RESEARCH ARTICLE

Discovery of Self-Assembling Small Molecules as Vaccine Adjuvants

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Abstract: Immune potentiators, termed adjuvant, trigger early innate immune responses to ensure the generation of robust and long-lasting adaptive immune responses of vaccines. Here we present study that takes advantage of a self-assembling small molecule library for the development of a novel vaccine adjuvant. Cell-based screening of the library and subsequent structural optimization led to the discovery of a simple, chemically tractable deoxycholate derivative (molecule **6**, also named cholicamide) whose well-defined nano-assembly potently elicits innate immune responses in macrophages and dendritic cells. Functional and mechanistic analyses indicate that the virus-like assembly is engulfed inside cells and stimulates the innate immune response through Toll-like receptor 7 (TLR7), an endosomal TLR that detects single-stranded viral RNA. As an influenza vaccine adjuvant in mice, molecule **6** was as potent as Alum, a clinically used adjuvant. The studies described here pave the way for a new approach to discovering and designing self-assembling small-molecule adjuvants against pathogens, including emerging viruses.

Introduction

Vaccines are the most successful medical interventions to prevent infectious diseases. Live attenuated vaccines derived from disease-causing pathogens have been serving as effective vaccines, primarily because they continuously stimulate both innate and adaptive immune responses [1]. The components of the attenuated pathogens stimulate the innate immune system, while the surface antigens engage in developing the adaptive immune responses. Recent understanding of Toll-like receptors (TLRs) and dendritic cells shed light on the essential roles of early innate immune responses in driving later antigen-specific responses and generating immunological memory.[2]

Despite the historical success of live attenuated vaccines, one safety concern is their potential to revert to a pathogenic form and cause diseases in vaccinees or their contacts. So-called subunit vaccines consisting of isolated antigens would be a safer and more cost-effective choice; however, they often lack sufficient immunogenicity and require inclusion of immune potentiators (termed adjuvants), which trigger early innate immune responses to ensure the generation of robust and long-lasting adaptive immune responses. Such adjuvants are also critical in particular during pandemic in increasing the response rate in low-responder individuals and decreasing the required dose of the vaccines.

Currently licensed adjuvants for human use, such as aluminum salts and oil-in-water emulsions, were empirically developed, and their modes of action remain incompletely understood[3]. Very few of the experimental innate immune potentiators have yet reached clinical approval as adjuvants, and they tend to have large, complex chemical structures, including emulsion, hydroxygel, crystal, micro- or nano-particle, which pose challenges for factory-scale, cost-effective chemical productions. This lack of progress in adjuvant development is partially due to the poor diversity of the approaches toward adjuvant discovery. We thus seek a new approach to increasing our portfolio of adjuvant candidates to meet the specific requirements of each particular vaccine against pathogens, especially newly emerging viruses.

The size of pathogen components often influences the level of innate immune responses. Large aggregates, such as actin filaments[4], monosodium urate crystals[5], and cholesterol crystals[6], and large polymers, such as lipopolysaccharide (LPS) and nucleic acids[7], have been documented to stimulate potent innate immune responses.[8] The potent activity of these aggregates or polymers compared to soluble single molecules can probably be explained by their ability to mimic the structures presented or condensed in the pathogens, which leads to clustering of multiple receptors of antigen-presenting cells (APCs) such as macrophages and dendritic cells and hence to higher levels of activation.

Such a large multivalent particle can be created by small molecules through molecular self-assembly, a process in which molecules spontaneously form an organized large structure through non-covalent interactions[9]. Here, we propose a library of self-assembling small molecules as a robust source of adjuvants. The approach enabled the rapid discovery of a novel small-molecule nano-assembly that stimulates innate immune

responses with a unique mode of action. Optimization of the seed molecule led to a simple, chemically tractable small molecule that served as an influenza vaccine adjuvant as potent as Alum, a clinically used adjuvant.

Results

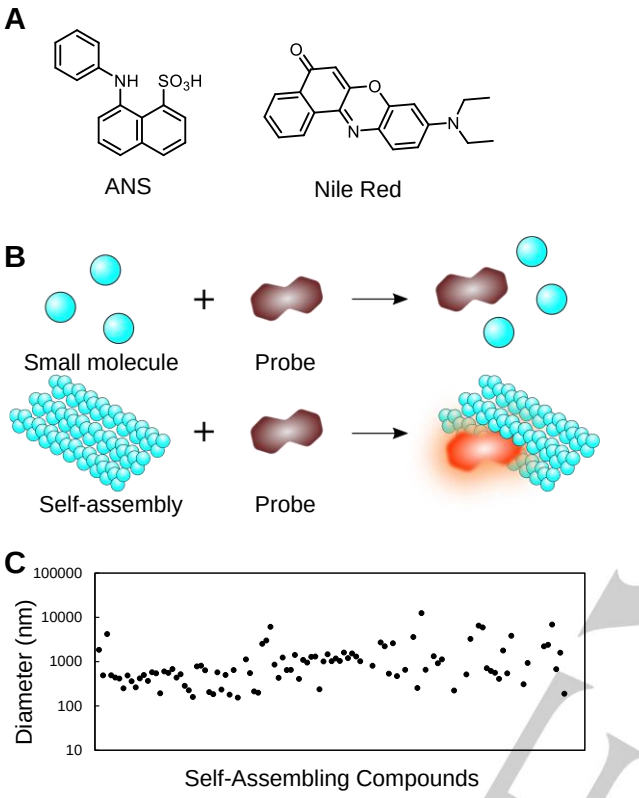

Fig. 1. Fluorescent-based screening for self-assembling molecules. (A) Chemical structures of ANS and Nile Red probes. (B) Illustration of the screening method. (C) Average hydrodynamic diameters of 116 self-assembling compounds as measured by DLS (ELS-Z2).

