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Enhancement of the Immunostimulatory Activity of a TLR7 Ligand by Conjugation to Polysaccharides

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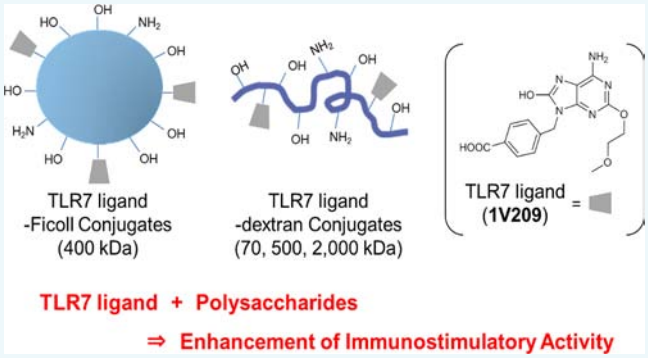
Supporting Information

ABSTRACT: Toll-like receptors (TLRs) in the innate immune system recognize specific pathogen-associated molecular patterns derived from microbes. Synthetic small molecule TLR7 agonists have been extensively evaluated as topical agents for antiviral and anticancer therapy, and as adjuvants for vaccine. However, safe and reproducible administration of synthetic TLR7 ligands has been difficult to achieve due to undesirable pharmacokinetics and unacceptable side effects. Here, we conjugated a versatile low molecular weight TLR7 ligand to various polysaccharides in order to improve its water solubility, enhance its potency, and maintain low toxicity. The synthetic TLR7 ligand, 2-methoxyethoxy-8-oxo-9-(4-carboxy benzyl)adenine, designated **1V209**, was stably conjugated to primary amine functionalized Ficoll or dextran using benzoic acid functional groups. The conjugation ratios using specified equivalents of TLR7 ligand were dose responsive and reproducible. The zeta potential value of the polysaccharides was decreased in inverse proportion to the ratio of conjugated TLR7 ligand. These conjugates were highly water-soluble, stable for at least 6 months at room temperature in aqueous solution, and easy to lyophilize and reconstitute without altering potency. In vitro studies with murine mononuclear leukocytes showed that the TLR7 agonist conjugated to polysaccharides had 10- to 1000-fold higher potencies than the unconjugated TLR7 ligand. In vivo pharmacodynamics studies after injection indicate that the conjugates induced systemic cytokine production. When the conjugates were used as vaccine adjuvants, they enhanced antigen specific humoral and cellular immune responses to a much greater extent than did unconjugated TLR7 ligands. These results indicated that small molecule TLR7 ligands conjugated to polysaccharides have improved immunostimulatory potency and pharmacodynamics. Polysaccharides can be conjugated to a variety of molecules such as antigens, peptides, and TLR ligands. Therefore, such conjugates could represent a versatile platform for the development of vaccines against cancer and infectious diseases.



INTRODUCTION

Toll-like receptors (TLRs) are pattern recognition receptors that play an essential role in host defense against pathogens. TLRs recognize specific pathogen-associated molecular patterns (PAMPs) conserved in viruses, bacteria, and other microbes. Various ligands interacting with TLRs have been identified such as lipoteichoic acid (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS, TLR4), flagellin (TLR5), single-stranded RNA (TLR7/8), and unmethylated CpG DNA (TLR9).[1] 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.[2,3] Most of the TLRs are expressed on the cell surface, whereas some of them including TLR7 are located mainly in the endosomal compartments of innate immune cells such as dendritic cells, macrophages, and B lymphocytes, and are activated after being proteolytically processed and transported into endosomes.[4]

In antimicrobial and anticancer vaccines, poor immunological responses due to weak host immune systems and poorly immunogenic antigens are often problematic. Currently, aluminum salts are commonly used as vaccine adjuvants.[5] However, their effects are nonspecific and limited after induction of protective immunity. TLR ligands therefore have gained substantial attention as targeted agents that are designed to activate innate adaptive immune responses in the host.[6] Several synthetic small molecule TLR7 ligands, including imidazoquinolines and purine-like molecules, have been extensively evaluated in preclinical and clinical studies.[6−10] Imiquimod (R837) is a

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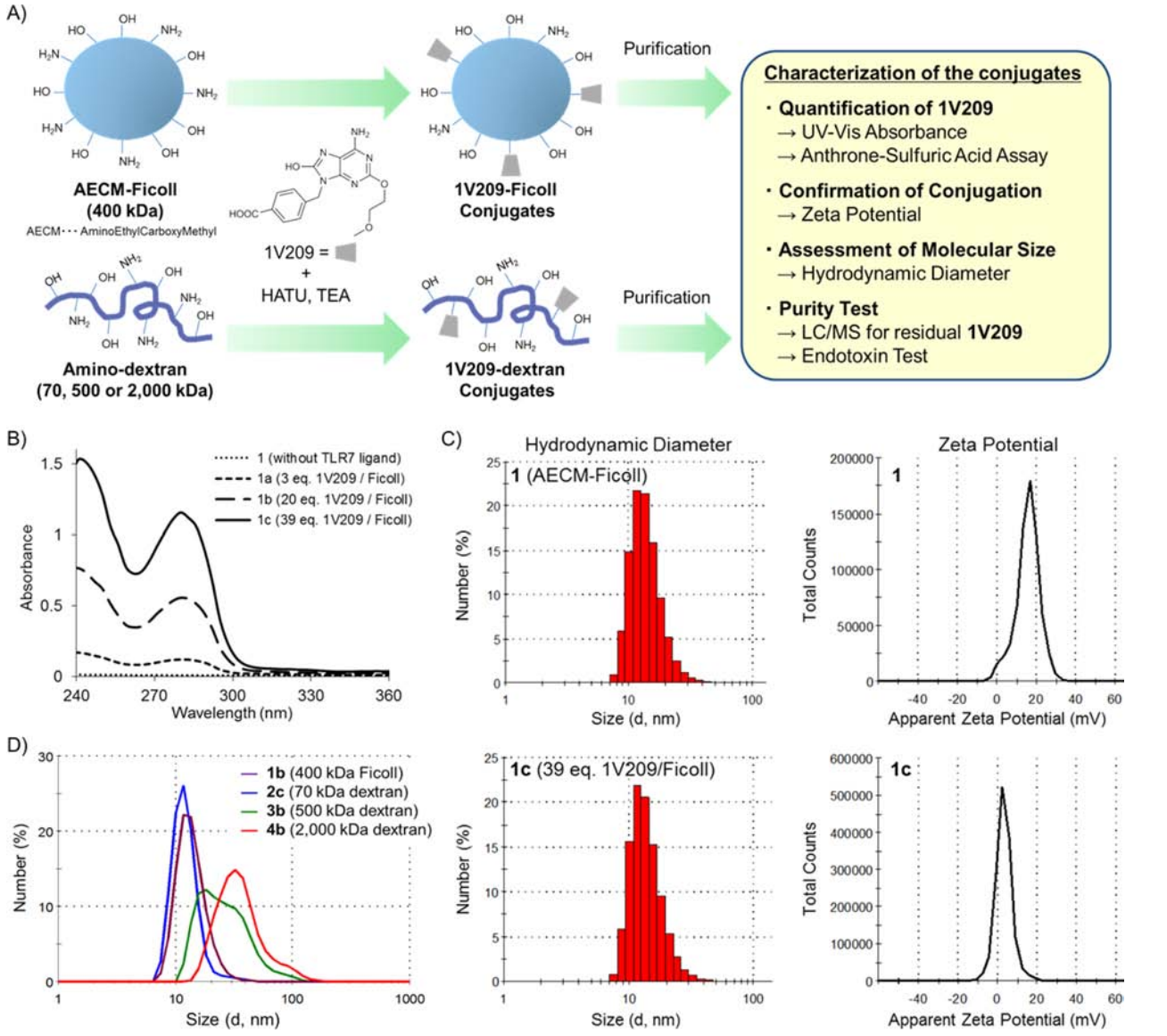

Figure 1. Preparation and characterization of TLR7 ligand−polysaccharide conjugates. (A) Schematic illustration of preparation of the conjugates. (B) UV absorption of the TLR7 ligand−Ficoll conjugates with different conjugation ratios (**1a**−**1c**). The concentration of the conjugates was adjusted to 10 μM correlated to Ficoll. (C) Hydrodynamic diameter (left) and zeta potential (right) of Ficoll before (**1**) and after conjugation of TLR7 ligand (**1c**). (D) Comparison of hydrodynamic diameter of TLR7 ligand−Ficoll conjugates with similar conjugation ratios (**1b**, **2c**, **3b**, and **4b**). Histogram of zeta potential and hydrodynamic diameter indicated in Table 1.

