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Development of TLR7 Ligand-Glycan-Immobilized Gold Nanoparticles as Novel Adjuvants

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Abstract

Adjuvants enhance the immune system during vaccination. Among FDA-approved adjuvants, aluminum salts are most commonly used for vaccines. Although aluminum salts enhance antibody production, they show a limited effect on the cell-mediated immune response. Thus, further development of adjuvants inducing T cell-mediated immune responses is significant. Toll-like receptors (TLRs) are immune-related receptors that recognize specific pathogen-associated molecular patterns. They play important roles in the activation of innate immunity, which is crucial to shaping adaptive immunity. Therefore, studies using TLR ligands as novel adjuvants for anti-microbial and anti-cancer immunotherapies have attracted much attention. Among them, a low molecular weight TLR7 ligand, Imiquimod, has been approved for clinical use, but its use is restricted for local administration due to unwanted adverse effects. Since TLR7 is mainly located in the endosomal compartment of immune cells, efficient transport of the ligand into the cell is important for activating TLR7. Our previous work indicated that the conjugation of a low molecular weight TLR7 ligand with serum albumin and polysaccharides can greatly enhance its potency. In this study, we examined gold nanoparticles (GNPs) as carriers, since GNPs are less toxic and can immobilize multiple molecules including antigens for pathogens and tumors. Furthermore, α-mannose for targeting antigen-presenting cells was also examined for the efficient delivery of GNPs. In this paper, we describe the preparation of a low molecular weight TLR7 ligand and α-mannose immobilized GNPs, and its *in vitro and in vivo* immunostimulatory activities.

Keywords

Adjuvant
Toll-like receptor
Gold nanoparticles
C-type lectin
Innate immunity

1. Introduction

Adjuvant is a generic term for substances (immunostimulants) that are administered with vaccine antigens to enhance their efficacy. In many cases, administration of antigen molecules alone is not sufficient to induce acquired immunity by a vaccine, and adjuvants are often used to enhance the immunogenicity of the vaccine. In recent years, adjuvants have also been used in immunotherapy to treat diseases such as cancer by activating the immune system, thus expanding its range of applications. On the other hand, aluminum salts, which are most clinically used as adjuvants, enhance humoral immunity but have a low induction capacity for cellular immunity, so their immunostimulatory activity is not high enough. Therefore, the development of new effective adjuvants is required, and ligands for Toll-like receptors (TLRs), which activate innate immunity, are attracting attention.

TLRs are a type of transmembrane pattern-recognition receptor that recognizes pathogen-associated molecular patterns (PAMPs) specific to pathogens such as viruses and bacteria. Ten types of TLRs have been identified in humans and act as sensors to detect pathogen infection. When PAMPs bind to TLRs and initiate signaling, inflammatory cytokines and type I interferon are produced, and innate immunity is activated. Subsequently, the induction of acquired immunity is promoted and TLRs play an important role in host defense mechanisms.

To date, several TLR ligands have been approved for clinical use. For example, the TLR4 ligand monophosphoryl lipid A is used as a mixed adjuvant adsorbed on aluminum salts for cervical cancer. Imiquimod, a synthetic small-molecule ligand for TLR7, is used as a therapeutic agent for condyloma acuminatum and cutaneous malignant melanoma. Various other TLR ligands have been clinically investigated and are expected to be used as adjuvants in vaccines and immunotherapy against infectious diseases and cancer [1,2]. In this article, we focus on TLR7 ligands and introduce recent studies.