Construction of a self-assembling molecule library

We initially developed a simple method to search for self-assembling compounds using two fluorescent probes, 8-anilino-1-napthlenesufonic acid (ANS) and Nile Red[10]. While these environment-sensitive probes are essentially non-fluorescent in aqueous solutions, their fluorescence increases in low polar solvents or upon binding to hydrophobic pockets[11]. Self-assembling or aggregating small molecules tend to form higher order structures with multiple hydrophobic pockets. Therefore, the increase of their fluorescence intensity can be used to monitor the presence of molecular condensates in aqueous solutions. Since self-assemblies of small molecules often form weak intermolecular interactions such as hydrophobic interaction, π-π stacking, and hydrogen bonding, we selected relatively large-sized 8,000 compounds (molecular weights >500) enriched in hydrophobic and/or aromatic functional groups from our collection of ~70,000 compounds. We screened the 8,000 molecules for the compounds that increased fluorescence of both Nile Red and ANS (Figure 1A) to create a focused library of 116 self-assembling compounds (Figure 1B).

To confirm the result of the fluorescence profiling, we measured the hydrodynamic sizes of the self-assembly structures in a phosphate buffer solution by dynamic light scattering (DLS) experiments (Figure 1C). All of the 116 molecules form measurable particles with diameters larger than 100 nm, and 53% of them have diameters smaller than 1 μm. Fluorescent confocal microscopy imaging of the different particles revealed a variety of particle morphologies such as micelle, fibers, crystals, and colloid aggregates (Figure S1).

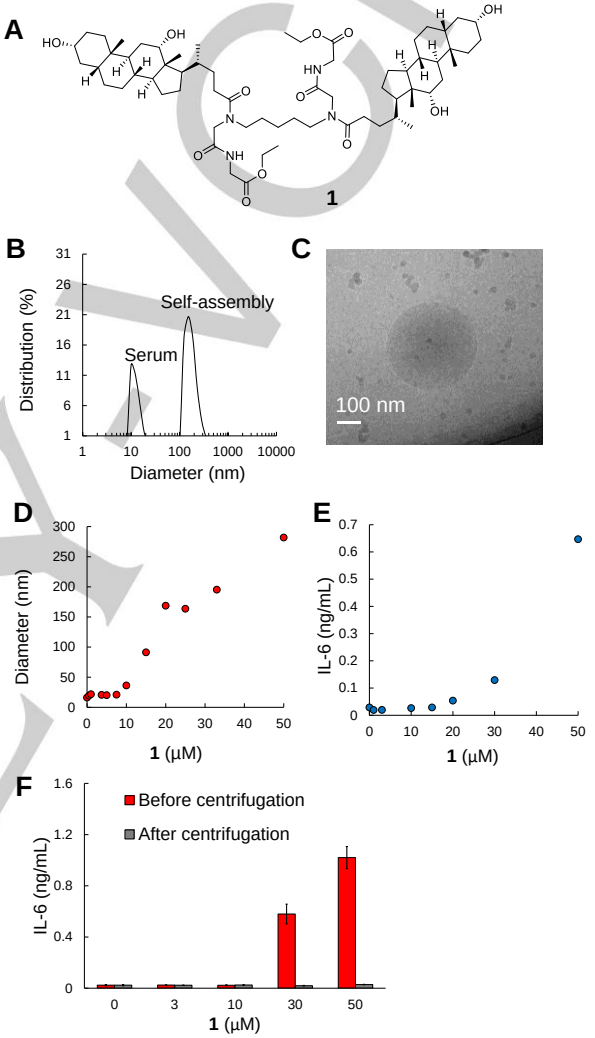

Fig. 2. Characterization of the nano-assembly of molecule **1**. (A) The chemical structure of molecule **1**. (B) Size distributions of molecule **1** (at 30 μM) in a serum-containing medium (DMEM containing 10% FBS and 1% penicillin-streptomycin) as measured by DLS (ELS-Z2). (C) A typical cryo-TEM image of **1** in DMEM. (D) Concentration-dependence of hydrodynamic diameters of the nano-assembly of **1** in DMEM. Results were recorded by DLS (ELS-Z2). (E) Concentration-dependent induction of the IL-6 by molecule **1** in RAW264.7 macrophages. The cells were treated with varied concentrations of molecule **1**, and IL-6 production was estimated by ELISA. (F) IL-6 induction in RAW264.7 cells by adding molecule **1** solution before or after centrifugation. The centrifugation eliminates the activity of molecule **1**. The IL-6 production was estimated by ELISA. Error bar is the standard deviation (n = 3).

Identification of molecule 1 as an immune-potentiating assembly

Among several effects of innate immune responses is the rapid burst of inflammatory cytokines and activation of antigen-presenting cells (APCs) such as macrophages and dendritic cells. These early responses shape the immune system for subsequent development of specific adaptive immune responses. We screened the 116 self-assembling molecules for their ability to elicit the expression and secretion of interleukin-6 (IL-6) in RAW264.7 macrophage cells. Enzyme-linked immuno-sorbent assay of interleukin-6 (IL-6 ELISA) identified molecule **1** as a weak but reproducible immuno-stimulatory compound. The IL-6-inducing activity of molecule **1** was also confirmed by mouse bone-marrow derived dendritic cells. Therefore, we focused our efforts on molecule **1** for further studies.