TLR7 agonist that has been approved by FDA as a topical agent for treatment of actinic keratosis.[11,12] Resiquimod, a TLR7/8 agent, has been evaluated in the clinic for topical use to treat viral skin lesions and skin cancer.[13−15] Both small molecules have been studied for their potential use as vaccine adjuvants.[6,16] However, oral applications were discontinued due to unacceptable side effects associated with cytokine release syndrome.[6,13−15]

In previous studies, we conjugated a synthetic small molecule purine-like TLR7 ligand to various hydrophilic macromolecules, including polyethylene glycol and serum albumin, to improve water solubility.[17,18] We demonstrated that conjugation to serum albumin increased the potency 10- to 100-fold in vitro and improved its pharmacodynamics in vivo.[17] However, serum albumin is a biological and may not be an ideal adjuvant due to its physical instability and potential microbial contamination.[19,20] Nanosize carrier molecules including metal nanoparticles, biodegradable polymers, and virus like particles are also utilized as carriers for TLR agonists though toxicity and mass-production of these carrier materials still remain challenges.[21−23] We therefore sought other safe, biocompatible, and mass-producible carrier molecules. Polysaccharides are abundant natural resources, and are known as stable, biocompatible, and hydrophilic molecules. Dextran has been clinically approved as a blood expander,[24,25] and modified dextrans have been used as a carrier material in drug delivery systems.[26] Moreover, Ficoll conjugated with a TLR9 ligand was recently shown to be a safe and effective vaccine adjuvant.[27] Hence, we hypothesized that polysaccharides would be superior carriers for a TLR7 ligand, and that conjugation of TLR7 ligand to polysaccharides would improve water solubility and enhance potency of the ligand. In addition, polysaccharides can potentially be conjugated with multiple ligands such as TLR agonists and various antigens. Several groups have reported that conjugation of a TLR ligand

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Table 1. Properties of TLR7 Ligand−Polysaccharide Conjugates

polysaccharide (M.W.)		equivalent of **1V209** for total amino groups in the reaction	moles of **1V209** per a mole of polysaccharide[a]	zeta potential[b] (mV)	hydrodynamic diameter[c] (nm)
AECM-Ficoll (400 kDa)	**1**	–	–	16 ± 5.3	14 ± 4.5
	1a	0.1	2.6 ± 0.0	15 ± 3.9	12 ± 3.9
	1b	0.5	20 ± 2.2	12 ± 5.6	14 ± 4.6
	1c	1.0	39 ± 3.5	3.6 ± 4.2	14 ± 4.7
Amino-dextran (70 kDa)	**2**	–	–	3.9 ± 5.8	9.2 ± 2.4
	2a	0.15	0.93 ± 0.07	4.0 ± 3.7	12 ± 3.5
	2b	0.75	5.9 ± 0.73	1.1 ± 4.6	12 ± 3.7
	2c	1.5	11 ± 0.63	−8.6 ± 4.4	12 ± 3.9
Amino-dextran (500 kDa)	**3**	–	–	15 ± 3.2	20 ± 7.0
	3a	0.1	3.8 ± 0.75	14 ± 4.5	27 ± 16
	3b	0.5	22 ± 1.8	9.3 ± 7.9	29 ± 17
	3c	1.0	44 ± 4.3	4.7 ± 6.8	25 ± 17
Amino-dextran (2000 kDa)	**4**	–	–	7.3 ± 3.9	47 ± 25
	4a	0.15	4.5 ± 1.7	6.8 ± 5.8	33 ± 22
	4b	0.3	16 ± 1.9	9.7 ± 4.7	37 ± 22
	4c	0.6	52 ± 2.7	7.2 ± 5.2	35 ± 23

[a]Average number of **1V209**. [b]Zeta-potential is the mean of data from three individual measurements. [c]Hydrodynamic diameter is the mean of data from three individual measurements.

with antigen in a single molecule improves antigen presentation in vitro and in vivo.[28−30] Moreover, the potency of the adjuvanted antigen in a polysaccharide conjugate may be adjusted by varying the ratio of TLR ligands to antigen. In the present study, the synthetic small molecule TLR7 specific ligand, 2-methoxyethoxy-8-oxo-9-(4-carboxy benzyl)adenine (**1V209**),[17] was covalently conjugated to primary amine functionalized Ficoll or dextran through the benzoic acid moiety. Because TLR7 expression is limited in human cell populations to mainly B cells and plasmacytoid dendritic cells, conjugates are expected to be less likely to cause severe cytokine release syndrome compared to imidazoquinolines such as resiquimod. The physical stability characteristics and immunologic potency of the resulting TLR7 ligand−polysaccharide conjugates were investigated.

RESULTS

Preparation of TLR7 Ligand−Polysaccharide Conjugates. Previous studies have shown that the in vitro and in vivo behavior of carrier materials such as metal nanoparticles, polymers, and proteins depends on size, shape, and net charge.[31−35] Therefore, the synthetic TLR7 ligand, 2-methoxyethoxy-8-oxo-9-(4-carboxy benzyl)adenine (**1V209**),[17] was conjugated to polysaccharides of different sizes and shapes, namely, 400 kDa aminoethylcarboxymethyl-Ficoll (AECM-Ficoll, **1**), 70 kDa amino-dextran (**2**), 500 kDa amino-dextran (**3**), or 2000 kDa amino-dextran (**4**), and the bioactivity of the purified conjugates were compared (Figure 1A). The conjugation was conducted by amide formation using primary amine groups of polysaccharides coupled to the benzoic acid group of the TLR7 ligand in the presence of HATU and triethylamine. The number of TLR7 ligands per polysaccharide was controlled by using specified equivalents of ligand and polysaccharide in the reaction (Table 1). After the conjugation, polysaccharides conjugated with TLR7 ligand were initially purified by precipitation using acetonitrile. Then, the precipitates were dissolved in water and further purified by centrifugal filtration using Amicon Ultra-10K membrane until the UV absorption of the filtrates was no longer detected. The absence of TLR7 ligand in the purified solution was confirmed by the lack of a detectable mass spectral peak in the LC/MS. The endotoxin levels of the conjugates were 0.1 to 1.1 EU/1 nmol of TLR7 ligand (Table 1S). All of the conjugates were highly water-soluble. In the case of the Ficoll conjugates, the solubility was greater than 80 mg/mL.

Characterization of TLR7 Ligand−Polysaccharide Conjugates. TLR7 ligand−polysaccharide conjugates were characterized by (1) the number of TLR7 ligands per polysaccharide and (2) the hydrodynamic size of the conjugates (Figure 1B−D). To evaluate the average conjugation number of TLR7 ligands per polysaccharide, the concentrations of TLR7 ligand and polysaccharides were determined by UV absorption of TLR7 ligand and anthrone-sulfuric acid assay for polysaccharides,[36] respectively. The average number of TLR7 ligands conjugated per polysaccharide was calculated by dividing the concentration of TLR7 ligand by the polysaccharide concentration. The average number of TLR7 ligands per polysaccharide increased in a dose-dependent manner, and the conjugation ratio was similar in three independent reactions (Figure 1B, Table 1). Conjugation of the TLR7 ligand to polysaccharides was further confirmed by zeta potential measurements because the net charge of the polysaccharide indirectly indicates the number of amine resides before and after conjugation.[37] Thus, the decrease in zeta potential value was inversely proportional to the number of TLR7 ligands per polysaccharide (Figure 1C, Table 1).

