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The use of nanolipoprotein particles to enhance the immunostimulatory properties of innate immune agonists against lethal influenza challenge

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ABSTRACT

Recent studies have demonstrated that therapies targeting the innate immune system have the potential to provide transient, non-specific protection from a variety of infectious organisms; however, the potential of enhancing the efficacy of such treatments using nano-scale delivery platforms requires more in depth evaluation. As such, we employed a nanolipoprotein (NLP) platform to enhance the efficacy of innate immune agonists. Here, we demonstrate that the synthetic Toll-like receptor (TLR) agonists monophosphoryl lipid A (MPLA) and CpG oligodeoxynucleotides (CpG) can be readily incorporated into NLPs. Conjugation of MPLA and CpG to NLPs (MPLA:NLP and CpG:NLP, respectively) significantly enhanced their immunostimulatory profiles both *in vitro* and *in vivo* compared to administration of agonists alone, as evidenced by significant increases in cytokine production, cell surface expression of activation markers, and upregulation of immunoregulatory genes. Importantly, enhancement of cytokine production by agonist conjugation to NLPs was also observed in primary human dendritic cells. Furthermore, BALB/c mice pretreated with CpG:NLP constructs survived a lethal influenza challenge whereas pretreatment with CpG alone had no effect on survival.

1. Background

Select classes of pathogenic organisms pose significant threats to public health due to the high levels of morbidity and mortality induced by very low infectious doses and their ease of transmissibility by aerosol [1]. For many of these organisms, efficacious therapies are not available or not optimal in the event of a bioterror attack or acute disease emergence. Vaccines, long considered the gold standard medical countermeasure for disease prevention, may not be available, or not suitable for widespread administration to the general public due to safety concerns [2]. Similarly, antimicrobial compounds may be insufficient to counter a wide range of unknown, deliberately altered, or drug-resistant pathogens and can potentially lead to resistance. As such, development of innovative approaches that allow for rapid responses to traditional and emerging pathogens is paramount. Recently, targeting the innate immune system to counter infection has been gaining attention as a therapeutic strategy [3–5]. The innate immune system has evolved over millennia to function immediately and non-specifically upon encountering pathogens [6], thus modulation of a single innate host resistance mechanism has the potential to offer broad-spectrum efficacy independent of pathogen identity. Furthermore, therapeutics targeting innate immunity would be less likely to promote the development of resistance, as the pathogen itself is not directly targeted.

Over the past fifteen years, pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs), have emerged as an intricate innate immune surveillance system that have evolved to detect a

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variety of invading pathogens expressing so-called pathogen-associated molecular patterns or PAMPs [6–8]. TLRs play a key role in the early steps of the immune response to infection, and robust TLR activation is critical for the downstream induction of a sustained adaptive immune response and pathogen clearance [9]. Importantly, the well-described association of TLRs with myriad infections underscores their importance as therapeutic targets and current research efforts are focused on clinically translating our understanding of TLRs and innate immunity [10–12]. Vaccine adjuvants are perhaps the most extensively explored application for TLR agonists, although there have been recent efforts exploring the use of TLR monotherapy to enhance host resistance to infection by a variety of pathogens [13–25]. However, there are significant limitations to the effectiveness of TLR agonists as stand-alone therapeutics, including marginal and short-lived protection, thus limiting their utility in both pre- and post-exposure scenarios. Furthermore, the dose required to afford protection in several of these studies was significantly higher than doses typically co-administered in combination with subunit antigens for vaccine applications [26], potentially raising toxicity concerns. Hence, strategies to improve the therapeutic index of TLR agonists *in vivo* are urgently needed.

Nanoparticles have been widely explored as delivery vehicles for immunotherapeutic applications [11,27], primarily to improve vaccine immunogenicity, reduce toxicities associated with high doses of adjuvants, improve pharmacokinetic profiles, and enhance stability of labile vaccine components. Although the functional indicator is ultimately heightened adaptive immune responses, nanoparticle-mediated enhancement of vaccine immunogenicity is likely due, at least in part, to heightened innate immune responses [28–31]. Indeed, several recent studies have demonstrated the prostimulatory effects of diverse nanoparticles on innate immune responses *in vitro* and *in vivo* [32–37]. In this study, we aimed to utilize nanolipoprotein particles (NLPs) to improve the efficacy of innate immune targeting therapeutics. NLPs are discoidal, nanometer-sized particles comprised of self-assembled phospholipid membranes and apolipoproteins [38,39]. Previously, we have described NLPs as a flexible vaccine platform for the co-localized administration of protein antigens and synthetic TLR agonists monophosphoryl lipid A (MPLA) and CpG oligodeoxynucleotides (CpG) [40]. Immunization with antigen:agonist:NLP complexes containing either influenza hemagglutinin or *Yersinia pestis* LcrV antigens and CpG or MPLA resulted in significantly higher antibody titers in mice against both antigens versus co-administration of antigens and agonists without NLP conjugation. Thus, NLPs have demonstrated effectiveness as a compatible platform for immune modulation strategies and have been used previously in other *in vivo* applications [41–44], supporting their broad utility.

Here, we investigated the interaction of NLP complexes with the innate immune system of mice and humans. Methods were developed for the incorporation and quantification of MPLA and CpG within NLPs. We then investigated the impact of NLP conjugation on innate immune responses to MPLA and CpG by measuring cytokine secretion, expression of immunoregulatory genes, and surface expression levels of key activation markers on antigen presenting cells (APCs). Stimulation of cytokine production was also investigated in primary human dendritic cells. Finally, we employed a mouse model of influenza in order to test the efficacy of CpG:NLP constructs at ameliorating infection.