The chemical structure of molecule **1** (Figure 2A) was confirmed by re-synthesis through Ugi reaction (Scheme S1): it is comprised of two deoxycholate moieties and two glycine esters connected via a diamine linker. Dynamic light scattering (DLS) measurement of **1** in a culture medium at 30 μM revealed two distributions of particles at average sizes of ~10 nm and 198.6 nm (Figure 2B). Comparison with culture medium alone (with and without 10% FBS; Figure S2) indicated that the 198.6-nm particle distribution was derived from self-assemblies of **1** while the small 10-nm particle distribution corresponded to serum in the culture medium. Cryo-transmission electron microscopy (cryo-TEM) revealed spherical particles with sizes consistent with the hydrodynamic diameter as measured by DLS (Figure 2C). The shape and size are reminiscent of those of the typical viruses that are readily captured by immune cells.

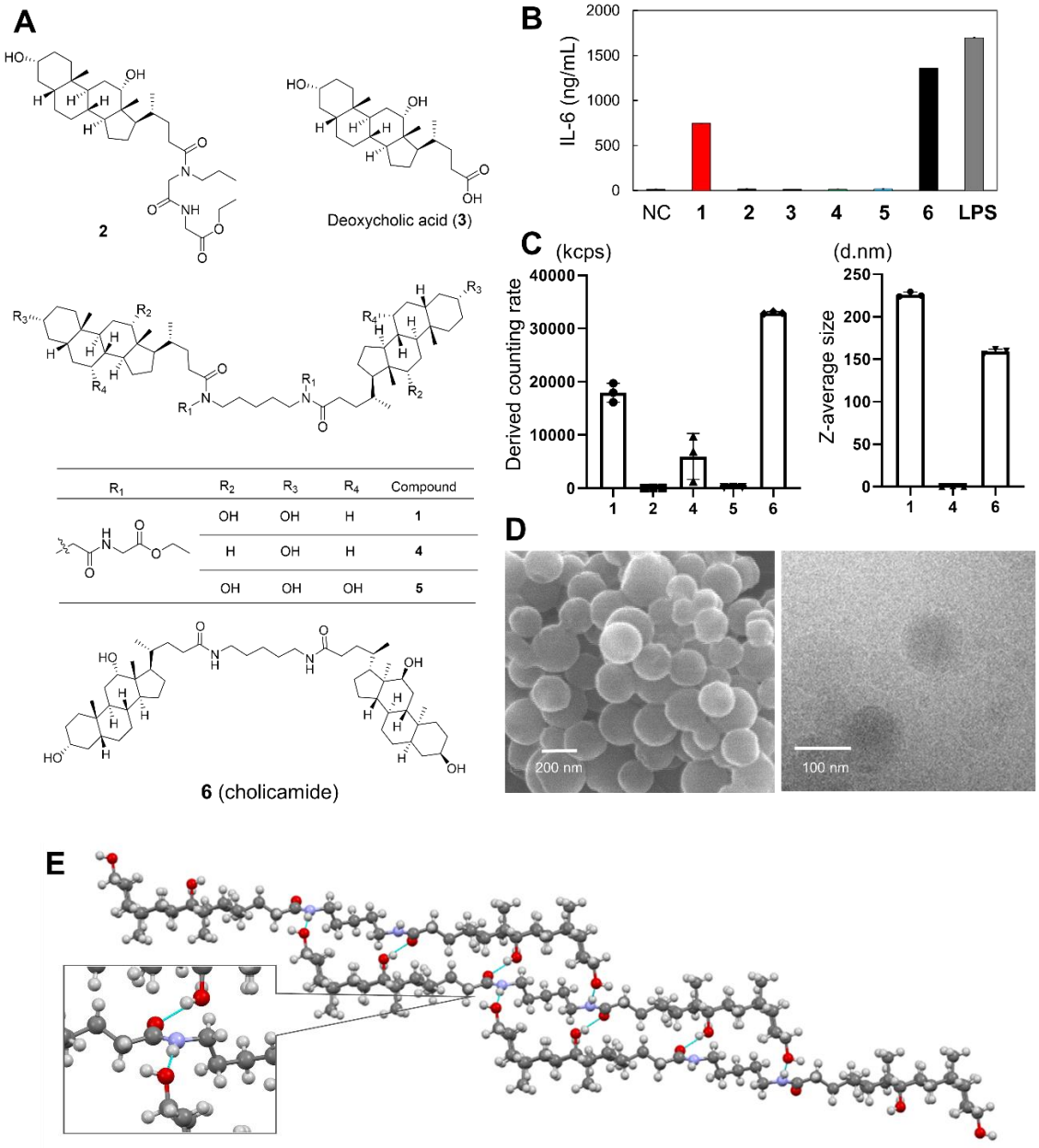

Fig. 3. Discovery of molecule **6** (cholicamide). (A) Chemical structures of molecules **1**-**6**. (B) Effects of molecules **1-6** on the IL-6 production of RAW246.7 macrophages. All compounds were used at 30 μM. LPS was used at 100 ng/mL. NC = no compound. Error bar is the standard deviation (n = 3). (C) Derived counting rate of molecules **1**-**6** and Z-average sizes of molecules **1**, **4** and **6** as measured by DLS (Zetasizer *Nano-s*). (D) FE-SEM (*left*) and cryo-TEM (*right*) images of molecule **6** particles. (E) Interaction between two molecules of **6** in the crystal structure. Water molecules are omitted for clarity. Molecule **6** forms eight intermolecular hydrogen bonds with neighboring molecules (blue-dashed lines).

