Pathogen-like Nanoassemblies of Covalently Linked TLR Agonists Enhance CD8 and NK Cell-Mediated Antitumor Immunity

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ABSTRACT: Therapies based on Toll Like Receptor agonists (TLRa) are emerging as a promising modality for cancer immunotherapy to recruit antitumor T-cells in unresponsive immunologically "cold" tumors. Often, combinations of agonists are employed to synergistically enhance efficacy. However, low efficacy and severe toxicities deter these TLR-based therapeutics from further clinical applications. Studies have suggested that the rapid systemic diffusion of agonists to nontarget tissues is the primary cause. To address this challenge, we developed supramolecular nanotherapeutics of covalently linked TLRs for multivalent, synergistic interactions by drawing inspiration from immune recognition of pathogens. This new nanotherapeutic increased stimulation of key pro-inflammatory cytokines and remarkably enhanced CD8 and NK cell-mediated antitumor response while exhibiting ultralow off-target toxicity in an aggressive B16.F10 tumor model. Results from our studies thereby indicate that such supramolecular immune-agonist therapeutics may be further developed as a viable treatment modality for cancer immunotherapy.

INTRODUCTION

Toll-Like Receptors (TLR) are a family of pattern recognition receptors present on innate immune cells that detect danger-associated molecular patterns.1 The ability of TLRs to recognize pathogens and tumors has been employed for disease treatments.2 Targeting TLRs by agonists (known as toll like receptor agonist (TLRa)) to stimulate innate immune cells have demonstrated promise for cancer immunotherapy.3 More recently, synergistic combinations of TLRa have been investigated as a promising cancer therapeutic to generate an amplified immune response by costimulation.4,5 Among the various TLR receptors, activation of TLR 2/6 and TLR 7/8 has been reported to induce the production of CD8+ T cells and NK cells, which are key players in antitumor immunity.6−8 Synergistic coactivation of these receptors has been further demonstrated to induce highly amplified immune stimulation,9,10 making them an ideal combination for the advancement of immunotherapy. However, the translation of prominent synergistic effects observed in vitro with combinations of TLRa to in vivo applications can often be challenging due to rapid systemic diffusion of agonists, leading to low efficacy and unacceptable levels of off-target toxicity.11

In this context, Klinman and co-workers noted that synergistic coactivation can only be realized in vivo if all the agonists can persist at clinically relevant concentrations for at least 24 h post administration.12 Thus, it is essential to consider molecular design constraints to restrict diffusion for generating local coactivation when employing a combination of agonists. In this context, covalently linking synergistic agonists together to generate agonist heterodimers can be a promising strategy to increase stimulation by cellular coactivation.13 Unfortunately, such molecules may still diffuse to the bloodstream because of low molecular weights that generate low efficacy and create off-target toxicity.13

In light of this, molecular design of covalently linked heterodimer molecules to generate nanostructures via self-assembly may provide a good solution via improved pharmacokinetics.14,15 Additionally, at a molecular level, agonist−receptor interactions often rely on multivalency to achieve enhanced binding. For example, in pathogen recognition, multiple copies of ligands are recognized, inducing an amplified synergistic response.16,17 Thereby, often in immunotherapeutic design, it is imperative to have multivalent synergistic interactions. Thus, multicomponent molecular assembly of amphiphilic heterodimer agonists could be used as a tool to harness localized amplified multivalent response via formation of supramolecular entities.17 We would hereby like to engineer this structure with a covalently linked heterodimer composed of a cell-surface-active peptide-based TLR 2/6a and an endosomal active small-molecule TLR 7/8a with the TLR...
2/6a largely exposed on the surface for immune recognition. Unfortunately, such small-peptide amphiphiles have limited capacity to generate stable structures via controlled hierarchical assembly. 

In this context, Stupp and co-workers demonstrated that interactions between a carbohydrate polymer and small-peptide amphiphiles can trigger coassembly and direct the formation of hierarchical structures. Thus, given the unique nature of the TLRα heterodimer amphiphile, we designed an amphiphilic carbohydrate polymer because of the flexibility it offered in controlling coassembly via stabilizing noncovalent interactions. We envisioned that such interactions can be harnessed to yield supramolecular nanotherapeutics having a multivalent display of synergistic TLRα enabling enhanced antitumor efficacy while reducing systemic off-target toxicity.

