Synthesis and Immunological Characterization of Toll-Like Receptor 7 Agonistic Conjugates

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Activation of toll-like receptors (TLRs) on cells of the innate immune system initiates, amplifies, and directs the antigen-specific acquired immune response. Ligands that stimulate TLRs, therefore, represent potential immune adjuvants. In this study, a potent TLR7 agonist was conjugated to phospholipids, poly(ethylene glycol) (PEG), or phospholipid-PEG via a versatile benzoic acid functional group. Compared to the unmodified TLR7 agonist, each conjugate displayed a distinctive immunological profile in vitro and in vivo. In mouse macrophages and human peripheral blood mononuclear cells, the phospholipid TLR7 agonist conjugate was at least 100-fold more potent than the free TLR7 ligands, while the potency of PEG−phospholipid conjugate was similar to that of the unmodified TLR7 agonist. When administered systemically in mice, the phospholipid and phospholipid−PEG TLR7 conjugates induced prolonged increases in the levels of proinflammatory cytokines in serum, compared to the unmodified TLR7 activator. When the conjugates were used as adjuvants during vaccination, only the phospholipid TLR7 agonist conjugates induced both Th1 and Th2 antigen-specific immune responses. These data show that the immunostimulatory activity of a TLR7 ligand can be amplified and focused by conjugation, thus broadening the potential therapeutic application of these agents.

INTRODUCTION

Toll-like receptors (TLRs) are pattern recognition molecules present on diverse cell types that recognize common ligands in microbes such as bacteria, viruses, and fungi (*1*). Various TLRs interact with lipoprotein (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS, TLR4), flagellin (TLR5), single-stranded RNA (TLR7/8), and unmethylated CpG DNA (TLR9). All TLRs, except TLR3, signal through the myeloid differentiation primary response gene 88 (MyD88) adapter protein, resulting in the activation of NF-κB and the cytokine genes that it regulates (*2*).

The active form of TLR7 is located mainly in the endosomal compartment of innate immune cells, including dendritic cells, mast cells, and B lymphocytes (*3, 4*). The natural ligand for TLR7 was identified as guanine and uridine-rich single-stranded RNA (*5*). In addition, several low molecular weight activators of TLR7 have been discovered, including imidazoquinolines, and purine-like molecules (*3, 6, 7*). Among the latter, 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (SM360320; designated here as **SM**) has been shown to be a potent and specific TLR7 agonist (*8*). In a previous study, we synthesized a derivative of SM-designated UC1V150, in which the aldehyde functional group on the benzyl moiety enabled coupling the agonist to different auxiliary chemical entities through a bifunctional linker molecule containing a hydrazine and *N*-hydroxysuccinimide activated ester function (*9*). Conjugation to mouse serum albumin (MSA) increased potency by 10- to 100-fold and improved in vivo pharmacodynamics, compared with the free drug (**9**). Moreover, the conjugate (MSA-UC1 V150) could be delivered effectively to the respiratory system by intranasal or intratracheal administration, without inducing a systemic cytokine syndrome. Drug delivery by the intranasal route was effective in reducing mortality in mouse models of bacterial and viral infection (*9*).

An important aspect of TLR biology is the connection between receptor localization, cell activation, and cytokine production. TLR7 and TLR9 are only active when proteolytically processed and loaded into endosomes (*10–12*). The conjugation of various chemical entities to phospholipids is known to facilitate endocytosis. Therefore, we hypothesized that conjugation of a TLR7 activator to a phospholipid would enhance innate immune activation. The conjugation of drugs to poly(ethylene glycol) can promote their circulation time in the blood and potentially increase solubility in water (*13*). It was therefore of interest to determine if conjugation of a TLR7 agonist to PEG could alter its in vivo pharmacodynamics.

EXPERIMENTAL PROCEDURES

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were purchased as at least reagent grade from Sigma-Aldrich (St. Louis, MO) without further purification. Solvents were purchased from Fischer Scientific (Pittsburgh, PA) and were either used as purchased or redistilled with an appropriate drying agent. Ovalbumin (OVA, grade V) was purchased from Sigma-Aldrich.

Endotoxin levels in the reagents and conjugates for experiments of immunological activities were measured using the QCL1000 end point chromogenic Limulus Amoebocyte Lysate (LAL) assay purchased from BioWhittaker (Walkerville, MD). Reagents that contained less than 1 pg endotoxin per μg protein or drug were used throughout the experiments. Since DOPE gave false positive results in the chromogenic endotoxin assay, bone marrow derived macrophages (BMDM) from lipopolysaccharide unresponsive mutant mice (C3H/HeJ) were used to test

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contamination by endotoxin (Supporting Information Figure 1). The levels of cytokine production by bone marrow derived mononuclear cells from C3H/HeJ mice were similar to the levels in cells derived from the wild-type C3H/HeOuJ strain, indicating that endotoxin contamination was negligible (Supporting Information Figure 1).

All conjugates were stored at −80 C° as dry powders. The conjugates were dissolved in DMSO at 100 mM and further diluted before immunological assays.

Instrumentation. Analytical TLC was performed using precoated TLC silica gel 60 F_{254} aluminum sheets purchased from EMD (Gibbstown, NJ) and visualized using UV light. Flash chromatography was carried out on EMD silica gel 60 (40−63 μm) using the specified solvent system. Chromatography and mass spectra (ESI) for compounds without phospholipids were recorded on a 1100 LC/MSD (Agilent Technologies, Inc., Santa Clara, CA) with a Supelco Discovery HS C18 column (Sigma-Aldrich) with purity above 98% by percent area. Mass spectra (ESI) of phospholipid-containing compounds were recorded on a Finnigan LCQDECA (Thermo Fisher Scientific Inc., Waltham, MA). ^{1}H NMR spectra were obtained on a Varian Mercury Plus 400 (Varian, Inc., Palo Alto, CA). The chemical shifts are expressed in parts per million (ppm) using suitable deuterated NMR solvents in reference to TMS at 0 ppm. UV absorbance for solubility experiments were measured using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE).