Increasing concentration of **1** led to the formation of larger particles up to a diameter of 300 nm (Figure 2D) with concomitant higher IL-6 production activity both in protein and mRNA levels (Figure 2E and Figure S3). To confirm that the assembly of **1** is responsible for the IL-6 production in macrophages, we compared the activity of a solution of **1** (0–50 μM) before and after centrifugation at 14,000 rpm. Although the samples before centrifugation induced the IL-6 production, the centrifugal removal of the particles abolished the IL-6 production (Figure 2F), suggesting that molecule **1** exerts its activity through self-assembly.

Discovery of cholicamide (molecule 6) thru structure-activity relationship studies

We set out to obtain the structure-activity relationship of molecule **1** (Figure 3A). The truncated half-structure of **1** (molecule **2**) or the endogenous deoxycholic acid (molecule **3**) exhibited no detectable assembly as measured by DLS and failed to induce secretion of IL-6 from RAW264.7 cells (Figure 3BC), suggesting that the symmetrical structure of **1** is required both for the self-assembly and the immune-stimulatory activity. We then synthesized two closely related derivatives of **1** (molecule **4** and **5**) in which the numbers of hydroxy groups were changed. These derivatives had no detectable activity to elicit IL-6 production (Figure 3B) and little ability to form assemblies (Figure 3C). Molecule **5** displayed no detectable derived DLS counting rate, and although molecule **4** showed a small counting rate, its averaged size was negligible (Figure 3C). These results underscored the importance of hydroxy groups both in the self-assembly process and in the IL-6-inducing activity.

Remarkably, removal of the peptide segment of **1** (molecule **6**) substantially increased the IL-6-inducing activity, which is comparable to that of 10 ng/μL of LPS. This simple conjugate of deoxycholate and diamine maintained self-assembly property: it formed particles with the averaged diameter of 150 nm, which is consistent with the virus-like spherical particles observed in field-emission scanning electron microscopy (FESEM) and cryo-transmission electron microscope (cryo-TEM) analyses (Figure 3D). To obtain insights into how molecule **6** forms assemblies in solution, we determined the structure of molecule **6** in a crystalline form. The three-dimensional structure showed the side-by-side parallel packing of molecule **6** where four hydroxy groups of each molecule are located within hydrogen-bonding distance with the amide NH or CO groups of neighboring molecules (Figure 3E and Figure S4). In order to determine whether these hydrogen bonds are essential for initiating the assembly process, short-trajectory (~200 ns) molecular dynamics simulations of molecule **6** were performed. During these simulations, molecule **6** quickly condensed into a cluster stabilized mainly by the hydrogen bond with the CO group. The hydrogen bond with the NH group also emerged, but was weaker than that with CO (Figure S5). This result strongly suggests that the hydrogen bond between CO and hydroxy groups plays a major role in initiating the coacervation of molecule **6**, consistent with the importance of hydroxy groups both in the assembly and IL-6-inducing activity of molecule **6** (Figure 3BC).