2. TLR7 ligand

TLR7 localizes to intracellular endosomes and selectively recognizes virus-specific single-stranded RNA. It also recognizes low-molecular-weight compounds with imidazoquinoline-like and purine-like skeletons [3]. The binding of ligand molecules to TLR7 promotes the production of type I interferon, which may be useful for the development of antiviral and anticancer drugs. Clinical studies using adjuvants for antiviral drugs and cancer immunotherapy are being conducted, as the binding of ligand molecules to TLR7 promotes the production of type I interferon. On the other hand, most synthetic small-molecule TLR7 ligands are rapidly diffused in the bloodstream when administered systemically, and thus risk causing serious side effects such as cytokine release syndrome. As TLR7 is mainly localized to endosomes in immune cells, efficient transport of the ligand molecule into immune cells by endocytosis is required to increase the potency of TLR7 ligands. Studies have therefore been conducted to improve the pharmacokinetics and immunostimulatory activity of synthetic small-molecule TLR7 ligands by complexing them with macromolecules such as proteins, polysaccharides, polymers, and nanoparticles [4]. We have previously developed a small molecule TLR7 ligand (1V209) with a purine-like skeleton and reported a 10- to 103-fold increase in immunostimulatory activity *in vitro* and *in vivo* when complexed with serum albumin or dextran [5,6].

3. TLR7 ligand-glycan-immobilized gold nanoparticles

We recently developed a novel adjuvant using gold nanoparticles (GNPs) as carrier molecules for synthetic small molecule TLR7 ligands [7]. GNPs are chemically stable nanoparticles with a diameter of a few to several hundred nm with characteristic optical properties. GNPs surfaces can easily immobilize biomolecules with thiol functional groups, and research into their application in biosensors and diagnostics is being developed [8].

Applications in the medical field, such as bioimaging, photodynamic therapy [9], and drug delivery [10] are also being investigated and are considered safe for living organisms [11]. Furthermore, we have used sugar chains as molecules to selectively transport GNPs to immune cells. The surface layer of antigen-presenting cells such as dendritic cells and macrophages, expresses mannose receptors and C-type lectin receptors such as macrophage galactose lectin [12] to capture foreign substances. Therefore, by immobilizing sugar chains that bind to these receptors on the surface of GNPs together with TLR7 ligands, the TLR7 ligands are immobilized on the surface of GNPs, which is thought to enable efficient intracellular transport of TLR7 ligands to antigen-presenting cells. In this paper, we present the preparation of GNPs co-immobilized with synthetic small molecule TLR7 ligands and α-mannose (αMan), a sugar chain component, and their immunostimulatory effects *in vitro* and *in vivo*.

3.1. Preparation of 1V209-αMan-GNPs

1V209 was used as the synthetic small molecule TLR7 ligand [5]. To immobilize 1V209 on the surface of GNPs, 4,7,10-trioxa-1,13-tridecanediamine (TTDDA) was modified with thioctic acid to synthesize the 1V209 derivative (1V209-TTDDA-TA). The synthesis of 1V209-αMan-GNP was based on a previous report [13]. The 1V209 derivative and the ligand complex with α-mannose (Manα1-6Glc-mono) were mixed to a molar ratio of 1:9 and co-immobilized onto the GNP surface, as shown in **Figure 1**. The resulting 1V209-αMan-GNPs were purified by dialysis (Spectra/Por® 3, fractional molecular weight: 3,500). 1V209-TTDDA-TA and Manα1-6Glc-mono immobilized on 1V209-αMan-GNP were quantified and found to be immobilized in a 1:19 molar ratio. 1V209-αMan-GNP in aqueous solution was determined by dynamic light scattering (DLS) and the mean particle size was 9.4 ± 2.1 nm. This is similar to the particle size measured by transmission electron microscopy (TEM) (mean particle size 4.4 ± 1.3 nm), suggesting that they are dispersed as a single particle in an aqueous solution.

3.2. Immunopotentiating activity of 1V209-αMan-GNP *in vitro*

The *in vitro* immunopotentiating activity of 1V209-αMan-GNP was investigated using mouse bone marrow-derived dendritic cells (BMDC), mouse macrophage cell line J774A.1 cells, and human peripheral blood mononuclear cells (PBMCs). After 18 hours of culture in the presence of 1V209-αMan-GNP, cytokines produced in the culture supernatant were quantified by ELISA to assess cytokine production capacity.