4-{[6-Amino-2-(2-methoxyethoxy)-8-oxo-7*H*-purin-9(8*H*)-yl]methyl}benzoic Acid (5). To 4-{[6-amino-8-methoxy-2-(2-methoxyethoxy)-9*H*-purin-9-yl]methyl}benzonitrile (**1**) (0.10 g, 0.28 mmol) was added 1:1 ethanol/10M NaOH (20 mL) and refluxed for 8 h. The reaction mixture was allowed to cool and acidified to pH 2 with conc HCl. The aqueous solution was extracted with dichloromethane (DCM) (3 × 20 mL), dried over $MgSO_4$, and evaporated in vitro to yield a mixture of the 8-oxo-9-benzoic acid (**5**), 8-methoxy-9-benzoic acid, and 8-oxo-9-ethyl benzoate. Once dried, the products were dissolved in CH_3CN (25 mL) and NaI (0.14 g, 0.96 mmol) was added. To this solution was added chlorotrimethylsilane (12 μL, 0.96 mmol) dropwise with stirring. The reaction mixture was heated at 40 °C for 4 h, then cooled, filtered, and washed with water (20 mL) and then diethyl ether (20 mL) to obtain a white solid in 85% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.33 (s, 1H), 7.89 (d, J = 8 Hz, 2H), 7.37 (d, J = 8 Hz, 2H), 6.65 (s, 2H), 4.92 (s, 2H), 4.24 (t, J = 4 Hz, 2H), 3.56 (t, J = 4 Hz, 2H), 3.25 (s, 3H). Retention time (Rt) on HPLC = 14.3 min. ESI-MS (positive ion mode): calculated for $C_{16}H_{17}N_5O_5$ m/z [M+1] 360.34; found 360.24.

2-(4-{[6-Amino-2-(2-methoxyethoxy)-8-oxo-7*H*-purin-9(8*H*)-yl]methyl}benzamido)ethyl 2,3-Bis(oleoyloxy)propyl Phosphate (6). To a solution of compound **5** (0.022 g, 0.06 mmol) in anhydrous *N*,*N*-dimethylmethanamide (DMF) (1 mL) was added *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) (0.026 g, 0.067 mmol) and anhydrous triethylamine (TEA) (17.0 μL, 0.12 mmol). A solution of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (0.05 g, 0.067 mmol) in anhydrous 1:1 DCM/DMF (1 mL) was prepared and slowly added to the reaction mixture. The reaction mixture was stirred at room temperature until completion and then evaporated in vitro. The product was purified by flash chromatography using 15% methanol (MeOH) in DCM to give 0.038 g of white solid in 58% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.7 (s, 1H), 7.87 (d, J = 8.3 Hz, 2H), 7.32 (d, J = 8.3 Hz, 2H), 6.61 (s, 2H), 5.30 (m, 4H), 5.05 (m, 1H), 4.88 (s, 2H), 4.26 (m, 4H), 4.06 (m, 1H), 3.77 (m, 4H), 3.57 (m, 2H), 3.35 (m, 2H), 3.26 (s, 3H), 2.23 (m, 4H), 1.95 (m, 8H), 1.46 (m, 4H), 1.22 (m, 40H), 0.83 (m, 6H). ESI-MS (negative ion mode): calculated for $C_{57}H_{92}N_6O_{12}P$ m/z [M−1] 1083.35; found 1083.75. HR-ESI-FT-MS (positive ion mode): calculated for $C_{57}H_{92}N_6O_{12}PNa$ m/z [M+Na+1] 1107.6481; found 1107.6477.

4-{[6-Amino-8-hydroxy-2-(2-methoxyethoxy)-9*H*-purin-9-yl]-methyl}-*N*-(32-azido-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontyl)benzamide (7). To a solution of compound **5** (0.100 g, 0.278 mmol) in anhydrous DMF (5 mL) was added HATU (0.117 g, 0.306 mmol) and anhydrous TEA (77.014 μL, 0.556 mmol). A solution of *O*-(2-aminoethyl)-*O*′-(2-azidoethyl)nonaethylene glycol (0.150 g, 0.306 mmol) in anhydrous DMF (1 mL) was prepared and slowly added to the reaction mixture. The reaction mixture was stirred at room temperature until completion and then evaporated in vitro. The product was purified by flash chromatography using 5% MeOH in DCM to give 0.224 g of an opaque oil in 93% yield. Rt = 12 min. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.01 (s, 1H), 8.45 (t, J = 5.6 Hz, 1H), 7.78 (d, J = 8.3 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 6.49 (s, 2H), 4.90 (s, 2H), 4.25 (t, J = 4 Hz, 2H), 3.57 (m, 4H), 3.5 (m, 36H), 3.4 (M, 6H), 3.26 (s, 3H). ESI-MS (positive ion mode): calculated for $C_{38}H_{61}N_9O_{14}$ m/z [M+1] 868.94; found 868.59.

3-{1-[1-(4-{[6-Amino-8-hydroxy-2-(2-methoxyethoxy)-9*H*-purin-9-yl]methyl}phenyl)-1-oxo-5,8,11,14,17,20,23,26,29,32-decaoxa-2-azatetratriacontan-34-yl]-1*H*-1,2,3-triazol-4-yl}propanoic Acid (8). Compound **7** (0.218 g, 0.251 mmol) and 4-pentynoic acid (0.074 g, 0.753 mmol) was dissolved in 1:1 *t*-butanol/H_2O (3 mL). Sodium ascorbate (0.02 g, 100 mmol) and $Cu(OAc)_2$ (0.009 g,50 mmol) in 1:1 *t*-butanol/H_2O (1 mL) was slowly added to the reaction mixture and stirred at room temperature until compound **7** was fully reacted by TLC. The product was extracted with DCM (10 mL) and H_2O (10 mL), and the organic layer was dried over $MgSO_4$ to give 0.230 g of an opaque oil in 95% yield. Rt = 11.5 min. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 13.48 (s, 1H), 7.76 (d, J = 8.29 Hz, 2H), 7.75 (s, 1H), 7.23 (d, J = 8.29, 2H), 4.88 (s, 2H), 4.41 (t, J = 5.12 Hz, 2H), 4.23 (t, J = 4 Hz, 2H), 3.74 (t, J = 5.12 Hz, 2H), 3.57 (t, J = 4 Hz, 2H), 3.51 (m, 8H), 3.42 (m, 36H), 3.26 (s, 3H), 2.79 (t, J = 7.56 Hz, 2H), 2.24 (t, J = 7.56 Hz, 2H). ESI-MS (positive ion mode): calculated for $C_{43}H_{67}N_9O_{16}$ m/z [M+1] 966.04; found 966.67.