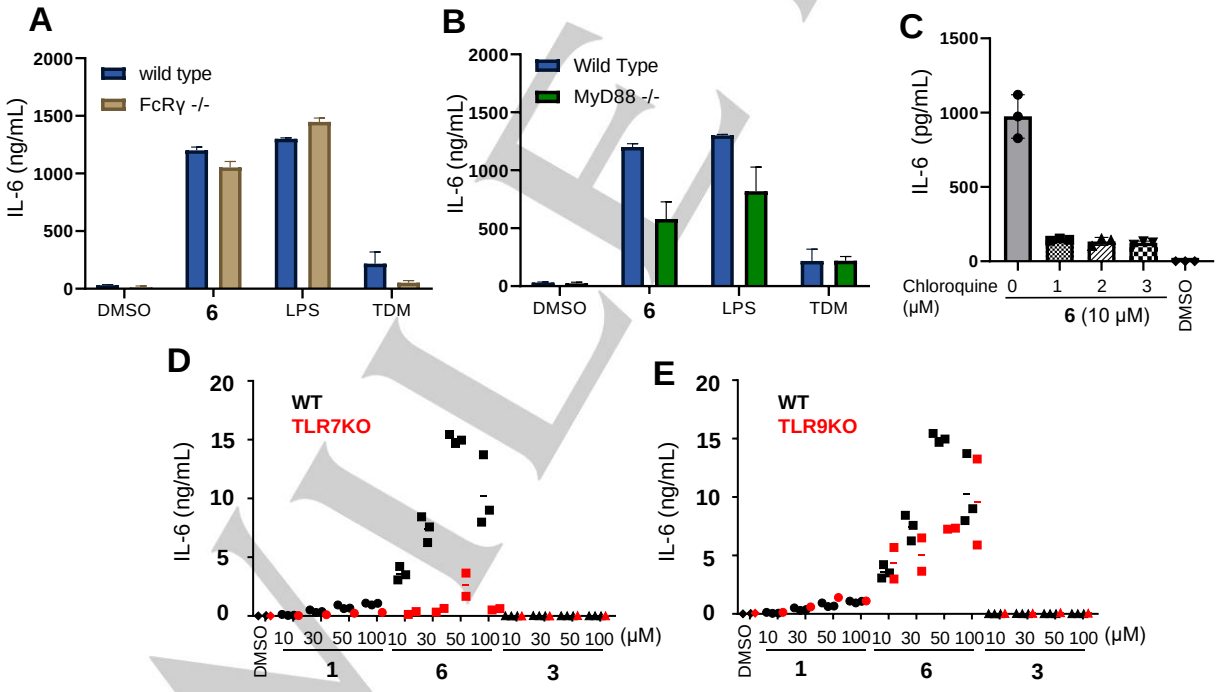

Fig. 4. Effects of mediator knockout on the activity of molecule **6** (cholicamide). (A) IL-6 production in wild type (WT) and FcRγ knockout (KO) bone-marrow-derived dendritic cells (BMDC). The cells were treated with molecule **6** (30 μM) for 24 h. TDM at 1 μg/well and LPS at 100 ng/mL were used as positive controls. (B) IL-6 production in WT and MyD88 KO BMDC. The cells were treated with molecule **6** (30 μM) for 24 h. TDM at 1 μg/well and LPS at 10 ng/mL were used as positive controls. Molecule **6** potently induced IL-6 production in WT BMDC, but displayed reduced IL-6 induction in Myd88 KO BMDC. All data points are mean ± SD. Error bar is the standard deviation (n = 3). (C) Induction of IL-6 by molecule **6** is chloroquine-sensitive. RAW264.7 macrophages were pre-incubated with 1, 2, or 3 μM chloroquine for 2 h and then treated with molecule **6** (10 μM) for 22 h. All data points are mean ± SD. Error bar is the standard deviation (n = 3). (D) IL-6 production in WT and mTLR7 BMDC. The cells were treated with molecule **1, 6** and **3** at four different concentrations for 24 h. The secreted amounts of IL-6 were quantified by ELISA. (E) IL-6 production in wild-type and mTLR9 KO BMDC. The cells were treated with molecule **1, 6** and **3** as described in (**A**). All data points are mean ± SD. Error bar is the standard deviation (n = 3).

Mechanism of action of cholicamide (molecule 6)

The simple structure and potent activity of molecule **6** inspired us to pursue extensive mechanistic studies. Given the particle size of the assembly of molecule **6**, we initially hypothesized that the molecule exerts its biological activity through interaction with membrane receptors. Two major classes of receptors are known to drive innate immune responses: C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). One of the essential downstream signal-proteins for CLRs is FcRγ, while the downstream signals of TLRs are mediated by myeloid differentiation primary response protein 88 (MyD88). To examine the involvement of CLRs or TLRs in the activity of **6**, we carried out ELISA for IL-6 using FcRγ/MyD88 knockout bone-marrow derived dendritic cells (Figure 4AB). LPS, a strong TLR4 agonist, maintained its ability to induce IL-6 in the FcRγ knockout cells, whereas the activity of TDM (trehalose dimycolate), an agonist of CLR, was compromised. By contrast, LPS displayed compromised IL-6 induction in the MyD88 knockout cells while TDM maintained its activity. Similar to LPS, the IL-6 induction of molecule **6** was compromised in the MyD88 knockout cells, whereas it was maintained in the FcRγ knockout cells (Figure 4AB). Taken together, these results indicate that the nano-assembly of molecule **6** exerts its immune stimulatory activity through a member of the TLR family.

TLRs are expressed in the distinct cellular compartments: TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular vesicles such as endosomes. To determine whether recognition of molecule **6** requires endocytosis and endosomal maturation, we treated RAW264.7 cells with the molecule in the presence of chloroquine, an inhibitor of endocytosis and endosomal maturation[12]. Chloroquine completely abrogated IL-6 induction by molecule **6** (Figure 4C). The inhibition did not appear to be due to drug toxicity or particle disassembly, because chloroquine had no measurable effects on macrophage viability (Figure S6A) and the self-assembly ability of molecule **6** (Figure S6B). The assemblies of molecule **6** appear to be stable under acidic pH conditions, which is analogous to late endosome environments (Figure S7). These results collectively suggest that endosomal TLR(s) recognize the molecule **6** assembly.

To identify the TLRs required for the activity of molecule **6**, we examined whether the molecule elicits IL-6 production in the mouse bone-marrow derived dendritic cells in which each of the two major endosomal Myd88-dependent TLRs (TLR7 and TLR9) was knocked out. The depletion of TLR7 in wild-type bone-marrow derived dendritic cells reduced the IL-6 induction of molecules **1** and **6** (Figure 4D), whereas the TLR9 depletion showed little effects (Figure 4E). These results are consistent with a primary role of TLR7 in mediating the induction of IL-6 by molecules **1** and **6**.