2. Materials and methods

2.1. Reagents

Phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (Ni-Lipid) and synthetic MPLA (PHAD™) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol-modified CpG oligonucleotides were purchased as a custom order from Biosearch Technologies (Novato, CA). All mouse experiments were done utilizing the following sequence for Class B CpG: cholesterol-5′-TCCATGACGTTCCTGACGTT-3′ – all are phosphorothioate linkages. Experiments done in human cells utilized the following sequence for Class A CpG: cholesterol-5′ G*G*G*GACGACGTCGTGG*G*G*G*G-3′ – * denotes phosphorothioate linkages. It is worth noting that the cholesterol modified CpG compounds will be referred to as CpG below.

2.2. ApoE422k protein production

The expression clone to produce apoE422k, the N-terminal 22 kDa fragment of apolipoprotein E4 (apoE4), as a 6xHis and thioredoxin-tagged fusion construct was kindly provided by Dr. Karl Weisgraber. The apoE422k was expressed and purified using a similar protocol as previously described [38,39]. Endotoxin contamination was removed by reverse phase high-performance liquid chromatography (HPLC). ApoE422k was loaded onto a Vydac C4 column (22 × 250 mm, 10 μm; Grace Davidson, Deerfield, IL) and eluted with a 30–100% water/acetonitrile gradient (0.05% TFA, 7 ml/min). Fractions containing apoE422k were lyophilized, resuspended in pyrogen-free water, aliquoted, and stored at −20 °C.

2.3. Assembly of agonist-loaded nanolipoprotein particles (agonist:NLPs)

MPLA:NLPs and CpG:NLPs were assembled as described previously [40]. Briefly, for the MPLA:NLP assembly, the appropriate amount of chloroform-solubilized Ni-Lipid (35 mol%), DOPC (60–64.5 mol%) and MPLA (0.5–5 mol%) were determined prior to the experiment and added to a glass reaction vial. Chloroform was then removed using a stream of N_2 under agitation to form a thin lipid film. Lipids were solubilized in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4) using 40 mM sodium cholate. ApoE422k was added to the solubilized lipid suspension to a final lipid:apoE422k ratio of 80:1 (typically 150 μM apoE422k was used in the final assembly volume). For the CpG:NLP assembly, the lipid constituents were prepared as described above. CpG (hydrated from a lyophilized state using pyrogen-free water) was added to the solubilized lipid together with the apoE422K. Assemblies were dialyzed overnight against PBS to remove cholate and then filtered through a 0.22 μm spin filter to remove any large particulate matter. Samples were subsequently analyzed and purified by size exclusion chromatography (SEC) (Superdex 200 pg, HiLoad 16/600 column, GE Healthcare, Piscataway, NJ) in PBS buffer (1.0 ml/min flow rate). The apoE422k concentration in the NLP samples was determined using the Advanced Protein Assay Reagent (Cytoskeleton Inc., Denver, CO), using BSA as the standard. Free MPLA was prepared by first solubilizing MPLA in powder form in 100% DMSO at a concentration of 10 mg/ml. The solution was then diluted to 1 mg/ml in water and sonicated with a tip sonicator (Branson Sonifer, Model 250, Branson Ultrasonics, Danbury, CT) at the highest power until the suspension reached clarity. All buffers, eppendorf tubes and vials used during the assembly process were endotoxin-free. It is worth nothing that lipids containing a nickel chelating head group (Ni-Lipid) were included in all NLP assembly reactions, and while the NLPs generated for this study did not require the Ni functional group, we wanted to ensure that results obtained from this study would be applicable to ongoing NLP-based vaccine studies, in which a protein antigen is attached via the Ni-His tag interaction [40]. Additionally, a long-term objective of the approach described herein is to examine the effects of multiple immune modulators on a single NLP; thus, the Ni-Lipid was included in order to evaluate NLPs equipped for the attachment of His-tagged adjuvants, such as His-tagged flagellin [45].

2.4. Quantification of MPLA incorporation in NLPs

Incorporation of MPLA into NLPs was assessed by reverse phase HPLC by modifying published procedures [46]. Briefly, 20 μl concentrated NLP stocks containing MPLA were speed-vacuumed until dry. Lipophilic components were extracted from the dry pellet with extraction buffer (90% MeOH, 10% $CHCl_3$) for 2 h under constant agitation. After centrifugation to pellet insoluble material, the solvent was transferred to glass injection vials for HPLC analysis (Shimadzu Precision HPLC system). MPLA standards were prepared in extraction buffer (5–150 μg/ml). Samples and standards were analyzed on a Luna C18 column (4.6 × 150 mm, 5 μm; Phenomenex, Torrence, CA) using a constant flow rate (1 ml/min, 30 °C). Gradient conditions of Buffer A (95% methanol, 5% water, 0.1% TFA) and Buffer B (100% isopropanol, 0.1% TFA) were as follows: 0 min, 5% B; 10 min, 40% B; 18 min, 40% B; 20 min, 80% B; 28 min, 80% B; 35 min, 5% B. MPLA was detected using an evaporative light scattering detector (ELSD) (40 °C, gain 11). Sample concentrations were calculated using a quadratic curve fit of calibration standards ($r^2 = 0.998$) using Shimadzu LabSolutions (v5.51) software. In a typical assembly, mass ratios of E422k to MPLA were *ca.* 15:1.

2.5. Quantification of CpG incorporation in NLPs

Incorporation of CpG into the NLPs was quantified using Quant-iT OliGreen dye (Invitrogen, Carlsbad, CA), which is widely used to quantify ssDNA. Briefly, standard