2-(3-{1-[1-(4-{[6-Amino-8-hydroxy-2-(2-methoxyethoxy)-9*H*-purin-9-yl]methyl}phenyl)-1-oxo-5,8,11,14,17,20,23,26,29,32-decaoxa-2-azatetratriacontan-34-yl]-1*H*-1,2,3triazole-4-yl}propanamido)ethyl 2,3-Bis(oleoyloxy)propyl Phosphate (9). To a solution of compound **8** (96 mg, 0.1 mmol) and HATU (42 mg, 0.11 mmol) in anhydrous DMF (1 mL) was added anhydrous TEA (2.7 uL, 0.2 mmol). A solution of DOPE (81.4 mg, 0.11 mmol) in 1:1 DCM/DMF (1 mL) was added dropwise to the reaction mixture and stirred at room temperature until completion. Upon completion, the product was isolated by evaporation in vitro followed by flash chromatography using 15% MeOH in DCM to give 155 mg of opaque oil in 92% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.5 (s, 2H), 8.39 (s, 1H), 7.79 (m, 3H), 7.33 (d, J = 6.23 Hz, 2H), 6.91 (s, 2H), 5.31 (m, 4H), 5.05 (m, 1H), 4.89 (s, 2H), 4.46 (m, 2H), 4.23 (m, 4H), 4.08 (t, J = 8 Hz, 2 H), 3.76 (m, 4H), 3.63 (t, J = 8 Hz, 2H), 3.56 (t, J = 8 Hz, 2H), 3.48 (m, 36H), 3.26 (m, 5H), 3.17 (m, 2H), 2.82 (t, J = 8 Hz, 2H), 2.39 (t, J = 8 Hz, 2H), 2.24 (m, 4H), 1.96 (m, 8H), 1.48 (m, 4H), 1.23 (m, 40H), 0.84 (m, 6H). ESI-MS (positive ion mode): calculated for $C_{84}H_{142}N_{10}O_{23}P$ m/z [M+1] 1691.05; found 1692.82.

Mice. 6−8 week old female C57BL/6 mice were purchased from Charles River Laboratories (San Diego, CA). C3H/HeJ and C3H/HeOuJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). TLR7 deficient mice were a gift from Dr. S. Akira, (Osaka University, Osaka, Japan) and were backcrossed ten generations onto the C57BL/6 background. Animals were bred and maintained at UCSD in rooms at 22 ±

0.5 °C on a 12/12 h light−dark cycle from 7 a.m. to 7 p.m. All procedures and protocols were approved by the Institutional Animal Care and Use Committee.

In Vitro Measurements of Cytokine Induction. The RAW264.7 (mouse leukemic monocyte macrophage cell line) was obtained from the ATCC (Rockville, MD) and cultured in DMEM (Irvine Scientific, Irvine, CA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin/100 μg/mL streptomycin. BMDM were prepared from C57BL/6, TLR7 deficient, C3H/HeJ, and C3H/HeOuJ mice as described (*9*). The cells (1×10^6/mL) were incubated with the conjugates for 18 h at 37 °C, 5% CO_2, and culture supernatants were collected. The levels of cytokines (IL-6, IL-12, or TNFα) in the supernatants were determined by ELISA (BD Biosciences Pharmingen, La Jolla, CA) (*12*). Minimum detection levels of these cytokines were 15 pg/mL, respectively.

Human blood peripheral mononuclear cells (PBMC) were isolated from human buffy coats obtained from The San Diego Blood Bank (San Diego, CA) as described previously (*11*). PBMC (1×10^6/mL) were incubated with various compounds for 18 h at 37 °C, 5% CO_2, and culture supernatants were collected. The levels of cytokines (IL-6, TNFα, or IFNα1) in the supernatants were determined by Luminex bead assays (Invitrogen, Carlesbad, CA) due to the relatively small sample volume. The minimum detection levels of IL-6, TNFα, and IFNα1 were 6 pg/mL, 10 pg/mL, and 15 pg/mL, respectively.

In Vivo Experimental Procedures. For in vivo pharmacokinetic experiments, 6 to 8 week old C57BL/6 mice were intravenously injected with the TLR7 agonist and its conjugates (40 nmol **4a** or 200 nmol **SM**, **6**, **8**, or **9** per mouse as described in the figure legends). The solubility was measured in 10% DMSO in saline at room temperature for 30 min at a maximum theoretical concentration of 1 mM. The aqueous concentrations of compounds **SM**, **6**, **8**, and **9** were measured by UV absorption, and the concentration was derived by comparison to standard curves with four calibration concentrations in DMSO (Supporting Information Table 1). The solutions were homogeneous, and no precipitates were noted prior to i.v. administration. Blood samples were collected 2, 4, 6, 24, or 48 h after the injections. Sera were separated and kept at −20 °C until use. The levels of cytokines in the sera were measured by Luminex bead microassay due to the small sample volume. The minimum detection levels of IL-6 and TNFα were 5 pg/mL and 10 pg/mL, respectively.