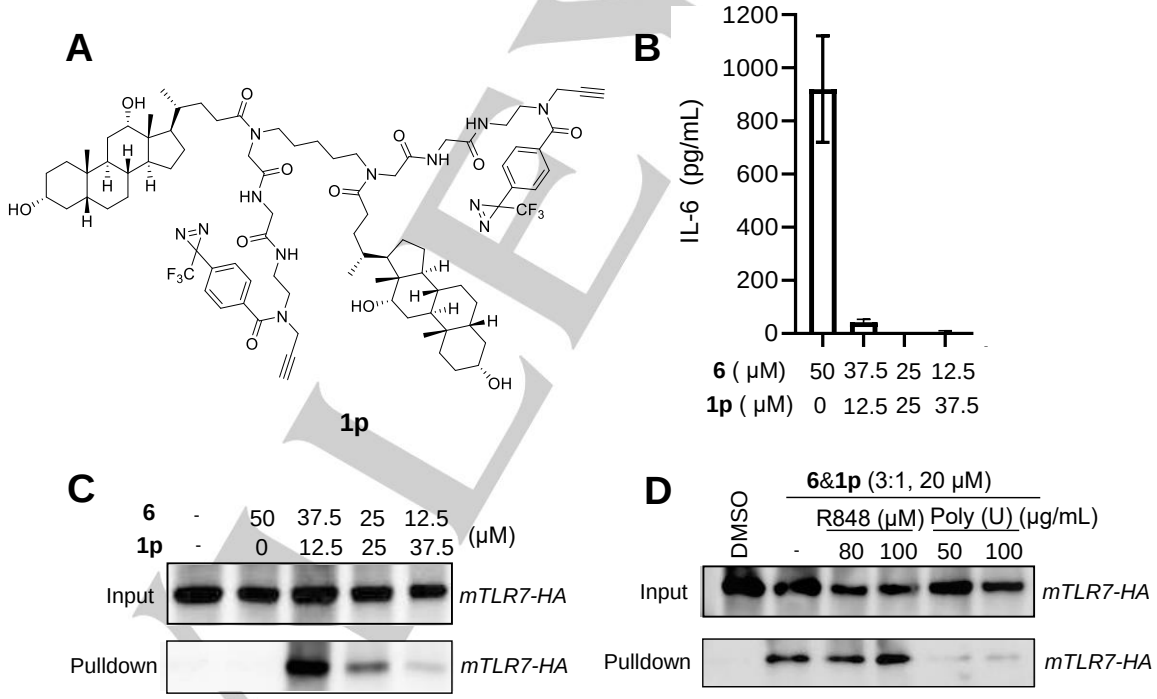

Fig. 5. Direct interaction between mTLR7 and a photo-affinity probe (**1p**). (A) Chemical structure of photoaffinity probe **1p**. (B) IL-6 production of RAW264.7 cells treated with mixtures of molecule **6** and **1p**. The cells were treated with varied ratios of molecule **6** and **1p**. The total concentration was fixed at 50 μM. The ratio of 3:1 displayed decreased but detectable induction of IL-6. All data points are mean ± SD. Error bar is the standard deviation (n = 3). (C) Western blot analysis of probe-mTLR7 interaction. HEK293 cells transfected with pUNO1-mTLR7-HA and treated with varied ratios of molecule **6** and **1p** under UV exposure. The photo-reacted mTLR7-HA is shown as "pulldown." (D) Competition experiments with two known TLR7 ligands, R848 (80 μM and 100 μM) and Poly (U) (50 μg/mL and 100 μg/mL). The transfected HEK293 cells were pretreated with each ligand for 2 h, followed by photo-reaction with the mixture of **1** and **6p** (3:1, 20 μM). The photo-reacted mTLR7-HA is shown as "pulldown."

We next examined the ability of molecules **1** and **6** to induce 23 representative immune cytokines in wild-type and TLR7-knockout bone-marrow derived dendritic cells (Figure S8). Bioplex analysis showed that molecule **6** stimulated the production of most of the cytokines tested in the wild-type cells and that the induction was mitigated in TLR7 knockout cells. The ability of molecule **6** to induce cytokines is more potent than that of molecule **1**. Note that, for a subset of cytokines including IL-6, molecule **6** displayed less potency at 100 μM than at 30 or 50 μM perhaps due to cytotoxicity at 100 μM.

TLR7 is a membrane-bound receptor expressed in endosomes that plays a role in stimulating both innate and adaptive immune signaling in response to single-stranded viral RNA[13]. After the attachment to the cell surface, viral particles are internalized into an endocytic compartment in which the low pH triggers a conformational change in hemagglutinin, allowing the exposure of viral RNA molecules to TLR7[12a]. To examine whether molecule **6** interacts directly with TLR7, we designed an alkyne-tagged photo-affinity probe (**1p**) (Figure 5A). We were guided in the design of **1p** by the finding that elimination of the peptide moiety in molecule **1** retains or even increases the IL-6-inducing activity. Thus, the peptide moiety of molecule **1** was replaced with an alkyne-containing short peptide. The resulting photo-affinity probe molecule (**1p**) retained its ability to self-assemble but failed to induce IL-6 in RAW264.7 cells. When **1p** was co-assembled with molecule **6** at a 3:1 ratio, the mixture elicited, although weakly, secretion of IL-6 from RAW264.7 cells (Figure 5B).