To enhance the potency of a TLR7 ligand, intracellular delivery through cellular uptake is important.[4,38] For efficient uptake by antigen presenting cells, carrier molecule size should preferably be larger than 10 nm.[31] Therefore, the hydrodynamic size of the conjugates was evaluated by dynamic light scattering (DLS). We found no significant difference in the hydrodynamic size between the conjugates and the unconjugated polysaccharides (Figure 1C, Table 1). The DLS data also showed that the conjugates with different molecular weights have different hydrodynamic sizes (Figure 1D). The hydrodynamic sizes of the conjugates which possess similar TLR7 ligand conjugation ratios were 14 ± 4.4 nm for the 400 kDa Ficoll conjugates (**1b**), 12 ± 3.5 nm for the 70 kDa dextran conjugates (**2c**), 29 ± 17 nm for the 500 kDa dextran conjugates (**3b**), and 37 ± 22 nm for the 2000 kDa dextran conjugates (**4b**).

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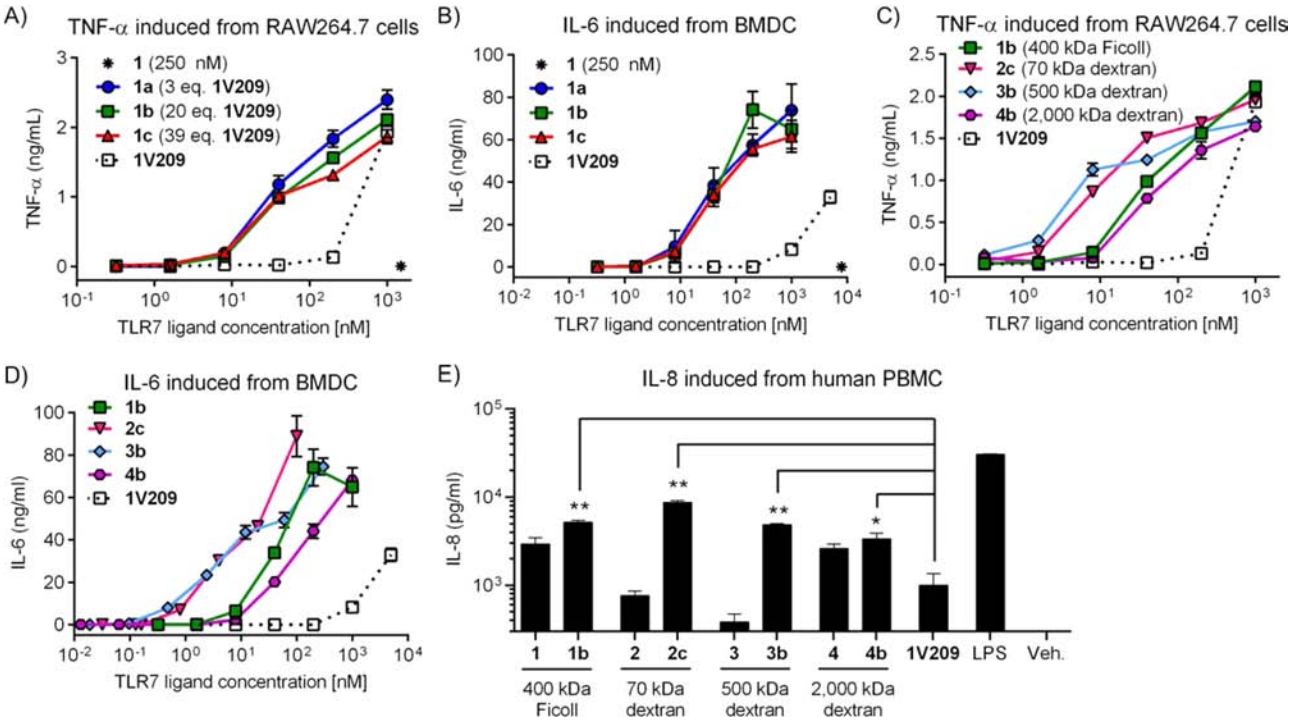

Figure 2. In vitro cytokine induction by TLR7 ligand−polysaccharide conjugates in murine and human cells. (A−D) RAW264.7 cells (1×10^4 cells/well, A and C) or BMDC (1×10^5 cell/well, B and D) were plated and incubated with serially diluted TLR7 ligand−polysaccharide conjugates (**1a**−**1c** or **1b**, **2c**, **3b**, and **4b**) or unconjugated TLR7 ligand (**1V209**) for 18 h. The control cells were treated with 250 nM Ficoll. TNF-α and IL-6 released in the culture supernatants were determined by ELISA. (E) Human PBMC were plated at 2×10^5 cells/well and incubated with 1 μM TLR7 ligand−polysaccharide conjugates (**1b**, **2c**, **3b**, or **4b**, concentration correlated to TLR7 ligand) or **1V209** for 18 h. Control cells were treated with unconjugated polysaccharides (**1**, **2**, **3**, or **4**, 50 nM), 100 ng/mL LPS or 0.5% DMSO. IL-8 released in the culture supernatants was determined by ELISA. All data shown are means ± SD of triplicate and are representative of three independent experiments. TLR7 ligand concentrations of the conjugates are equivalent to unconjugated **1V209**. *$P < 0.05$, **$P < 0.01$ by one way ANOVA with Dunnett's post hoc testing. Minimum detection levels of TNF-α, IL-6, and IL-8 were 39.1, 68.6, and 781 pg/mL, respectively.

In Vitro Agonistic Activities of TLR7 Ligand−Polysaccharide Conjugates. In vitro agonistic activities of the TLR7 ligand−polysaccharide conjugates were evaluated in two murine cell types, the RAW264.7 macrophage cell line and primary bone marrow-derived murine dendritic cells (BMDCs). The 50% maximal effective concentrations (EC_{50}) of TLR7 ligand conjugated to polysaccharides and the maximum levels of cytokine induction (I_{max}) were assessed by ELISA (Figure 2, Table 2). TLR7 ligand-Ficoll conjugates (**1a**−**c**) in murine cells showed 10- to 100-fold higher potencies, as assessed by tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) release, compared to the unconjugated TLR7 ligand (Figure 2A,B). Equivalent concentrations of Ficoll without TLR7 ligand induced minimal or no cytokine release. The number of TLR7 ligands per Ficoll relative to TLR7 ligand did not significantly influence the in vitro potencies, whereas the potencies increased when comparison was based on Ficoll concentration (Figure 1S). Ficoll conjugates whose amine residues were modified by *N*-acetylation using acetic anhydride indicated that residual amine groups of the conjugates also have no significant effect on the potencies (Figure 2S). A similar in vitro potency of the TLR7 ligand was observed after conjugation to the dextran (Table 2). Three types of amino-dextran with molecular weights of 70, 500, and 2000 kDa were used to study whether the molecular size of polysaccharide influences immunopotency of the conjugates. The 70 kDa dextran (**2c**) and the 500 kDa dextran (**3b**) conjugates showed approximately 10-fold higher potency than the 2000 kDa dextran conjugates (**4b**). Our studies also revealed that the shape of the polysaccharide affects immunostimulatory potency. When the potency of 400 kDa Ficoll conjugates (**1b**) with near-spherical shape and 500 kDa dextran conjugates (**3b**) with linear shape were compared, the 500 kDa dextran conjugates (**3b**) showed approximately 10-fold higher potency (Figure 2C,D).