For the adjuvanticity experiments, mice were subcutaneously immunized with 20 μg ovalbumin (OVA) with 10 nmol of TLR7 equivalent dose per mouse in various TLR7 conjugates on days 0 and 7. A TLR9 activating immunostimulatory oligonucleotide sequence (ISS-ODN, 1018) was used as a positive control for a Th1 inducing adjuvant (*10*). Sera were collected on days 0, 7, 14, 21, 28, 42, and 56. Mice immunized with saline or OVA mixed with vehicle served as controls. Mice were sacrificed on day 56, and the spleens were harvested. Approximately 2.5×10^6/mL spleen cells were aliquoted into round-bottom tissue culture microtiter plates in triplicate in a total volume of 200 μL RPMI 1640 complete medium [RPMI1640 (Irvine Scientific, Irvine, CA) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 100 U/mL penicillin/100 μg/mL streptomycin] and restimulated with either 100 μg/mL OVA or medium alone. Cultures were then incubated at 37 °C, 5% CO_2, and supernatants harvested after 72 h. The levels of IFNγ in the culture supernatants were measured by ELISA (BD Bioscience PharMingen) as per the manufacturer's instructions (*14*). The average total spleen cell numbers in each group were calculated and compared to the control group to monitor spleen cell proliferation.

Anti-OVA antibodies of the IgG subclasses, IgGl and IgG2a, were measured by ELISA, as described (*12*). Each ELISA plate contained a titration of a previously quantitated serum to generate a standard curve. The titer of this standard was calculated as the highest dilution of serum that gave an absorbance reading that was double the background. The various sera samples were tested at a 1:100 dilution. The results are expressed in units per mL, calculated based on the units/mL of the standard serum, and represent the mean ± SEM of five animals in each group.

In some experiments, the site of injection was examined 24 h after immunization for signs of inflammation or local reaction. Mice were observed for locomotor activity to assess a "sickness" response to immunization and then weighed weekly.

Histological Examination. In the adjuvanticity experiments, the spleens, lungs, livers, hearts, and kidneys were collected on day 56. The organs were fixed in 10% buffered formalin (Fisher Scientific) and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin and eosin (H&E) and evaluated under the microscope.

Statistical Analysis. A software package (*Prism 4.0*, GraphPad, San Diego CA) was used for statistical analyses including regression analyses. Data were plotted and fitted by nonlinear regression assuming a Gaussian distribution with uniform standard deviations between groups. The statistical differences were analyzed by two-way ANOVA with Bonferroni's post hoc, Dunnett's post hoc, or Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

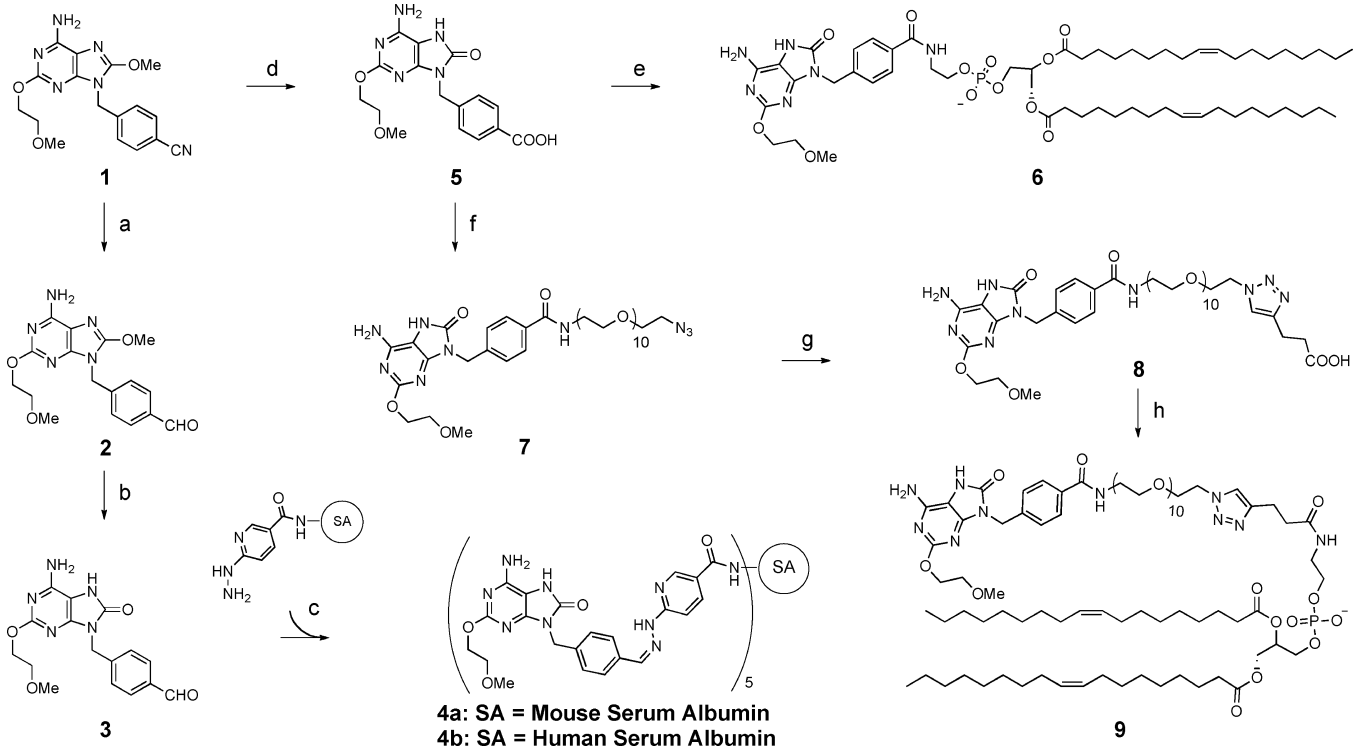
RESULTS

Chemical Synthesis. The synthesis of compound **4** from compound **1** yielded a consistent conjugation ratio of 5:1 UC1 V150 to MSA protein (*9*). Basic hydrolysis of the 9-benzylnitrile of compound **1** (*8*) provided a versatile benzoic acid functional group (*5*) in 85% yield and allowed for the assembly of conjugates **6**, **8**, and **9** (Scheme 1). The benzoic acid was coupled with DOPE by activation with HATU in the presence of TEA in anhydrous DMF to give compound **6** in 58% yield. Due to the difficulty in dissolution of compound **6** in suitable solvents for testing, a PEG spacer was coupled to provide improved solubility. A readily available amine/azide bifunctional PEG was coupled to the benzoic acid by activation with HATU in the presence of TEA in anhydrous DMF to give compound **7** in 93% yield. The formation of a 1,2,3-triazole through a copper(I)-catalyzed azide−alkyne cycloaddition with 4-pentynoic acid gave compound **8** in 95% yield. Finally, compound **9** was prepared by HATU activated amide formation with DOPE and compound **8** (92% yield).