When the production capacity of interleukin-6 (IL-6) was evaluated using mouse BMDCs, a concentration-dependent IL-6 production of 1V209 derivatives immobilized on GNPs was observed. Since IL-6 was not produced in αMan-GNPs without an immobilized 1V209 derivative, it was concluded that IL-6 production was induced by the 1V209 derivative immobilized on GNPs, as shown in **Figure 2A**. The cytotoxicity of 1V209-αMan-GNP was also assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay, no concentration-dependent cell death of the 1V209 derivative was observed as displayed in **Figure 2B**, suggesting that it is a low cytotoxic adjuvant.

Next, the ability to produce IL-6 was assessed using J774A.1 cells. The results in **Figure 2C** showed that the production capacity of IL-6 was significantly reduced compared to the 1V209 derivative. This may be because undifferentiated J774A.1 cells express very low levels of the mannose receptor [14], thus mannose receptor-mediated uptake of 1V209-αMan-GNP does not occur and TLR7 cannot be activated. Therefore, it is considered important that 1V209-αMan-GNP is transferred into the cell by mannose receptor-mediated endocytosis for TLR7 activation. The ability to produce tumor necrosis factor-α (TNF-α) in human

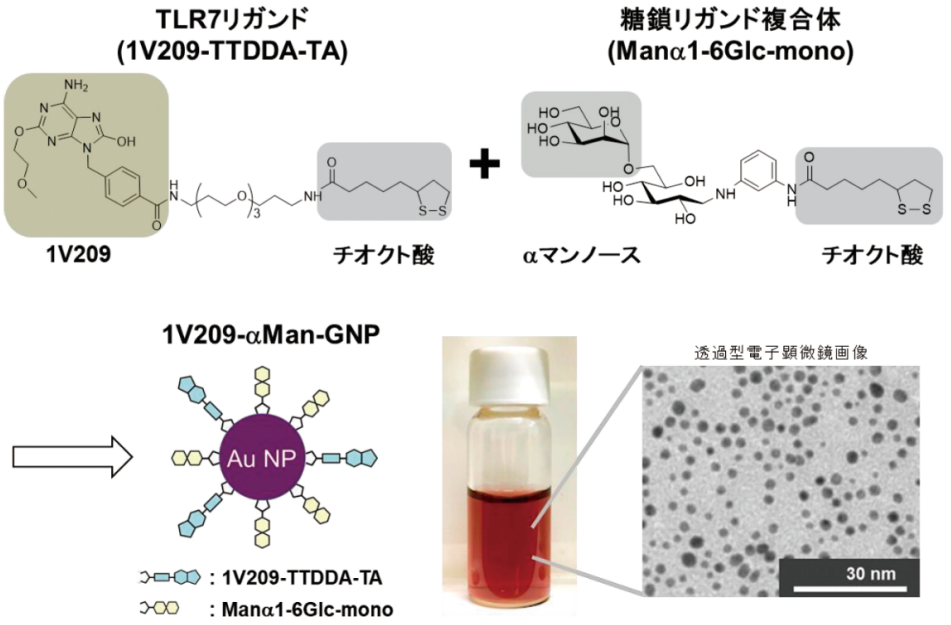

Figure 1. Preparation of 1V209-αMan-GNP

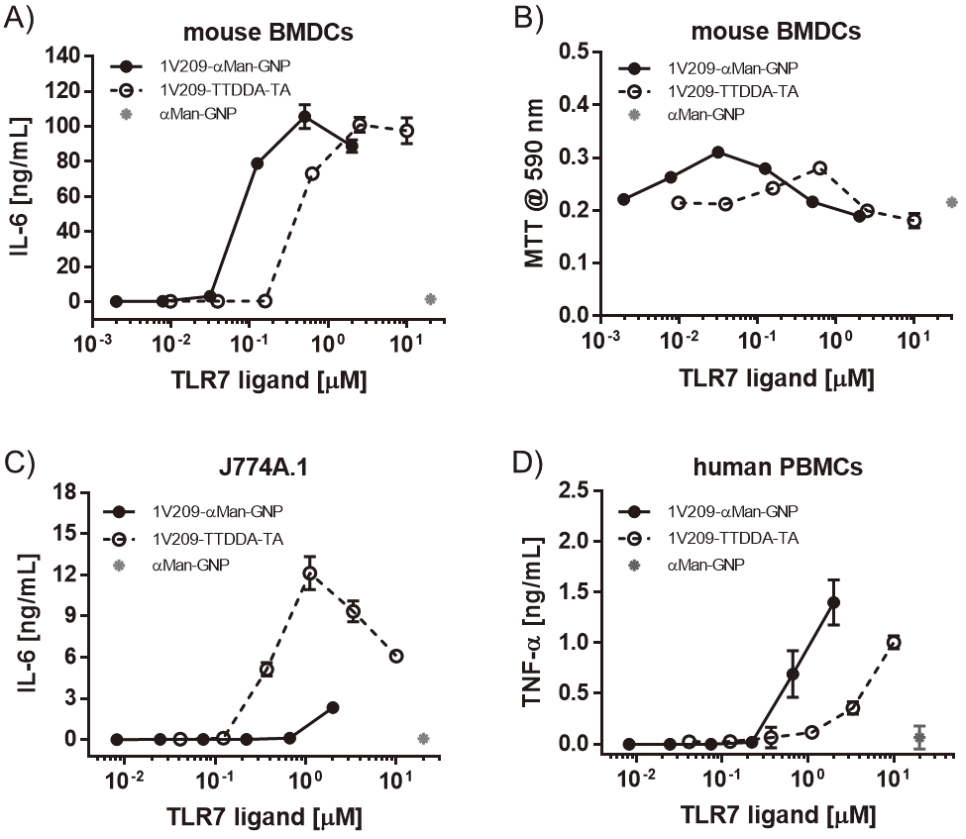