Table 2. In Vitro Proinflammatory Properties in RAW264.7 Cells and Murine Primary BMDC

		EC_{50}[a] (nM)		I_{max}[a] (ng/mL)	
polysaccharide (M.W.)		RAW264.7	BMDC	RAW264.7	BMDC
AECM-Ficoll[e] (400 kDa)	**1a**	48.2	41.2	2.4 ± 0.19	75 ± 4.1
	1b	61.6	38.3	2.3 ± 0.14	76 ± 4.6
	1c	53.3	36.5	1.7 ± 0.22	65 ± 2.2
Amino-dextran[e] (70 kDa)	**2a**	10.7	24.5	2.3 ± 0.11	109 ± 2.7
	2b	7.23	8.42	2.0 ± 0.06	80 ± 3.8
	2c	9.72	17.9	1.9 ± 0.06	102 ± 6.4
Amino-dextran[e] (500 kDa)	**3a**	15.6	35.8	2.1 ± 0.27	77 ± 2.4
	3b	4.64	6.07	1.7 ± 0.09	66 ± 3.0
	3c	4.62	3.20	1.8 ± 0.10	74 ± 4.6
Amino-dextran[e] (2000 kDa)	**4a**	94.6	188	1.8 ± 0.09	80 ± 1.4
	4b	46.6	132	1.6 ± 0.08	77 ± 1.7
	4c	20.1	27.1	1.3 ± 0.07	63 ± 1.3
1V209		N.D.[b]	N.D.[b]	1.9 ± 0.11[c]	17 ± 1.4[d]

[a]EC_{50} and I_{max} was calculated using *Prism* software. [b]N.D.: not determined. [c]I_{max} calculated at 1 μM. [d]I_{max} calculated at 5 μM. [e]Polysaccharide alone induced minimal or no cytokine induction

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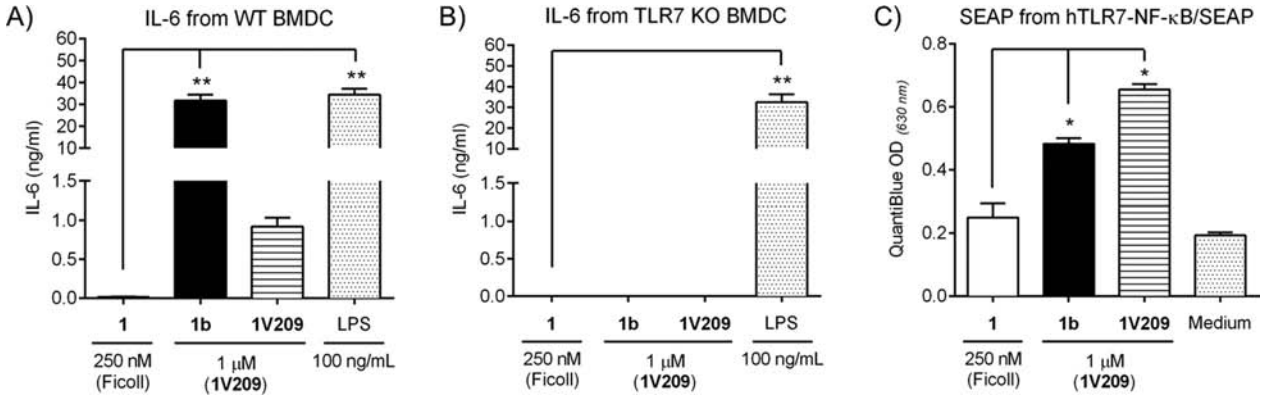

Figure 3. TLR7 specificity of TLR7 ligand−polysaccharide conjugates. (A and B) BMDC prepared from WT (A) or TLR7 deficient (B) mice were plated at 1×10^5 cells/well and incubated with 1 μM TLR7 ligand−polysaccharide conjugates (**1b**) or unconjugated TLR7 ligand (**1V209**) for 18 h. Control wells were treated with 250 nM Ficoll (**1**) or 100 ng/mL LPS. IL-6 released in the culture supernatants was determined by ELISA. (C) Human TLR7-NF-κB/SEAP reporter transfected HEK293 cells were plated at 5×10^4 cells/well and incubated with 1 μM **1b** or **1V209** for 24 h. Control cells were treated with 250 nM **1** or medium. The level of NF-κB induced SEAP was determined using QuantiBlue by reading the OD at 630 nm. All data shown are mean ± SD of triplicate and are representative of two independent experiments. TLR7 ligand concentrations of the conjugates are equivalent to unconjugated **1V209**. *P < 0.05, **P < 0.01 by one way ANOVA with Dunnett's post hoc testing compared to **1**.

A)

Storage	Hydrodynamic Diameter (nm)	Zeta Potential (mV)
AECM-Ficoll	14 ± 4.5	16 ± 5.3
Fresh	14 ± 4.6	12 ± 6.1
Room Temperature	15 ± 5.2	11 ± 4.3
Lyophilized	14 ± 4.3	12 ± 8.1

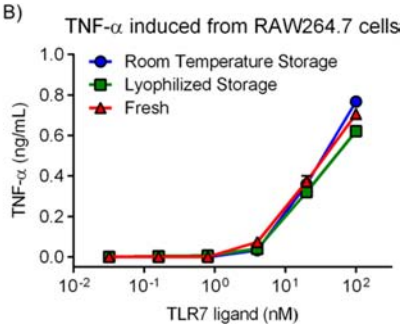

Figure 4. Physicochemical stability of TLR7 ligand−Ficoll conjugates. (A) Hydrodynamic diameter and zeta-potential of TLR7 ligand−Ficoll conjugates (**1b**) after storing for 6 months at room temperature or lyophilized. (B) RAW264.7 cells (1×10^4 cells/well) were plated and incubated with serially diluted **1b** after storing for 6 months at room temperature or lyophilized.

To evaluate whether the covalent conjugation of TLR7 ligand and polysaccharides is required for enhancement of immune potencies, the cells were incubated with TLR7 ligand and amino-dextran (**3**) mixture. TLR7 ligand and amino-dextran (**3**) mixture showed similar potency to TLR7 ligand alone, indicating covalent attachment of TLR7 ligand to polysaccharide is required for the enhanced potency and polysaccharides did not act as a vector to deliver TLR7 ligand to intracellular compartments (Figure 3S).

To assess the potency of the conjugates in human cells, peripheral blood mononuclear cells (PBMCs) were stimulated with various conjugates having similar TLR7 ligand conjugation ratios (10−22 ligands per polysaccharide, **1b**, **2c**, **3b**, and **4b**). All the conjugates induced higher levels of interleukin-8 (IL-8), IL-6 or interferon gamma induced protein 10 (IP-10) when compared to the unconjugated TLR7 ligand (Figures 2E and 4S). These results showed that the potency of the TLR7 ligand was enhanced by conjugation to polysaccharides, and that the molecular size and shape of the polysaccharides influence potencies.

In Vitro Receptor Specificity of TLR7 Ligand−Polysaccharide Conjugates. It is conceivable that conjugation of a TLR7 ligand to polysaccharides could alter its receptor specificity. To rule out this possibility, the TLR7 ligand receptor specificity of the conjugates was confirmed in BMDC isolated from TLR7 deficient mice and in human TLR7-NF-κB/SEAP reporter transfected HEK293 cells (Figure 3). Cytokine induction assays using the 400 kDa Ficoll conjugates (**1b**) in TLR7 deficient BMDC showed that neither **1b** nor unconjugated TLR7 ligand induced detectable IL-6 (Figure 3B). On the other hand, the levels of NF-κB induced SEAP from HEK293 cells were increased by both **1b** and unconjugated TLR7 ligand in the SEAP reporter assay (Figure 3C). These results indicate that TLR7 ligand conjugated to polysaccharide preserved receptor specificity for TLR7.