### RESULTS AND DISCUSSION

**Multicomponent TLR Agonist Assembly.** With the goal of generating a supramolecular TLRα assembly, we designed a TLRα heterodimer amphiphile 5 and a carbohydrate amphiphile 12. The heterodimer 5 was synthesized by linking TLR2/6a and TLR7/8a via flexible linkers, that is, (PEO)₄ and (CH₂)₅ (Scheme 1, see SI for synthesis). The heterodimer agonist was hereby referred to as 2/6_7a. We next designed a carbohydrate amphiphile capable of interacting with the TLR heterodimer amphiphile to form a supramolecular structure. For this, we employed a nonimmunogenic sugar poly-(orthoester) scaffold (SPOE) which provided an amphiphile (OL-DSPOE, Scheme 1B) uniquely compatible with the TLR2/6_7a assembly properties. The synthesis of OL-DSPOE 12 was performed by grafting oleyl moieties through “click” conjugation with SPOE followed by selective deprotection of acetates (Scheme 1, see SI for synthesis and characterization).

With these amphiphiles in hand, we next individually tested their self-assembly behavior. The amphiphilic nature of the 2/6_7a led to generation of nonuniform random coiled aggregates upon transitioning from DMSO to PBS (Figure 1A). Such aggregate formation can be possibly attributed to a relatively short hydrophilic segment unable to shield the hydrophobic segments from water molecules. In compar-
ison, the carbohydrate amphiphile OL-DSPOE (m:n = 1:5, Scheme 1) gave well-defined nanomicelles (TEM 10.6 ± 1.7 nm, Figure 1B). This small nanomicelle formation is possibly due to a relatively large hydrophilic segment of the carbohydrate amphiphile.\(^\text{19}\) We thereby attempted the formation of multicomponent assembly using various molar ratios of OL-DSPOE to 2/6\(_7\)a. It was observed that a gradual increase in the molar ratio of OL-DSPOE to enhance hydrophilicity of the blend assisted in the formation of more organized structures (see SI, Figure 12S). To our delight, well-defined discrete particles were obtained at an optimized molar ratio (0.5:1) of OL-DSPOE to 2/6\(_7\)a (Figure 1C, SI for experimental details), generating noncovalent stabilization and forming stable structures. TEM analysis indicated that the majority of particles thus obtained ranged in length from 50−75 nm, and their widths varied from 30 to 45 nm (Figure 1D,E). Multiangle light scattering (MALS) measurements indicated a rms radius of 69.4 ± 1.5 nm for the multicomponent particles (see SI, Figure 13S). This distribution falls within the range of particles that drain to tumor-sentinel lymph nodes (20−200 nm).\(^\text{14,22}\) UV−vis analysis of multiple batches of particles indicated a molar ratio of (0.43 ± 0.09):1 of OL-DSPOE to 2/6\(_7\)a in the supramolecular entity. Notably, the particles were very stable, and no significant structural changes were observed when stored at 4 °C in PBS for at least 8 weeks (Figure 1D,E). To simplify the language, we will hereby refer to this material as multicomponent TLRa assembly (MTA).