Cytokine Induction by a Phospholipid-Conjugated TLR7 Agonist. The in vitro potency of the phospholipids TLR agonist conjugate (**6**), PEG-TLR7 agonist conjugate (**8**), PEG-phospholipid (**9**) conjugate, and the MSA-TLR7 agonist conjugate were compared using the murine macrophage cell line, RAW264, and primary bone marrow derived macrophages (BMDM) (Figure 1A,B). The respective cells were stimulated for 18 h with serially diluted TLR7 agonist conjugates, and the levels of cytokines released in the media were measured by ELISA and compared to the unconjugated TLR7 agonist (**SM**).

As previously shown, the MSA-TLR7 agonist conjugate (**4a**) was approximately 100-fold more potent as a cytokine inducer compared to the unconjugated agonist. In the same system, the phospholipid-TLR7 conjugate was 10-fold more potent, when normalized to the molar level of the pharmacophore ($P < 0.0001$ compared to unconjugated TLR7 agonist in RAW cells and BMDM). Although the PEG-TLR7 agonist conjugates (**8**) showed less potency compared to the unconjugated TLR7 agonist (**SM**) ($P < 0.001$ in RAW cells), the hybrid phospho-

Scheme 1. Synthesis of the Phospholipid- (6), PEG- (8), Phospholipid-PEG (9) TLR7 Conjugates[a]



[a] (a) Lithium *N,N′*-methylethylenediaminoaluminum hydrides THF, 0° C; (b) NaI, chlorotrimethylsilane, CH_3CN, r.t; (c) PBS, r.t.; (d) NaOH: EtOH 1:1, reflux; (e) DOPE, HATU, triethylamine,DMF/DCM 1:1, r.t.; (f) *O*-(2-Aminoethyl)-*O*$_2$-(2-azidoethyl)nonaethylene glycol, HATU, triethylamine, DMF, r.t.; (g) 4-pentyonic acid, sodium ascorbate, $Cu(OAc)_2$, t-BuOH/H_2O/THF 2:2:1, r.t; (h) DOPE, HATU, triethylamine, DMF/DCM 1:1, r.t.

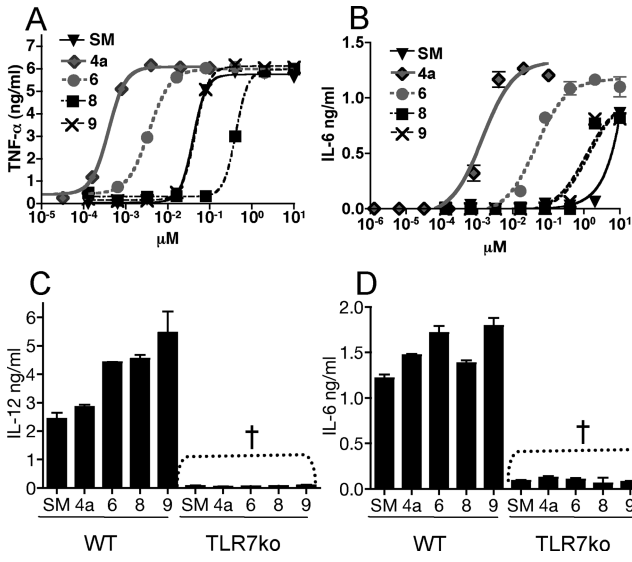


Figure 1. In vitro immunological characterization of TLR7 conjugates in murine macrophages. 1×10^6/mL RAW 264.7 cells (A), or 0.5×10^6/mL BMDM derived from wild-type (B−D) or TLR7 deficient mice (C,D) were incubated with serially diluted TLR7 conjugates for 18 h. Serial dilutions of **SM** (triangle), **6** (gray circle), **8** (solid square), and **9** (ex) were prepared (steps of 1:5) starting from 10 μM. **4a** (gray rhombus) was serially diluted (steps of 1:5), starting from 0.1 μM. The levels of TNFα, IL-6, and IL-12 in the culture supernatants were measured by ELISA. Data are mean ± SEM of triplicates and are representatives of three independent experiments. $P < 0.001$ by two-way ANOVA with Bonferroni post hoc test in comparison between **SM** and **6**. * denotes $P < 0.01$ by Student's t test compared to the corresponding data of wild-type macrophages.

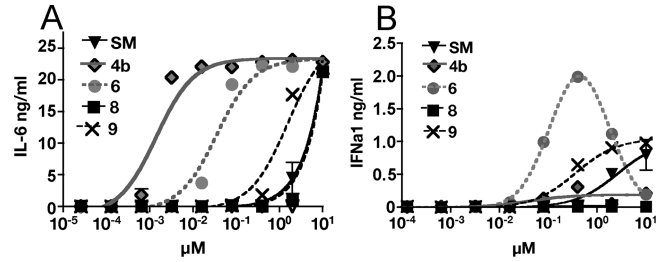


Figure 2. In vitro immunological characterization of TLR7 conjugates in human PBMC. 2×10^6/mL human PBMC were incubated with serially diluted TLR7 conjugates for 18 h. Serial dilutions of SM (triangle), 6 (gray circle), 8 (solid square), and 9 (ex) were prepared (steps of 1:5) starting from 10 μM. **4a** (gray rhombus) was serially diluted (steps of 1:5), starting from 0.1 μM. The levels of IL-6 (A) and IFNα1 (B) in the culture supernatants were determined by Luminex assay in duplicate. Data are means ± SEM and are representative of three independent experiments. The order of potency is **4b** > **6** > **9** > **SM** = **8** ($P < 0.0005$ by two-way ANOVA test for compounds **4b**, **6**, **9**, and **SM**; $P = 0.2$ by two-way ANOVA test for compound **8**).

lipid-PEG-TLR7 agonist was as potent as the unconjugated drug (**SM**). Equivalent concentrations of MSA, phospholipid, or PEG, processed without TLR7 conjugation, induced minimal or no cytokine levels in RAW264.7 cells and BMDM, respectively (data not shown).