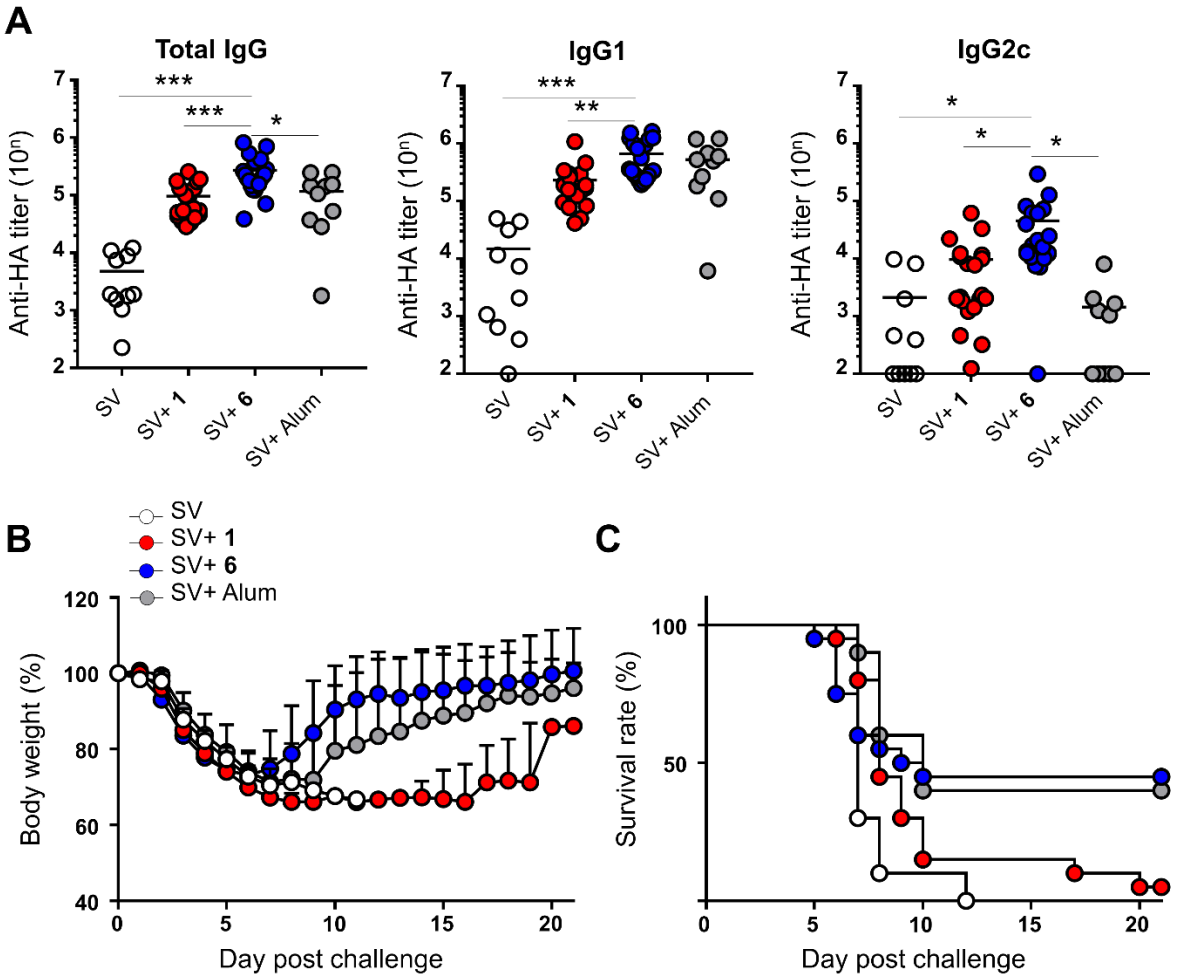

Fig. 6. Molecule **6** serves as a vaccine adjuvant. (A) Anti-HA IgG analysis. C57BL/6 mice were immunized twice (day 0 and day 14) with influenza split vaccine (SV; A/New Caledonis/20/1999 strain) simultaneously with molecules **1** (100 μg/head), **6** (100 μg/head), or Alum (500 μg/head). Three weeks after first immunization, blood of the mice was taken for analysis of IgG titer. (B-C) Body weights and survival rates of the mice challenged with PR8. After immunized with molecule **1**, **6**, or Alum for four weeks, the mice were challenged with PR8 ($30LD_{50}$). Their body weights and survival rates were monitored for three weeks. The body weights and survival rates of the mice treated with molecule **6** were higher than the other groups. (A-C) Each dot represents an individual mouse (n = 10 for SV and SV+Alum groups, and n = 20 for SV+**1** and SV+**6** groups) in two independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to molecule **6** (one-way ANOVA with Bonferroni's comparison test).

The co-assembled mixture above was added to the culture of HEK293 cells overexpressing HA-tagged TLR7, and the proteins that bind to the probe were purified through a click reaction and analyzed by Western blots with an HA antibody (Figure 5C). The results suggest that the probe molecule binds directly to TLR7. TLR7 is known to have two distinct agonist binding sites: one for single stranded RNA and the other for nucleosides. Excess amounts of Poly(U) decreased the interaction between the probe and TLR7, whereas R848, an agonist for the nucleoside binding site, failed to do so at a detectable level, suggesting that molecule **6** immune

stimulatory activities are achieved by interacting with the single stranded RNA-binding site of TLR7 (Figure 5D).

The selectivity of the probe was accessed for other TLRs. The co-assembled mixture of molecule **6** and the probe (**1p**) exhibited photo-reaction with TLR9, another endosomal Myd88-dependent TLR; however, the reaction appears weaker than that with TLR7 and was not competed with excess amounts of ODN1585, an oligonucleotide agonist of TLR9 (Figure S9). Given that TLR9 is dispensable for the activity of molecule **6**, the detected binding may not be productive enough to drive the activation of the receptor. Alternatively, the endosomal localization of the probe co-assembly led to the non-specific labeling of TLR9. Indeed, similar photo-affinity experiments with two cell surface TLRs, TLR2 and TLR4, displayed no detectable photo-reaction with the co-assembled mixture of the probe (Figure S9).

Cholicamide (molecule 6) as a potent vaccine adjuvant in mice

To validate the efficacy of molecule **6** as a vaccine adjuvant, we immunized mice twice (day 0 and day 14) with influenza split vaccine (SV; A/New Caledonis/20/1999 strain) simultaneously with molecules **1** or **6** (100 μg/head). The HA-specific IgG antibody titer was significantly higher in the mice injected with SV and molecule **6** than in those immunized with SV alone (Figure 6A). Immunization with SV and molecule **6** increased the titers of the HA-specific IgG1 as much as with SV immunization with Alum, a clinically used vaccine adjuvant. Importantly, HA-specific IgG2c production, an indicator of type-1 immune responses, was induced by molecule **6** more potently than by Alum.