MTA Enhances Immune Activation in Vitro. With MTA in hand, we then investigated its ability to elicit immune responses. Initial in vitro studies were performed to examine stimulation of transcription factor NF-κB activity with RAW
Blue macrophages to obtain a broad measure of immunogenicity. For comparison, parallel studies were performed by incubating equivalent amounts of unlinked agonist mixture (referred as 2/6a + 7a) and the linked 2/6_7a in PBS. Our studies indicated MTA induced significantly enhanced levels of NF-κB activity compared to 2/6_7a or the unlinked agonist mixture (Figure 2A). Encouraged by these results, we further analyzed the secretion of a series of pro-inflammatory cytokines following activation of bone-marrow derived dendritic cells. It was observed that, compared with other agonist-treated groups, MTA induced much higher secretion of two key cytokines: IFN-β (a type-I interferon) and IL-12 (Figure 2B,C; for other cytokines, see SI Figure 15S), which plays a major role in cancer immunosurveillance and promote CD8 T-cell and natural killer (NK) cell mediated immunity.23,24 The enhancement in activity for MTA verified our hypothesis that multivalent concentrated display of synergistic agonists in the multicomponent assembly significantly influenced innate immune responses. Remarkably, we did not observe a significant decrease in immunological activity of MTA particles over 8 weeks when stored at 4 °C (SI, Figure 14S), indicating that MTA was functionally stable.
MTA Enhances Antitumor Efficacy. With the exciting in vitro analysis, we were motivated to evaluate the efficacy of MTA in a B16.F10 tumor challenge model. This model has been widely studied as a poorly immunogenic and highly aggressive murine model of human melanoma.25 B16.F10 tumors are resistant to various immune therapies and have demonstrated poor efficacy compared with other traditional tumor models on treatment with immune agonists.26−28 Even combination of immune checkpoint therapy and chemotherapy, which has been widely used in clinical studies, have failed to inhibit the growth of B16.F10 tumors.29 Thereby, we studied the effect of MTA in reducing tumor burden and prolonging survival over a period of one month. MTA was administered via peritumoral injection (17.5 nmole) on day 9 post tumor inoculation after the tumor volume reached ∼100 mm3. For comparison, we performed parallel studies by injecting equivalent amounts of unlinked agonist mixture or 2/6a + 7a. Treatments were repeated on day 15 and day 21. To our delight, administration of MTA reduced tumor burden and prolonged survival beyond 31 days in 50% of challenged animals (Figure 3 A−D, SI Figure 16S). In contrast, the unlinked agonists and 2/6_7a resulted in median survival of 22 days and 26 days, respectively. Further, to understand the role of each therapeutic, we isolated tumors 2 days after the second injection (day 17) from a cohort of treated mice and analyzed for tumor-infiltrating immune cell populations. Compared with other groups, the MTA significantly enhanced tumor-infiltrating leukocytes (TIL) in each tumor (Figure 3F). Among various TILs, cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells can inhibit tumor development through the release of immunostimulatory cytokines like IFN-γ.30−32 Notably, MTA significantly augmented the percentage of tumor-infiltrating CD8+ CTLs and NK cells (Figure 3G,H) compared with other treated groups (see SI, Figure 17S for representative plots).32 Such infiltration of CD8+ T cells and NK cells into the tumor microenvironment in MTA-treated animals generates improved host survival by enhanced tumorspecific immunity. These results thus indicate that MTA serves as a promising anticancer therapeutic by significantly enhancing antitumor efficacy via stimulation of immune cell populations.

MTA Reduces Off-Target Toxicity. An important attribute of TLRa therapeutics that prevents further clinical translation is unacceptable levels of off-target toxicity.12,33 Hence, we next evaluated off-target toxicity of MTA in the B16.F10 model. We performed toxicity analysis by monitoring the reduction in cellular populations in blood such as WBCs, lymphocytes, monocytes, neutrophil, thrombocytes, and RBCs as a reflection of toxicity caused by systemic diffusion of
agonists into the bloodstream. Here, the blood was collected 2 days post first and second injection and whole blood analysis were performed to evaluate for any significant reduction of blood cell populations compared with PBS controls (Figure 4). To our delight, post first injection, MTA maintained similar cellular counts compared to PBS showing only a moderate decrease in thrombocytes. On the contrary, unlinked agonists significantly reduced WBCs including neutrophils, lymphocytes, monocytes along with severe reduction in thrombocytes, indicating severe hematological toxicities. Likewise, 2/6_7a gave significantly reduced cellular counts. Thus, from this whole blood count analysis, we concluded that MTA prevented off-target hematological toxicity compared with either unlinked agonists or 2/6_7a.

Further evaluation of hematological toxicity post second injection indicated that MTA continued to maintain normal blood cell counts (see SI Figure 20S). Meanwhile 2/6_7a displayed decreased lymphocyte counts. Similarly, the unlinked agonist-treated mice displayed decreased lymphocyte and thrombocyte counts. Furthermore, mice in this group displayed reduced RBC counts and low hemoglobin levels indicating symptoms of hemolytic anemia. These studies thereby indicate mitigation of diffusion induced off-target systemic toxicity by MTA.