Physicochemical Stability of the TLR7 Ligand−Polysaccharide Conjugates. In vaccine development, long-term physicochemical stability is a crucial issue for widespread clinical deployment. To assess the long-term stability of the TLR7 ligand−polysaccharide conjugates, the 400 kDa Ficoll conjugates (**1b**) in aqueous solution or in lyophilized form were stored at room temperature in the dark for 6 months. The lyophilized **1b** was easily reconstituted in water, and did not precipitate over time. The long-term stability of the conjugates was evaluated by hydrodynamic size, net charge, and in vitro cytokine induction activity (Figure 4). DLS analysis indicated that both hydrodynamic size and zeta-potential values after storage were consistent with those of the freshly prepared conjugates (Figure 4A). TNF-α release assay in RAW264.7 cells showed that the potency of the stored conjugates (**1b**) was comparable to that of the freshly prepared conjugates (Figure 4B). These data reveal that TLR7 ligand−polysaccharide conjugates are highly stable

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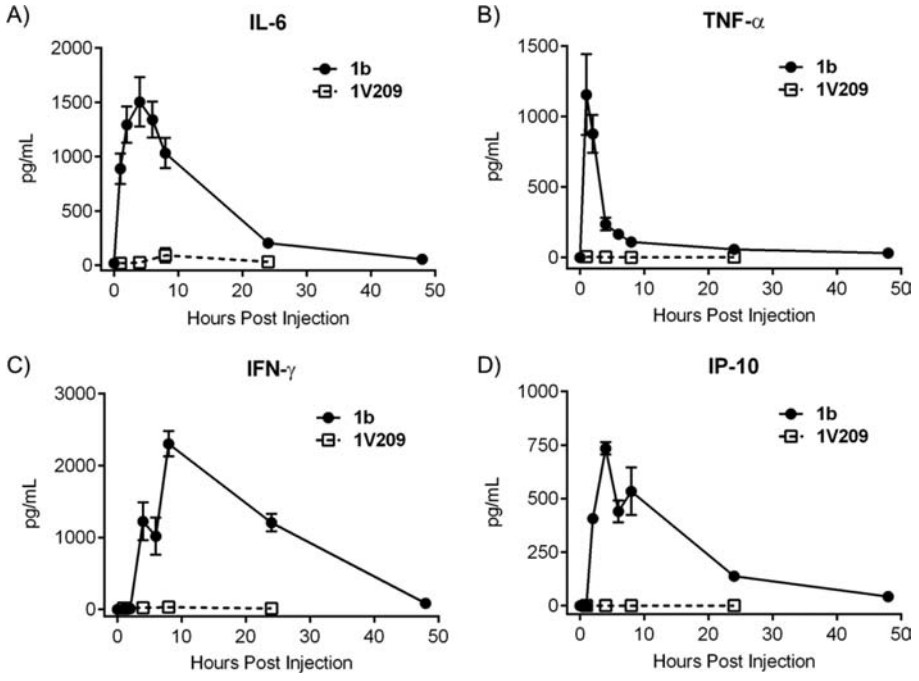

Figure 5. In vivo pharmacodynamics of TLR7 ligand−polysaccharide conjugates. (A−D) C57BL/6 mice (n = 3) were intravenously administrated with 100 nmol of **1b** or unconjugated TLR7 ligand (**1V209**). In vivo kinetics of IL-6 (A), TNF-α (B), IFN-γ (C), and IP-10 (D) were determined by Luminex beads assay. Serum samples were collected at 0, 1, 2, 4, 6, 8, 24, and 48 h after the injection. TLR7 ligand concentrations of the conjugates are equivalent to unconjugated **1V209**.

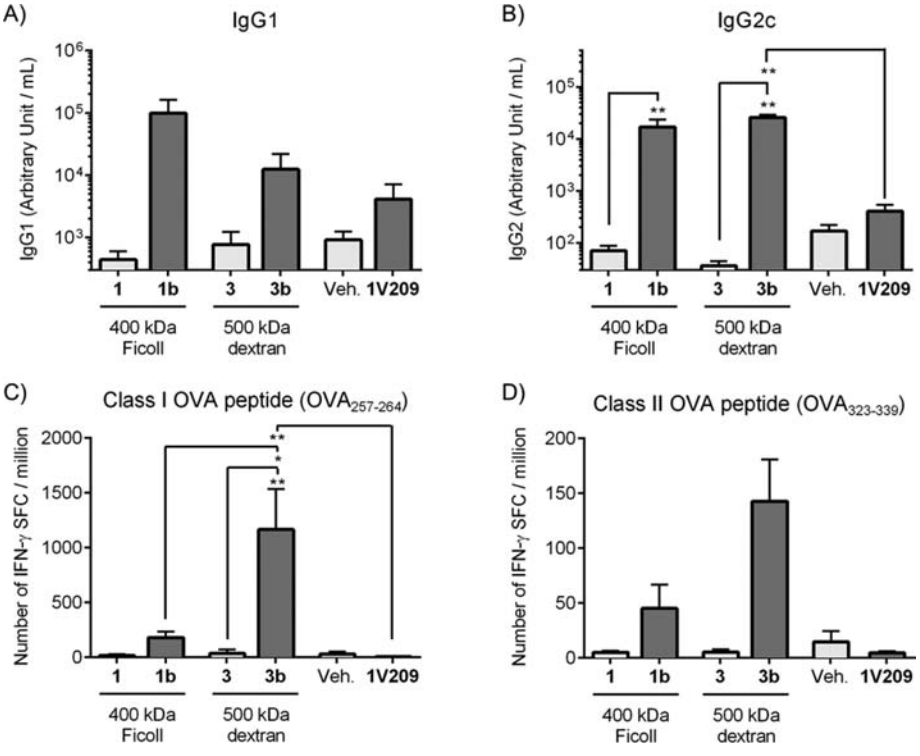

Figure 6. Immunization study of TLR7 ligand−polysaccharide conjugates with OVA as an antigen. (A−D) C57BL/6 mice (n = 4 or 5) were intramuscularly immunized with 20 μg of OVA and 0.2 nmol of **1b**, **3b** or unconjugated TLR7 ligand (**1V209**) on days 0 and 14, and sacrificed on day 35. Sera were collected at 35 days after the injection. The levels of serum anti-OVA IgG1 antibody (A) or IgG2c antibody (B) were determined by ELISA. Splenocytes were stimulated for 18 h at 37 °C, 5% CO_2 on anti-IFN-γ antibody coated ELISpot plates with 10 μg/mL OVA class I ($OVA_{257-264}$) peptide (C) or class II ($OVA_{323-339}$) peptides (D). IFN-γ spot-forming cells (SFC) per million cells were detected with the IFN-γ ELISpot ALP Kit. *P < 0.05, **P < 0.01 by one way ANOVA with Dunnett's post hoc testing.

and can be stored in aqueous solution or in lyophilized form for at least 6 months.

In Vivo Cytokine Induction by TLR7 Ligand−Polysaccharide Conjugates. The experiments described above

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showed that polysaccharide conjugation significantly enhanced the in vitro immune potency of a TLR7 ligand. Previous experiments indicated that conjugation of TLR7 ligands to macromolecules altered in vivo behavior of the TLR7 ligand.[18] To study the pharmacodynamics (PD) of the conjugates in vivo, C57BL/6 mice were intravenously injected with 100 nmol, based on the concentration of TLR7 ligand, of the 400 kDa Ficoll conjugates (**1b**). Sera were collected at 0, 1, 2, 4, 6, 8, 24, or 48 h thereafter. The level of cytokines in the serum was evaluated by Luminex bead assay specific for detection of IL-6, TNF-α, interferon-γ (IFN-γ), and IP-10. Cytokine induction by **1b** in serum following systemic administration was at least 500-fold more potent than unconjugated TLR7 ligand (Figure 5A−D).