Figure 2. Immunopotentiating activity and cytotoxicity of 1V209-αMan-GNP *in vitro*. After 18 hours of incubation in the presence of 1V209-αMan-GNP or 1V209-TTDDA-TA, cytokines produced in the culture supernatant were quantified. (A) IL-6 production from mouse BMDC; (B) MTT assay of mouse BMDC; (C) IL-6 production from J774A.1 cells; (D) TNF-α produced from human PBMCs. All results show the means and standard deviations of three independent experiments.

PBMCs was subsequently evaluated. The results in **Figure 2D** showed that TNF-α production was induced at lower concentrations compared to the 1V209 derivative. Therefore, it was suggested that 1V209-αMan-GNP has a high cytokine-producing capacity in human cells.

3.3. *In vivo* adjuvant activity of 1V209-αMan-GNPs

The *in vivo* immunopotentiating activity of 1V209-αMan-GNP was evaluated using C57BL/6 mice. The mice were intradermally injected with a model protein antigen, ovalbumin (OVA), and the adjuvant 1V209-αMan-GNP in the tail on days 0 and 14. On day 38 of immunization, blood was collected from the orbital venous plexus, and the production titers of IgG1 and IgG2c antibodies to OVA in the blood were evaluated. Based on **Figure 3**, the results showed that the production titer of IgG2c antibodies was 103 times higher than that of 1V209 alone, suggesting a high ability to induce cellular immunity. This result was similar to that of the previously developed complex of 1V209 and dextran (1V209-dextran) [6]. However, when comparing the ratio of spleen weight to body weight of mice on day 38 of immunization, the spleen of 1V209-dextran-treated mice was enlarged, whereas that of 1V209-αMan-GNP remained almost unchanged. These results indicate that 1V209-αMan-GNP is an effective adjuvant that does not induce excessive immune responses such as B cell proliferation.