To confirm that the conjugated forms of the TLR7 agonist stimulated exclusively via TLR7 activation, BMDM derived from TLR7 deficient mice were analyzed (Figure 1C,D). Compounds **4a**, **6**, **8**, **9**, and **SM** induced little or no IL-12 and IL-6 production in the TLR7 deficient cells, whereas these conjugates were active in wild-type BMDM. To assess the immunological effects of the conjugates in human cells, PBMC from three separate donors were treated with the different agents, and the levels of IL-6 and IFNα1 released into the supernatants were determined (Figure 2A,B). Human serum albumin (HSA) conjugated to the TLR7 agonist (**4b**) was used instead of MSA-

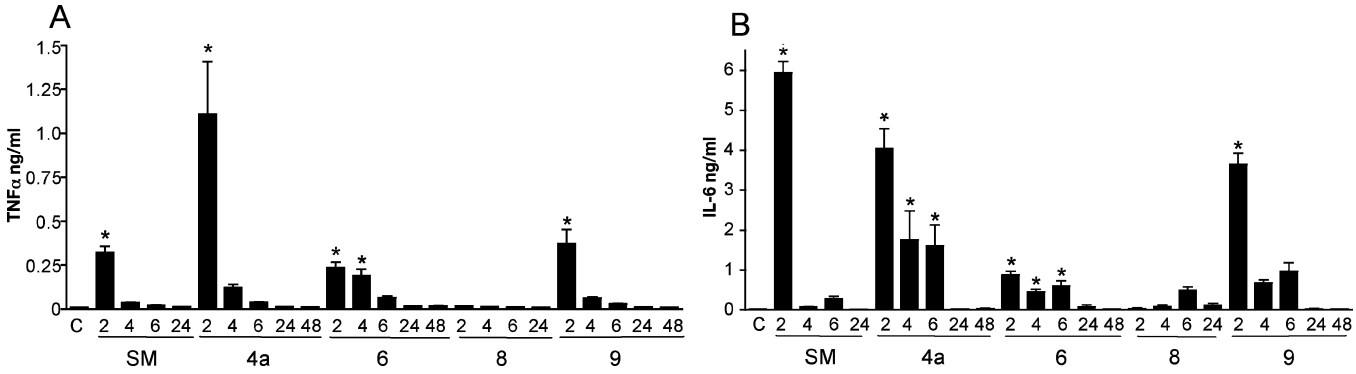


Figure 3. Kinetics of proinflammatory cytokine induction by TLR7 conjugates in vivo. C57BL/6 mice ($n = 5$ per group) were intravenously injected TLR7 conjugates (**SM**, 200 nmol; **4a**, 40 nmol; **6**, 200 nmol; **8**, 200 nmol; **9**, 200 nmol). Serum samples were collected 2, 4, 6, 24, and 48 h after injection. The levels of TNFα (A) and IL-6 (B) were measured by Luminex assay. Data are means ± SEM of five mice and are representative of two independent experiments. (C) Control naïve mice. * denotes $p < 0.05$ compared to the naïve mice by one-way ANOVA tests with Bonferroni post hoc testing.

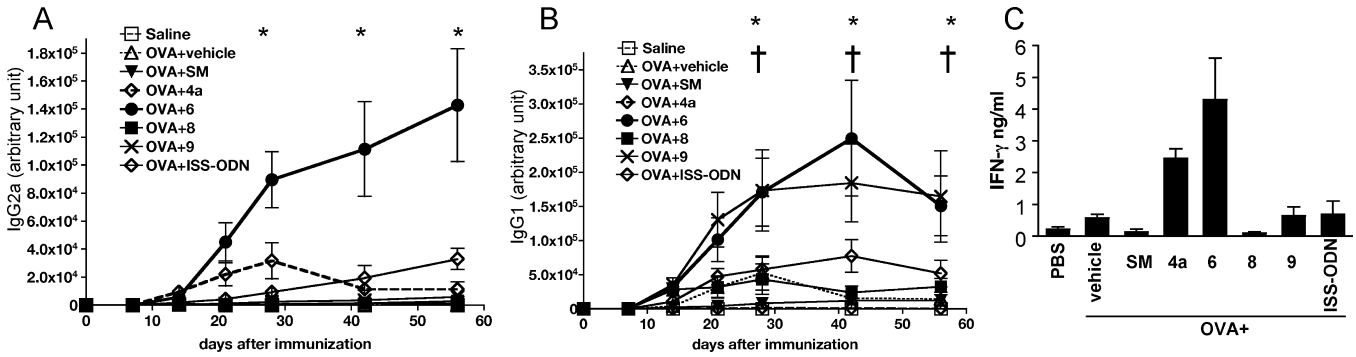


Figure 4. Adjuvanticity of TLR7 conjugates in vivo. Groups of C57BL/6 ($n = 5$ per group) were subcutaneously immunized with 20 μg OVA mixed with TLR7 conjugates (10 nmol TLR7 equivalent dose per mouse) on days 0 and 7. Sera were collected days 0, 7, 14, 21, 28, 42, and 56. OVA specific IgG1 and IgG2a were measured by ELISA (A and B). On day 56, mice were sacrificed, and splenocytes were cultured with OVA (100 μg/mL) in RPMI1640 for 3 d. IFNγ level in the supernatant was measured by ELISA (C). Data are means ± SEM of 5 mice/group and are representative of 3 independent experiments. * and † denote $P < 0.05$ and $P < 0.01$ by one-way ANOVA with Dunnett's post hoc comparison to the mice immunized with OVA mixed with vehicle, respectively.

conjugates (**4a**) for this experiment, to avoid any effects of anti-MSA antibodies. The order of potency of the conjugates was similar in human PBMC and mouse BMDM (**4b** > **6** > **9** > **SM** = **8**) (Figure 2A). Compound **4b** induced minimal levels of IFNα1 in all of the human PBMC samples tested (Figure 2B).

