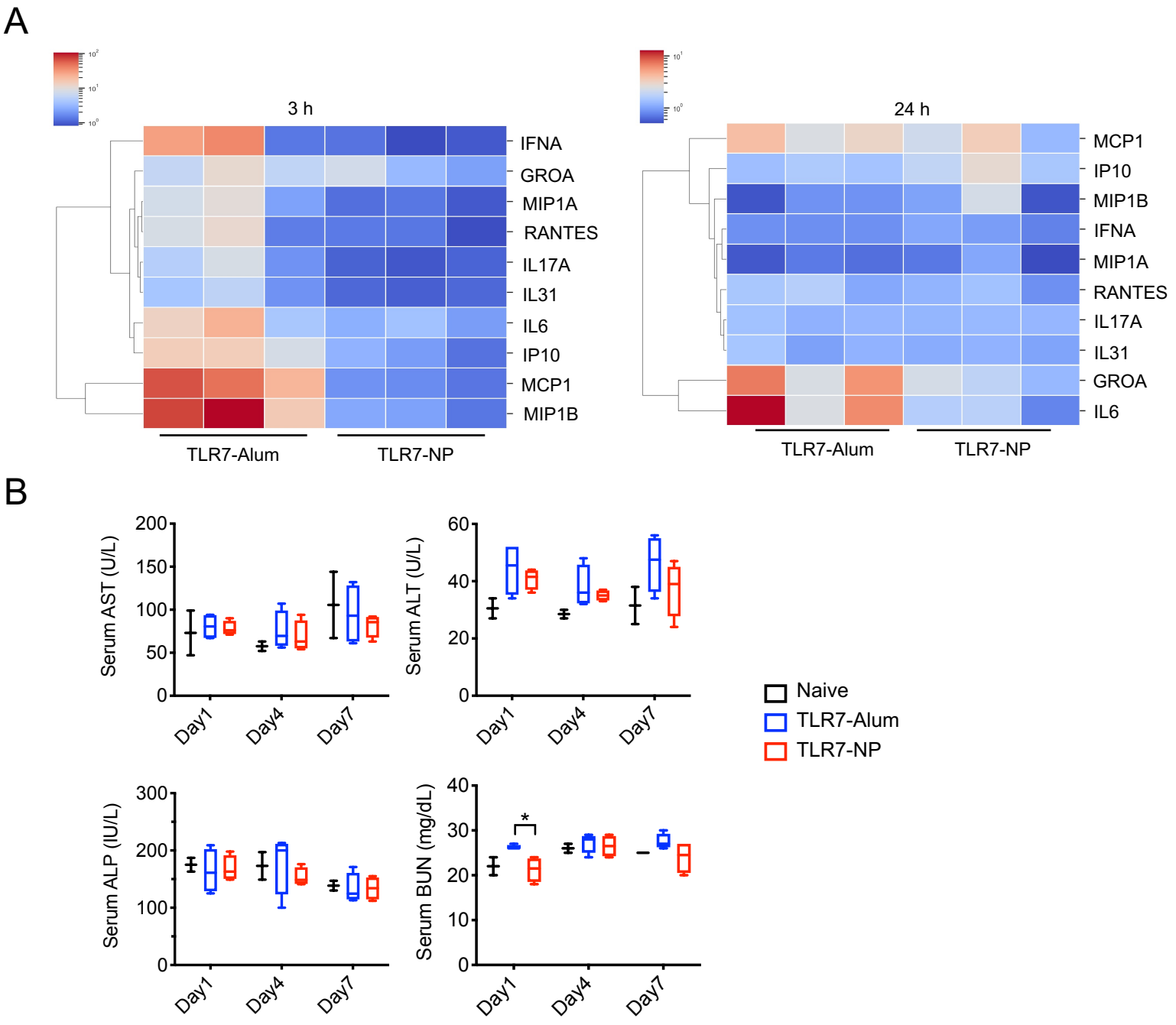


Figure S6: TLR7-NPs adjuvant minimizes systemic immune toxicity. (a)The expression of top-10 elevated cytokines in the serum of C57BL/6 mice 3 hours or 24 hours after a single immunization of NP-OVA protein (50 μg) plus TLR7 agonist (20 μg) in two different platforms (TLR7-Alum, or TLR7-NPs). (b) The clinical chemistry of immunized mice at Day1, Day 4 and Day7 post immunization.