Immunization Study of TLR7 Ligand−Polysaccharide Conjugates with Ovalbumin As Antigen. To test whether TLR7 ligand−polysaccharide conjugates can serve as vaccine adjuvants, the conjugates were tested in vivo using ovalbumin (OVA) as a model antigen. Systemic administration of high concentrations TLR7 ligands in rodents can induce splenomegaly due to enhanced proliferation of hematopoietic cells.[39,40] We therefore selected a dose of 0.2 nmol which did not induce significant splenomegaly in the preliminary experiments. C57BL/6 mice were intramuscularly immunized with 20 μg OVA together with 0.2 nmol of the 400 kDa Ficoll conjugates (**1b**) or the 500 kDa dextran conjugates (**3b**) at days 0 and 14, and were sacrificed at day 35 relative to the first immunization. Sera were then collected and splenocytes were harvested. Unconjugated 400 kDa AECM-Ficoll (**1**), 500 kDa amino-dextran (**3**), and unconjugated TLR7 ligand (**1V209**) were used as controls. The levels of serum immunoglobulins against OVA were evaluated by ELISA (Figure 6A,B). Immunoglobulin levels on day 35 in sera showed that **3b** induced significantly higher anti-OVA IgG2c antibody titers than **3** (Figure 6B). In contrast, neither **1b** nor **3b** significantly enhanced IgG1 production (Figure 6A). Unconjugated TLR7 ligand did not enhance the antibody production compared to vehicle control. When splenocytes from mice immunized with **1b** or **3b** were restimulated with 10 μg/mL of class I OVA peptides for 18 h, both splenocyte samples contained significantly increased frequencies of IFN-γ producing T lymphocytes (Figure 6C). In addition, splenocytes from mice immunized with **3b** contained significantly higher frequencies of IFN-γ producing T cells than **1b** as adjuvant. When the splenocytes were restimulated with class II OVA peptides, increased frequency of IFN-γ producing T lymphocytes were observed (Figure 6D). These results indicate that the 500 kDa dextran conjugates (**3b**) could enhance antigen specific humoral and cellular immune responses, particularly in $CD8^+$ T cell responses, to a greater extent than the 400 kDa Ficoll conjugates (**1b**) or unconjugated TLR7 ligands.

DISCUSSION

The TLR7 ligand, imiquimod, is one of only a few TLR7 ligands approved for clinical use.[6,11,14] Improvement of immunostimulatory potencies of small molecule TLR7 ligands has been achieved by conjugation and formulation.[17,18,41,42] In previous studies, we demonstrated that conjugation of macromolecules, such as albumin, phospholipids and polyethylene glycol, to small molecule TLR7 ligands enhanced the immunostimulatory activities and efficacy as vaccine adjuvants.[17,18] Kedl et al. also reported that the conjugation of TLR7 ligand to proteins is necessary for enhancement of immunostimulatory activity.[41] Polysaccharides have been used to increase immunostimulatory potency of TLR9 ligands.[27] In this study, we used polysaccharides as carriers for small molecule TLR7 ligand, **1V209**. We selected **1V209** over imiquimod, or its relative resiquimod, for our studies because the structure of **1V209** contains a molecular "handle" in the 9-benzyl moiety of the purine ring that allows for substantial modification, such as conjugation to macromolecules, without abrogating immune activity, as we previously showed.[17,18] Imiquimod and resiquimod do not contain such a "handle" and the effects of modification of the magnitude we required here are unknown.

Because polysaccharides of various sizes and shapes functionalized with primary amines, AECM-Ficoll and amino-dextran, were commercially available, we investigated the optimal sizes or types of polysaccharides that could maximize the potency of the TLR7 ligand. In vitro studies showed that conjugation of the TLR7 ligand to any of the polysaccharides increased agonistic potency compared to the unconjugated TLR7 ligand. The number of TLR7 ligands per polysaccharide molecule did not significantly influence the immune potencies, while the shape and molecular size of carrier polysaccharides did affect their potencies. In vivo pharmacodynamics studies showed that TLR7 ligand−polysaccharide conjugates had enhanced and prolonged cytokine-inducing activities compared to **1V209**.

The conjugation process of the TLR7 ligand to polysaccharides is simple, and the conjugates are highly water-soluble and stable. The conjugations can be performed in water at room temperature and do not require strict reaction conditions, and the conjugates are simply purified by acetonitrile precipitation followed by centrifugal filtration. Conjugation of TLR7 ligand to polysaccharides was confirmed by UV and zeta potential analysis (Figure 1). The distinct UV absorption of the benzoic acid moiety of **1V209** at 280 nm is useful for quantification of **1V209**.[17] Zeta potential values correlate with the net charge of the polysaccharides, which indirectly indicate the amount of amine residues. As expected, a decrease in zeta potential value was inversely proportional to the number of TLR7 ligands per polysaccharide except for the 2000 kDa dextran conjugates. The zeta potential values of the 2000 kDa dextran conjugates (**4a−c**) were not changed by conjugation of the TLR7 ligand. Because the 2000 kDa amino-dextran (**4**) possesses 300 to 400 primary amines per molecule, the zeta potential was not sensitive enough to detect changes of less than 20% of the amine residues. Conjugation ratios of TLR7 ligand to polysaccharides were controllable by using specified equivalents of TLR7 ligand and polysaccharide in the reaction. In drug development, the long-term stability of the chemical entity is crucial. The zeta potential value, hydrodynamic size, and potency of the 400 kDa Ficoll conjugates (**1b**) were unaltered during storage in aqueous solution or in lyophilized form at room temperature for at least 6 months (Figure 4).

To investigate the in vitro immune potency of the conjugates, assays were conducted in both murine and human cells. In murine cells, the potency of the TLR7 ligand was significantly enhanced by conjugation to polysaccharides, which retained receptor specificity for TLR7 (Figures 2 and 3). The conjugates were also active in human blood cells (Figures 2E and 4S). However, 400 kDa AECM-Ficoll (**1**) and 2000 kDa amino-dextran (**4**) showed relatively high IL-8 as well as IL-6 induction. There are two possible reasons for relatively high backgrounds. The first possibility is that relatively large molecular weight polysaccharides may enter the cells through a phagocytic process that can release pro-inflammatory cytokines and chemokines.[43] Another possibility is that endotoxin in the samples may influence the immunostimulatory activity. Because the endotoxin

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levels of 400 kDa AECM-Ficoll (**1**) and 2000 kDa amino-dextran (**4**) are slightly higher than other dextrans (0.19 and 1.1 EU/1 nmol of the TLR7 ligand conjugates equivalent, respectively), the endotoxin in the samples might contribute to the observed activities.

To investigate the influence of the shape of carrier molecules in vitro, the potency of the 400 kDa Ficoll (**1b**) and the 500 kDa dextran (**3b**) conjugates containing similar conjugation ratios of TLR7 ligand were compared. Thus, the 500 kDa dextran (**3b**) showed approximately 10-fold higher cytokine-inducing activities than the 400 kDa Ficoll (**1b**). These data indicate that conjugation to a linear polysaccharide, dextran, increased agonistic potency to a greater extent than to spherical polysaccharide, Ficoll. To further seek the optimal size of dextran for enhancement of agonistic potency, the dextran conjugates containing similar conjugation ratios (11−22 ligands/polysaccharide) were compared (Figure 2). The 70 kDa (**2c**) and 500 kDa dextran conjugates (**3b**) were approximately 10-fold more potent than the 2000 kDa dextran conjugates (**4b**). These data suggest that lower molecular weight dextran is a preferred size of dextran for enhancement of potency. However, the detailed mechanism that determines potency is still unclear. Thus, further analysis is needed to reveal this size dependency for activity of the dextran conjugates.