In Vivo Kinetics of Induction of Pro-Inflammatory Cytokines by TLR Agonist Conjugates. To compare the in vivo immunological properties of the different TLR7 agonist conjugates, C57BL/6 mice were injected intravenously with the agents, and cytokine levels in sera were assayed at various time intervals (Figure 3A,B). On the basis of the results of a previous study (*9*), compound **4a** was used at a lower concentration (40 nmol per animal) than compounds **SM**, **6**, **8**, and **9** (200 nmol per animal). The maximum induction of TNFα and IL-6 was observed 2 h after injection with all the TLR7 conjugates (Figure 3A,B). Although the levels of the cytokines induced by the unconjugated TLR7 agonist (**SM**) declined rapidly after 2 h, cytokine induction by compounds **4a**, **6**, and **9** was sustained for up to 6 h. Compound **8** induced only a low level of IL-6, and undetectable TNFα, 6 h after injection (Figure 3B). Sera from control mice that received saline, MSA, or DOPE revealed little or no detectable cytokine levels (data not shown).

Phospholipid-TLR7 Agonist Conjugates Promote Rapid and Long-Lasting Humoral Immune Responses. The adjuvanticity of the different conjugates was assessed by the levels and isotypes of antigen-specific IgG induced after immunization of mice with ovalbumin (OVA) and drug (*15*), as well as by antigen-induced synthesis of interferon γ (IFNγ). Groups of C57BL/6 ($n = 5$ animals per group) were subcutaneously injected with OVA mixed with the various TLR7 conjugates. A TLR9 activating immunostimulatory oligonucleotide sequence (ISS-ODN, 1018) is known to stimulate Th1 immune response and was used as a positive control for a Th1 inducing adjuvant (*10*). The kinetics of OVA-specific serum IgG1 and G2a on days 0, 7, 14, 21, 28, 42, and 56 were monitored by immunoassay (Figure 4A,B). Induction of IgG was observed as early as 14 days in mice immunized with OVA and compound **4a** or compound **6** (Figure 4A). The anti-OVA IgG2a levels increased thereafter in mice immunized with OVA and compound **6**, while the levels in mice immunized with OVA and compound **4a** mixture declined progressively. The spleen cells of mice immunized with OVA combined with compound **4a** or **6** displayed enhanced OVA-specific IFNγ secretion, compared to mice immunized with OVA alone or OVA and a TLR9 agonist (Figure 4C).

Adverse Effects. We previously reported that 150 nmol of the TLR7 agonist **SM** induced anorexia and weight loss in mice after intranasal administration (*16*). We, therefore, monitored both body weights and local skin inflammation in mice immunized with the phospholipid-TLR7 agonist conjugate. At the dose of TLR7 agonist that was an effective adjuvant (10 nmol per mouse), no weight loss or behavioral changes were noted. Chronic administration of TLR7 agonists can also stimulate hematopoietic cell proliferation, leading to spleen and lymph node hyperplasia (*17*). However, there were no significant differences in the numbers of splenocytes or myeloid cells between mice immunized with OVA alone, compared to TLR7 agonist conjugates and the saline control mice (Figure 5A).

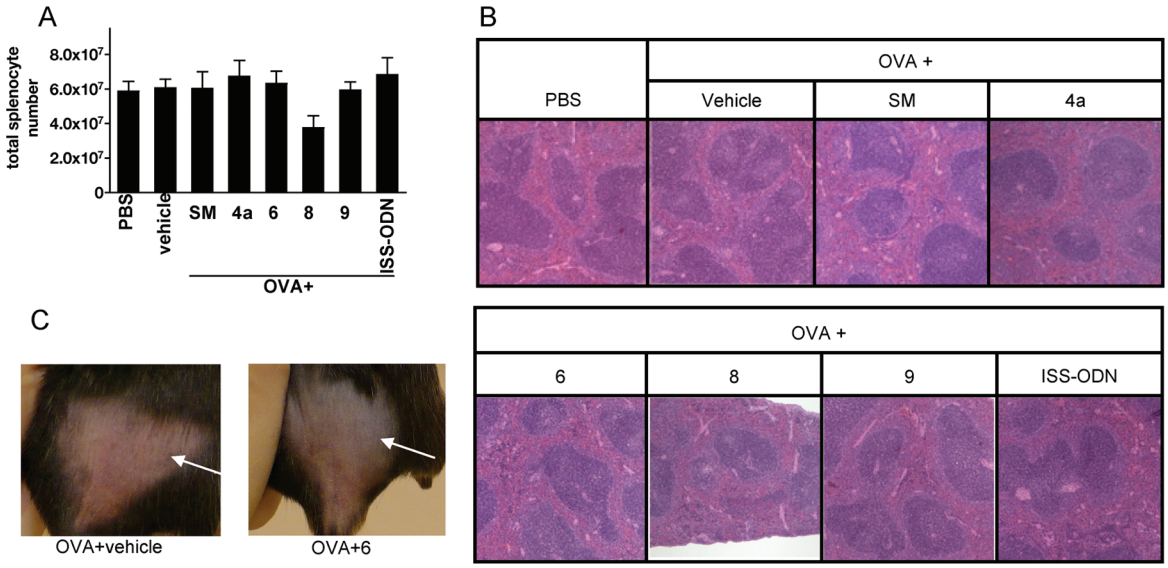


Figure 5. Evaluation of possible adverse effects of TLR7 conjugates. C57BL/6 mice were immunized with OVA 20 μg with TLR7 conjugate. On day 56, mice were sacrificed, and the number of total splenocytes was determined (A). The spleens were collected and submitted for histological examination ($\times$100) (B). The skin at injection sites was inspected 24 h after injection (C). There was no significant difference in the splenocyte number between the mice immunized with OVA plus TLR7 conjugates and the mice immunized with OVA alone (A). Histological examination of the spleens from mice immunized with OVA mixed with TLR7 conjugates did not show any disruption of the white pulps or increased cellularity in red pulp (B). The skin at injection sites did not have visible redness (C).