To examine the protective efficacy, mice were vaccinated with the influenza vaccine antigen and molecules **1** or **6**, allowed to establish a vaccine response for 2 weeks, and then challenged with a lethal dose ($30LD_{50}$) of the PR8 strain of influenza A virus (H1N1). All the mice vaccinated only with the antigen lost weight and died (Figure 6BC). Mice vaccine-immunized together with molecule **1** were partially protected, exhibiting a survival rate of 5%. Vaccination together with molecule **6** exhibited protection with a survival rate of 45%, which is comparable with that of Alum (Figure 6C). These mice also showed relatively quick recovery of body weight from day 7 after infection as observed with the mice treated with Alum. We observed no detectable *in vivo* toxicity of molecules **1** and **6** (100 μg/head) as measured by the activities of two toxicity marker enzymes in serum, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Figure S10). The results demonstrate that the molecule **6** can act as a potent adjuvant for the influenza vaccine in mice.

Discussion

Many of TLRs mainly detect bacterial products that are unique to bacteria. In contrast, TLRs 3, 7, 8 and 9 detect viruses by recognizing nucleic acids and free nucleosides, which are not unique to viruses. Safeguard mechanisms must be instituted to maximize the distinction between self and non-self nucleic acids. One such mechanism is for TLRs to bind RNA or DNA ligands within endosomes and thereby evade recognition in the extracellular space.

Our study demonstrates that the molecule **6** nano-assembly is engulfed by APCs and recognized within endosomes primarily by TLR7, an endosomal TLR that detects single-stranded viral RNA. However, unlike viral particles, the condensate of molecule **6** does not contain RNA or nucleosides, raising an interesting question of how TLR7 mediates the signal of molecule **6**. Our results suggest that the molecule **6** assembly occupies the RNA-binding pocket of TLR7 to stimulate immune response. How the deoxycholate assembly mimics or competes with single-stranded RNA remains unknown. Determining the co-crystal X-ray structure of TLR7 with bound molecule **6** would be of particular interest in understanding the biochemical underpinnings of the interaction.

The use of TLR7 potentiators is a promising approach to improving the immunogenicity of a variety of antigens exploited in vaccine strategies. Imidazoquinolines, including R848, are particularly noteworthy because they are the first small, drug-like compounds shown to be TLR agonists. The imidazoquinolines stimulate cytokine production from immune cells by binding to the nucleoside binding site of TLR7 and have been documented as vaccine adjuvants and immunotherapeutic agents for cancer and infectious diseases[14]. The present study led to the discovery of another small-molecule activator of TLR7. Unlike the imidazoquinolines, molecule **6** is a non-nucleoside deoxycholate derivative that self-assembles to interact with the RNA-binding site of TLR7. Molecule **6** (cholicamide) is a simple, chemically tractable compound that enables cost-effective production and serves as a starting point for developing a new class of non-nucleoside vaccine adjuvants. Moreover, molecule **6** is a conjugate of two molecules of deoxycholic acid, a highly abundant secondary bile acid that is produced in the liver and circulates in the gut. The unique structure of molecule **6** as a TLR7 agonist suggests the existence of hitherto unidentified endogenous bile acid metabolites as TLR7 modulators.

Self-assembly or aggregation of small molecules notoriously leads to promiscuous inhibition in high-throughput and virtual screening, causing a major source of false positive. A number of small molecules, including bioactive molecules and approved drugs, are also known to form colloidal aggregates in aqueous solutions at micromolar concentrations thereby reducing their biological activities[15]. These aggregation propensities of bioactive molecules have long been considered as undesirable properties that need to be eliminated during lead optimization. On another front, a new class of non-peptidic assembling compounds has been emerging as biologically active small molecules, postulating that such molecules are capable of modulating selected biological processes including cell death[16], cell survival[17], and organelle functions[18]. Our study suggests that expansion of self-assembling compound libraries might provide a robust source of synthetic small molecules that mimic or modulate biological assemblies including viruses, nucleic acids, and protein condensates.

In conclusion, the present study represents, to the best of our knowledge, the first report of constructing a self-assembling small molecule library for biological purposes and, importantly, the first demonstration of its use for vaccine adjuvant discovery. It ushers in a new and timely approach for the advancement of small molecule-dependent adjuvants against emerging viruses.

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Keywords: Chemical biology, Self-assembly, Toll-like receptor

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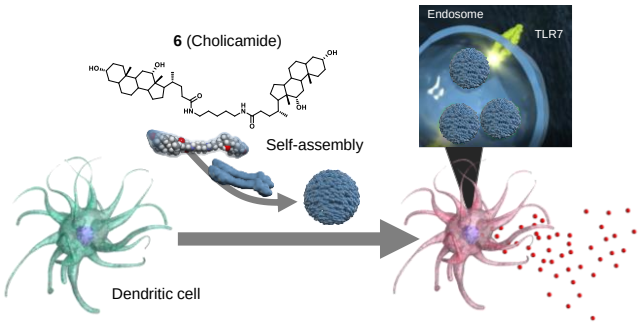

Vaccine adjuvants are required for the generation of robust and long-lasting immune responses of antigen vaccines. Screening of a newly constructed self-assembling chemical library led to the rapid discovery of molecule **6**, also named cholicamide, as a potent vaccine adjuvant. Just like viruses, the nano-assembly of cholicamide is engulfed inside cells and recognized by an endosomal toll-like receptor to elicit potent innate immune responses.

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