Enhancement of the potency of the TLR7 ligand may be the result of more efficient uptake of the conjugates by phagocytic antigen presenting cells. TLR7 is mainly located in the endosomal compartments of plasmacytoid dendritic cells.[4,38] Thus, the intracellular delivery of a TLR7 ligand is a critical modulator of its agonistic activity. For the efficient intracellular delivery in phagocytic antigen presenting cells, small molecules are often conjugated to macromolecules to enhance endocytosis.[31] In the case of our polysaccharide conjugates, when endocytosis was inhibited by cytochalasin D,[44] the cytokine induction of the conjugates was significantly inhibited, but not in the case of TLR7 ligand alone (Figure 5S). Furthermore, in TLR7-NF-κB/SEAP reporter assay using HEK293 cells which do not have phagocytic activity, the immunostimulatory activity of the TLR7 ligand and the conjugates (**1b**) were similar. These findings suggest that increased potency of TLR7 ligand−polysaccharide conjugate is due to increased cellular uptake. It should be noted that hydrodynamic sizes of all the conjugates prepared in this study were within the size range reported for efficient uptake by antigen presenting cells.[31]

Because the cell surface is typically negatively charged,[45,46] ionic interaction can strongly influence the uptake of macromolecules.[33,47−50] The structure of **1V209** has a carboxylic acid functional group bearing a net negative charge at physiological pH, which may reduce the receptor binding, and thereby reduce lysosomal uptake and subsequent cytokine induction. One possible mechanism of increased potency of TLR7 ligand is due to efficient intracellular delivery by conversion of the net charge of molecules from negative to zero or positive. To evaluate whether the net charge of the molecules affect the immune potency of TLR7 ligand−polysaccharide conjugates, residual amines on the conjugates were converted to *N*-acetyl groups by treatment with acetic anhydride. The potency of the conjugates was not changed by this modification, suggesting that the net positive charge did not influence the potencies TLR7 ligand−polysaccharide conjugates (Figure 2S). Furthermore, the potency as indicated by EC_{50} value in RAW264.7 cells of the uncharged TLR7 ligand (1087 nM), 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (designated as **1V136**[18]), and negatively charged **1V209** (1194 nM) were similar (Figure 6S). Thus, increased immunostimulatory potency of the conjugates was not simply due to removal of net negative charge of the ligand, but also by other factors, such as an increase in intracellular delivery of TLR7 ligands, irrespective of charge.

Conjugation of a TLR7 ligand to polysaccharide greatly enhanced in vivo immunostimulatory activity confirming earlier observations with CpG oligonucleotides conjugated to Ficoll.[27] In an immunization study using OVA as a model antigen, the 400 kDa Ficoll conjugates (**1b**) and 500 kDa dextran conjugates (**3b**) were compared (Figure 6). On day 35 after immunization with the conjugates and OVA, splenocytes isolated from mice immunized with antigen plus **1b** or **3b** had significantly increased frequency of major histocompatibility complex (MHC) class I OVA peptide specific IFN-γ producing T cells. In this system, the dextran conjugates (**3b**) were even more potent than the Ficoll conjugates (**1b**). MHC class I recognizing $CD8^+$ T cells have a unique ability to specifically kill cancer and virus infected cells. Thus, the dextran conjugates could be useful vaccine adjuvants for both preventive and therapeutics applications.

■ CONCLUSION

In this study, we prepared small molecule synthetic TLR7 ligand conjugated polysaccharides, and investigated the immunostimulatory activity of the conjugates in vitro and in vivo. The TLR7 ligand−polysaccharide conjugates were prepared by amide formation using primary amines of polysaccharides and benzoic acid functional groups of the TLR7 ligand. The resulting conjugates were highly water-soluble, reproducible, and stable. The in vitro and in vivo agonistic activity of the TLR7 ligand was greatly enhanced by conjugation to polysaccharides. A vaccine immunization study showed that TLR7 ligand−500 kDa dextran conjugates elicited strong Th1 biased immune responses, accompanied by expansion of antigen specific $CD8^+$ T cells. Polysaccharides are biocompatible and mass-producible materials, and can be conjugated to various molecules such as peptides, proteins, and TLR ligands. Therefore, polysaccharides could be practical and versatile carriers for development of cancer and infectious disease vaccines.

■ EXPERIMENTAL PROCEDURES

Reagents. 400 kDa aminoethylcarboxymethyl-Ficoll (AECM-Ficoll) was purchased from Biosearch Technologies Inc. (Cat. No. F-1100-1, Petaluma, CA). 70 kDa and 500 kDa amino-dextran were purchased from Life Technologies (Cat. No. D-1862 and D-7144, Carlsbad, CA). 2000 kDa amino-dextran was purchased from Fina Biosolutions LLC (Cat. No. AD2000−350, Rockville, MD). All polysaccharides are functionalized with primary amine unless otherwise noted. 2-Methoxyethoxy-8-oxo-9-(4-carboxy benzyl)adenine (**1V209**) was synthesized in our laboratory by previously reported procedures.[17] All other reagents were purchased as at least reagent grade from Sigma-Aldrich (St. Louis, MO) without further purification. Solvents were purchased from Fisher Scientific (Pittsburgh, PA). Milli-Q water (18.2 MΩ cm^{-1}) was used in all experiments unless otherwise noted.

Endotoxin levels of the conjugates used for experiments on immunological activity were measured using Limulus Amoebocyte Lysate (LAL) Test Cartridges with the Endosafe-PTS portable Test System purchased from Charles River Laboratories (Wilmington, MA). RPMI 1640 or DMEM (both from Life Technologies, Carlsbad, CA) media were supplemented with

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10% fetal bovine serum (Omega Scientific, Tarzana, CA) and 100 U/mL penicillin−streptomycin (Life Technologies) to prepare complete RPMI or DMEM media.

Instrumentation. UV/Vis absorption was measured using NanoDrop ND-1000 spectrophotometer (Wilmington, DE) or Infinite M200 plate reader (TECAN, Männedorf, Switzerland). Mass spectra were measured using an Agilent 6420 Triple Quad LC/MS (Agilent Technologies, Inc., Santa Clara, CA). Hydrodynamic size and zeta-potential were measured using Zetasizer Nano ZS (Malvern, Worcestershire, UK).

Animals. All procedures and protocols were approved by the University of California, San Diego Institutional Animal Care and Use Committee (UCSD IACUC, Protocol Number: S09331 and S00028). Seven- to eight week-old-female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR7 deficient mice (C57BL/6 background) were a gift from Dr. S. Akira (Osaka University, Osaka, Japan) and were backcrossed for 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.

Conjugation of 1V209 to Polysaccharides. The TLR7 ligand (**1V209**, 0.1 to 1.5 equiv to primary amine of polysaccharides) and HATU (1.1 equiv to **1V209**) were dissolved in anhydrous DMF (50 μL) (Figure 1A). These solutions were mixed, and TEA (2.0 equiv to **1V209**) was added. The mixed solution was stirred for 10 min at room temperature. The solutions of primary amine functionalized polysaccharide were prepared by dissolving 400 kDa Ficoll (7 mg, 1.0 nmol) in anhydrous DMF (300 μL) or 70 kDa dextran (5.0 mg, 71 nmol), 500 kDa dextran (5.0 mg, 10 nmol) or 2000 kDa dextran (7.0 mg, 3.5 nmol) in Milli-Q water (100 μL). The polysaccharide solution was added rapidly to the reaction mixture. The reaction mixture was stirred overnight at room temperature. For purification of the conjugates, the reaction mixture was dropped slowly into acetonitrile under vigorously stirring. The wet precipitates were collected and dissolved in Milli-Q water (500 μL). The obtained solution was purified by centrifugal filtration (14 000*g*, 7 min) using an Amicon Ultra 10K (Millipore, Billerica, MA), and the residue was washed 4 times with Milli-Q water. Then, Milli-Q water was added to prepare a solution of TLR7 ligand−polysaccharide conjugates. All conjugates were stored as 50 to 100 μM, which correlates to **1V209** concentration, aqueous solution at 4 °C and further diluted before immunological assays. For the blocking of amine residues of Ficoll after **1V209** conjugation, the conjugates (7.0 mg, 1.0 nmol) were treated with acetic anhydride (1.0 μL, 10 μmol) in 0.1 M sodium phosphate buffer (1 mL, pH 6.5−7.5) on ice. After overnight stirring, the conjugates were purified by the same procedure as described above.

Characterization of TLR7 Ligand−Polysaccharide Conjugates. The average number of TLR7 ligands conjugated per polysaccharide was calculated by dividing the concentration of TLR7 ligand by polysaccharide concentration in the aqueous solution. The **1V209** concentration was determined from UV absorption at 280 nm, and serially diluted **1V209** dissolved in methanol was used for the standard curve. Polysaccharide concentration was determined by anthrone-sulfuric acid assay.[36] In this assay, serially diluted polysaccharide aqueous solutions were used as standard. The hydrodynamic size of TLR7 ligand−polysaccharide conjugates was analyzed by dynamic light scattering (DLS) in phosphate buffered saline. The zeta-potential value of the conjugates was evaluated by DLS in water.