Histological examination of spleens from mice immunized with OVA plus TLR7 agonist conjugate showed no structural disruption of the white pulp and no increased cellularity in the red pulp (Figure 5B). Moreover, no significant differences were observed in the histological appearance of the liver, lung, heart, and kidney samples collected from each group (data not shown). There was also no significant erythema or warmth at the sites of injection (Figure 5C).

DISCUSSION

TLR7 agonists are not active unless delivered to endosomal vesicles where the active receptor resides (*18*). Previous experiments showed that the conjugation of a TLR7 agonist to serum albumin greatly increased potency, compared to the unconjugated drug (**9**). Presumably, the modified albumin quickly interacted with scavenger receptors on macrophages and dendritic cells, followed by endocytosis. However, modified proteins are not generally suitable for adjuvants, as they are difficult to standardize, store, and administer, and may be immunogenic. Therefore, we conjugated a TLR7 agonist to phospholipids (**6**), which are widely used to deliver drugs to cells. The phospholipid-TLR7 conjugated was 10-fold more potent as a cytokine inducer in mouse and human cells, compared to the unconjugated TLR7 agonist. More importantly, the phospholipid conjugate was a rapidly acting and potent adjuvant for vaccination. It induced antibody responses by 14 days after primary immunization and stimulated both antibody production and antigen specific interferon production. In contrast, the conjugation of the same TLR7 agonist to PEG (**8**) reduced its potency in vitro, unless a phospholipid moiety was included in the conjugated molecule (**9**). Experiments using TLR7 deficient mouse cells confirmed that all the conjugates used in this study stimulated immune responses exclusively through TLR7.

One would normally expect that the same TLR7 activating pharmacophore, conjugated to different delivery molecules, would induce comparable patterns of cytokine synthesis. However, this was clearly not the case. The TLR7 agonist albumin conjugates (**4b**) induced high levels of IL-6 in human blood leukocytes but very low levels of type I interferons. In contrast, the TLR7 agonist phospholipid conjugate (**6**) induced abundant IL-6 and type I interferon. In humans and mice, the major source of type 1 interferon is plasmacytoid dendritic cells (pDC) (*19*), while other dendritic cells, mast cells, and B lymphocytes can produce IL-6. Furthermore, it is mainly TLRs in early, newly formed endosomes that are linked to type I interferon synthesis (*20*). Interaction of a TLR agonist and its receptor in the early endosome triggers the interferon induction pathway, whereas interaction in the late endosome induces cytokines dependent on the NF-κB pathway, such as TNFα and IL-6 (*20*). The results emphasize the complexity of TLR7 signal transduction, which may be regulated by target cell type, endosomal localization, and receptor occupancy time. The conjugation of a TLR7 pharmacophore to different accessory molecules could affect all these parameters. In particular, the TLR7 phospholipid conjugate could promote uptake by plasmacytoid dendritic cells or enhance intracellular persistence of the ligand in early endosomal vesicles.

It is well-established that the antibody class and subtype induced by vaccination can be influenced by the type of coadministered adjuvant (*21*). In mice, IgG2 production is a surrogate marker for activation of Th1-type cells, which also promote delayed hypersensitivity responses. In contrast, Th2-type cells preferentially enhance IgG1 synthesis and do not foster delayed hypersensitivity. However, both antibody and T-cell mediated delayed hypersensitivity responses are optimally needed to control many infections. The TLR7 agonist conjugated to albumin or phospholipid caused rapid elevation of IgG2a antibodies. However, levels of IgG2a in the mice receiving the albumin conjugate (**4a**) declined by three weeks after the last immunization, while the mice immunized with the phospholipid conjugates sustained and even increased antigen-specific IgG2a levels without further boosting. One possible explanation for the divergent effects is that the phospholipid conjugate acted as a depo, delivering the TLR7 activating pharmacophore over a more prolonged period.

The unconjugated TLR7 active SM molecule is very poorly soluble in aqueous solutions. Conjugation to poly(ethylene glycol) (PEG) improved its solubility but decreased its ability to induce cytokines (Figure 3). However, a hybrid conjugate

containing both PEG and a phospholipid retained cytokine inducing activity. Vaccination with the hybrid conjugate as adjuvant induced a significant Th2 immune response but a minimal Th1 response.

Imiquimod, a specific but relatively weak TLR7 agonist, is currently approved for treatment of skin disorders such as viral warts and superficial basal cell carcinomas (*22, 23*). The TLR7 phospholipid conjugate described here is approximately 1000-fold more potent as a cytokine inducer. Although the hybrid conjugate containing both phospholipids and PEG was somewhat less potent, its water solubility renders it useful for systemic administration. In contrast, the phospholipid conjugate may be preferable for local administration, where persistent stimulation of in situ immune cells is desirable. The phospholipid moiety may also facilitate drug penetration through the epidermal barrier. Thus, the conjugation of a TLR7 agonist to phospholipid or PEG moieties is a promising strategy to expand the clinical uses of these agents for infectious, malignant, or atopic diseases.

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Supporting Information Available: Figure S1. Evaluation of endotoxin contamination using LPS unresponsive mutant and wild type macrophages. Table S1. Solubililty of the compounds used in vivo were measured by UV absorbance. This material is available free of charge via the Internet at http://pubs.acs.org.

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