4. Conclusion

In this paper, we presented our recent studies focusing on TLR7 ligands. GNPs, used as carriers for synthetic

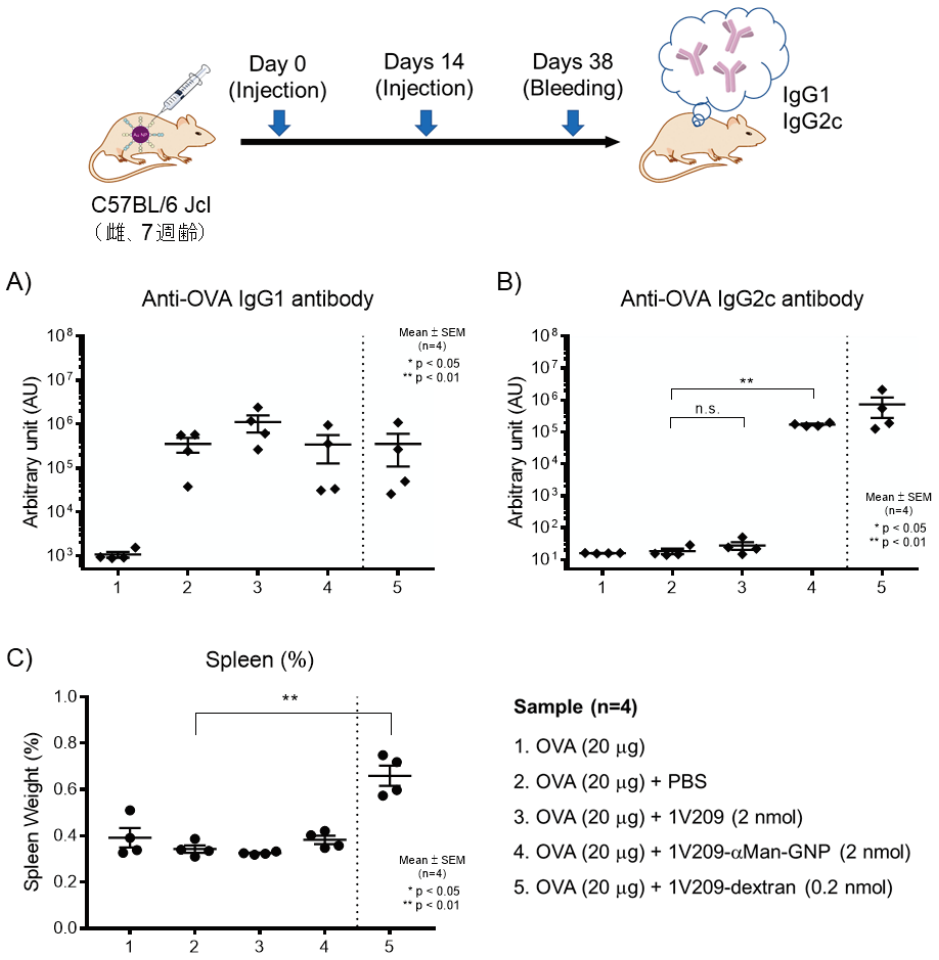

Figure 3. *In vivo* adjuvant activity and toxicity of 1V209-αMan-GNP. OVA (20 μg) and 1V209-αMan-GNP (2 nmol), 1V209 (2 nmol), or 1V209-dextran (0.2 nmol) administered intradermally into the tail of C57BL/6 mice, 38 days later. (A) The production titer of the anti-OVA IgG1 antibody; (B) the production titer of the anti-OVA IgG2c antibody; (C) the percentage of the spleen to mouse weight.

small molecule TLR7 ligands, are characterized by their ability to easily immobilize a variety of molecules containing thiol groups. As it has been reported that modification of antigen molecules and adjuvant compounds to a single molecule improves vaccine efficacy, GNPs, which can co-immobilize not only TLR ligands but also a variety of molecules, are expected to be a powerful molecular platform for the development of vaccines against a variety of diseases. In addition, 1V209-αMan-GNPs have high immunopotentiating activity, which may lead to various applications, such as their use as adjuvants for immunotherapy.

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Disclosure statement

The authors declare no conflict of interest.

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