In Vitro Cytokine Induction in RAW264.7 Cells. The RAW264.7 cells (mouse macrophage cell line) were obtained from the ATCC (Rockville, MD) and cultured in complete DMEM (Gibco, Carlsbad, CA). After the culture reached 70% confluency, the cells were harvested using 0.25% Trypsin-EDTA. Then, the cells were plated in 96-well plates at 150 μL (1×10^4 cells) per well and incubated overnight at 37 °C, 5% CO_2. Aqueous solutions of TLR7 ligand−polysaccharide conjugates were serially diluted with medium containing 2% DMSO. Unconjugated **1V209** dissolved in DMSO was also serially diluted with assay medium. Each solution (50 μL) was added to the wells, and the final concentration of DMSO was adjusted to 0.5%. Then, the cells were further incubated for approximately 18 h at 37 °C, 5% CO_2. The culture supernatants were collected, and the level of TNF-α in the supernatants was determined by ELISA (BD bioscience, La Jolla, CA).[51] In the endocytosis/phagocytosis inhibition assay, RAW264.7 cells (1×10^4 cell/well) were plated and pretreated with 5 μM cytochalasin D for 45 min. Then, the cells were incubated with 200 nM TLR7 ligand−dextran conjugates (**3b**) or unconjugated TLR7 ligand (**1V209**) for 3 h. TNF-α released in the culture supernatants was determined by ELISA. Minimum detection level of TNF-α was 39.1 pg/mL.

In Vitro Cytokine Induction in BMDC. BMDC were prepared from C57BL/6 mice or TLR7 deficient mice (C57BL/6 background) as described previously.[51] The cells were harvested and resuspended in complete RPMI medium (Gibco, Carlsbad, CA). Then, the cells were plated in 96-well plates at 200 μL (1×10^5 cells) per well with the serially diluted TLR7 ligand−polysaccharide conjugates or unconjugated **1V209** containing 0.5% DMSO. After 18 h incubation at 37 °C, 5% CO_2, the culture supernatants were collected and the level of IL-6 in the supernatants was determined by ELISA. Minimum detection level of IL-6 was 68.6 pg/mL.

In Vitro Activities in PBMC. Human PBMC were isolated from buffy coats obtained from the San Diego Blood Bank (San Diego, CA) as described previously.[52] The cells were harvested and resuspended in complete RPMI medium. Then, the cells were plated in 96-well plates at 200 μL (2×10^5 cells) per well with 1 μM TLR7 ligand−polysaccharide conjugates or unconjugated **1V209** containing 0.5% DMSO. After 18 h incubation at 37 °C, 5% CO_2, the levels of IL-8, IL-6, and IP-10 in the culture supernatants were determined by ELISA. Minimum detection level of IL-8, IL-6, and IP-10 were 781, 7.8 and 15.6 pg/mL, respectively.

In Vitro Measurements of NF-κB Activation Using NF-κB-hTLR7 HEK293 Cells. Human TLR7 NF-κB/SEAP-(SEAPorter) HEK293 cells were purchased from Novus Biologicals, LLC (Littleton, CO). The cells were resuspended in complete DMEM. Then, the cells were plated in 96 well plates at 200 μL (5×10^4 cells) per well with serially diluted TLR7 ligand−polysaccharide conjugates or unconjugated **1V209** solution containing 0.5% DMSO. After 24 h incubation at 37 °C, 5% CO_2, the culture supernatants were collected, and the level of NF-κB induced SEAP was determined using QUANTI-Blue (Invivogen, San Diego, CA) by reading the OD at 630 nm using a plate reader.

In Vivo Pharmacodynamics Studies. C57BL/6 mice were intravenously injected with the TLR7 ligand−polysaccharide conjugates (**1b**, **2c**, **3b**, or **4b**) or unconjugated **1V209** (2 nmol or 100 nmol, concentration correlating to **1V209**). Blood samples were collected at 0, 1, 2, 4, 6, 8, 24, or 48 h after injections. Sera were separated and kept at −20 °C until use. The levels of IL-6, TNF-α, IFN-γ, and IP-10 in the sera were

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measured by Luminex beads assay (Life Technologies, Carlsbad, CA).

In Vivo Immunization Studies Using OVA as an Antigen. C57BL/6 mice were immunized by intramuscular injection to gastrocnemius with 20 μg of ovalbumin (OVA, Sigma, St. Louis, MO) with TLR7 ligand−polysaccharide conjugates (**1b** or **3b**, 0.2 or 2 nmol/animal) or unconjugated **1V209** (0.2 or 2 nmol/animal) on days 0 and 14, in a total volume of 50 μL. Vehicle (Veh., 10% DMSO in saline) and unconjugated polysaccharides (**1** or **3**, 0.01 or 0.1 nmol/animal) were used as controls. Sera were collected on day 35. Mice were sacrificed on day 35, and the spleens were harvested. Splenocytes were stimulated for 18 h at 37 °C, 5% CO_2 in anti-IFN-γ antibody coated ELISpot plates with a pool of 10 μg/mL OVA class I ($OVA_{257-264}$, Invitrogen) or class II ($OVA_{323-339}$, Invitrogen) peptides. IFN-γ spot-forming cells (SFC) per million cells were detected with the IFN-γ ELISpot ALP Kit (Mabtech AB, Nacka Strand, Sweden).

Measurement of OVA Specific Antibodies. Anti-OVA antibodies of the IgG subclasses, IgGl and IgG2c, were measured by ELISA as previously described.[51] Each ELISA plate contained a titration of a previously quantified serum to generate a standard curve. The titer of this standard was determined as the highest dilution of serum that gave an absorbance reading that was double the background. Serum samples were tested at a 1:100 to 1:125 600 dilutions and reported as U/mL based on comparison with the standard curve.

Statistical Analysis. *GraphPad Prism 6* (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses including regression analyses. EC_{50} values were calculated by nonlinear regression assuming a sigmoidal dose distribution with uniform standard deviations between groups. The statistical differences were analyzed by one-way ANOVA with Dunnett's post hoc testing. A value of $P < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

Table 1S: Endotoxin levels of the conjugates. Figure 1S: In vitro cytokine induction by TLR7 ligand-Ficoll conjugates in mouse bone marrow dendritic cells. Figure 2S: Zeta-potential measurement of primary amine blocked Ficoll and its in vitro cytokine induction activity. Figure 3S: Covalent attachment of TLR7 ligands to polysaccharide requires enhancement of in vitro cytokine activity by TLR7 ligand-polysaccharide conjugates. Figure 4S: In vitro cytokine induction by TLR7 ligand-polysaccharide conjugates in human cells. Figure 5S: In vitro cytokine induction by TLR7 ligand-dextran conjugates in RAW264.7 cells treated with cytochalasin D. Figure 6S: In vitro potencies of known TLR7 ligands. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00285.

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The authors declare no competing financial interest.

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■ ABBREVIATIONS

BMDC, Bone marrow derived dendritic cells; **DLS**, Dynamic light scattering; **DMF**, Dimethylformamide; **DMSO**, Dimethyl sulfoxide; **ELISA**, Enzyme-linked immunosorbent assay; **HATU**, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate; **IFN**, Interferon; **IL**, Interleukin; **IP-10**, Interferon gamma-induced protein-10; **LPS**, lipopolysaccharide; **NF-κB**, Nuclear factor kappa-light-chain-enhancer of activated B cells; **OVA**, Ovalbumin; **PBMC**, Peripheral blood mononuclear cell; **PD**, Pharmacodynamics; **SEAP**, Secreted embryonic alkaline phosphate; **TEA**, Triethylamine; **TLR**, Toll like receptor; **TNF**, Tumor necrosis factor; **1V209**, 2-methoxyethoxy-8-oxo-9-(4-carboxy benzyl)adenine

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