The results from the hematological analysis showed great promise. We thus measured spleen sizes as another indicator of toxicity as splenomegaly (enlarged spleens) is a stereotypical response to chronic systemic immune activation. As expected, MTA did not result in splenomegaly indicating localized immune activation (Figure 5). On the contrary, administration of unlinked agonists or 2/6_7a resulted in severe splenomegaly in animals indicating significant systemic immune activation. These results thus corroborated our previous findings from hematological toxicity analysis.

To understand the effect of MTA formulation in reducing toxicity, we performed additional studies to analyze for secretion of systemic cytokines post injection of agonists. Systemic cytokines can be secreted in the blood as a result of systemic diffusion of immune agonists from the site of injection. In this study, blood was collected by submandibular bleed at 2, 6, 24, and 48 h post injection of agonists at the tumor site on day 9 and analyzed for level of serum cytokines (Figure 6A,B). It was observed that MTA formulation did not induce significant systemic cytokine secretion indicating better localization. On the contrary, animals treated with unlinked agonist formulation or 2/6_7a significantly enhanced the generation of systemic cytokines in blood indicating diffusion of agonists from the site of injection. The generation of significant amounts of systemic cytokines in animals treated with unlinked agonists or the linked heterodimer also resulted in significant weight loss (Figure 6C). The results thereby validated our previous observations on reduced toxicities with MTA formulation.

Figure 5. Toxicity analysis. (A) Spleens from mice of respective groups. (B) Surface area of spleens using measurements at the widest dimensions (n = 5, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, statistical analyses were performed using ANOVA with Tukey’s multiple comparisons test). 2/6a + 7a: unlinked mixture, 2/6_7a: linked heterodimer, MTA: multicomponent TLRa assembly.

Figure 6. Systemic cytokine analysis. Time-course analysis of systemic cytokine secretion for (A) TNF-α and (B) IL-6. (C) Change of weight in treated animals 24 h post agonist injection (day 9) Statistical analyses were performed using ANOVA with Tukey's multiple comparisons test. 2/6a + 7a: unlinked mixture, 2/6_7a: Linked heterodimer, MTA: multicomponent TLRa assembly.
SUMMARY
In conclusion, we designed and synthesized a novel multi-component immune agonist assembly based on noncovalent stabilizing interaction between a TLRa heterodimer amphiphile and an amphiphilic carbohydrate polymer. Our studies on MTA in comparison with unlinked agonists and linked TLR heterodimer indicate that such an assembly can largely enhance therapeutic efficacy and mitigate off-target toxicity—which are a critical roadblock in clinical translation of TLR agonists. This study with amphiphilic TLRa heterodimers thereby assists us to understand how to generate multi-component assemblies from other similar potent agonist combinations through molecular design for generating efficacious immunotherapeutic modalities with reduced off-target toxicity, thereby expanding and advancing the TLR-based immunotherapy. Further studies on structure-based design of immunomodulatory combinations, are currently underway in our lab.

MATERIALS AND METHODS
Complete details of reagents and methods for cell culture, chemical synthesis, cell assays, and in vivo experiments are provided in the SI, Materials and Methods section.

Safety Statement. No unexpected or unusually high safety hazards were encountered. Researchers should take precaution when handling immunostimulants to avoid potential adverse side effects.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensci.0c01001.

Synthesis and characterization of TLR heterodimer and SPOE polymer, synthesis and characterization of multicomponent assembly, in vitro immunogenicity assays, representative flow cytometry plots for cellular analysis, hematological toxicity analysis (PDF)

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ABBREVIATIONS
TLR, Toll-Like Receptor; TLRa, Toll-Like Receptor Agonist; SPOE, Sugar poly(orthoester); DSPOE, Deprotected Sugar poly(orthoester); MTA, Multicomponent TLR Agonist Assembly; TEM, Transmission Electron Microscopy; MALS, Multi-Angle Light Scattering; NF-kB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; IL-12, Interleukin 12; IFN-β, Interferon β; TNF-α, Tumor Necrosis Factor alpha; CCL2, chemokine (C–C motif) ligand 2; IL-6, Interleukin 6; IL-10, Interleukin 10; BMDC, Bone Marrow Derived Dendritic Cell; CD8+ T cell, Cytotoxic T cell; NK cell, Natural Killer Cell

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