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Artificial bacterial biomimetic nanoparticles synergize pathogen-associated molecular patterns for vaccine efficacy

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ABSTRACT

Antigen-presenting cells (APCs) sense microorganisms via pathogen-associated molecular patterns (PAMPs) by both extra- and intracellular Toll-like Receptors (TLRs), initiating immune responses against invading pathogens. Bacterial PAMPs include extracellular lipopolysaccharides and intracellular unmethylated CpG-rich oligodeoxynucleotides (CpG). We hypothesized that a biomimetic approach involving antigen-loaded nanoparticles (NP) displaying Monophosphoryl Lipid A (MPLA) and encapsulating CpG may function as an effective "artificial bacterial" biomimetic vaccine platform. This hypothesis was tested in vitro and in vivo using NP assembled from biodegradable poly(lactic-co-glycolic acid) (PLGA) polymer, surface-modified with MPLA, and loaded with CpG and model antigen Ovalbumin (OVA). First, CpG potency, characterized by cytokine profiles, titers, and antigen-specific T cell responses, was enhanced when CpG was encapsulated in NP compared to equivalent concentrations of surface-presented CpG, highlighting the importance of biomimetic presentation of PAMPs. Second, NP synergized surface-bound MPLA with encapsulated CpG in vitro and in vivo, inducing greater pro-inflammatory, antigen-specific T helper 1 (Th1)-skewed cellular and antibody-mediated responses compared to single PAMPs or soluble PAMP combinations. Importantly, NP co-presentation of CpG and MPLA was critical for $CD8^+$ T cell responses, as vaccination with a mixture of NP presenting either CpG or MPLA failed to induce cellular immunity. This work demonstrates a rational methodology for combining TLR ligands in a context-dependent manner for synergistic nanoparticulate vaccines.

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1. Introduction

Despite incomplete mechanistic understanding, one of the most effective vaccines ever formulated is the live attenuated yellow fever vaccine 17D (YF-17D), inoculated in 400 million people for over 65 years. Its efficacy likely results from robust activation of Dendritic Cell (DC) subsets through Toll-like Receptors (TLRs), specifically TLR2, TLR7, and TLR9 to increase secretion of pro-inflammatory cytokines IL-12p40, IL-6, and IFN-α [1]. TLRs link innate and adaptive immunity by both inducing Antigen-Presenting Cell (APC) maturation for T cell activation and by attenuating suppressor functions of $CD4^+CD25^+$ regulatory T cells [2]. The TLR-triggering mechanism underlying this vaccine's polyvalent immune response motivated the design of newer strategies using multiple Pathogen-Associated Molecular Patterns (PAMPs) for combinatorial TLR stimulation to overcome the failures of several commercially available vaccines to induce cellular immunity [3,4]. For example, in both human and mouse DCs, activation of TLR3 and TLR4 together with TLR7, TLR8, and TLR9 improved and sustained T helper type 1 (Th1) responses, demonstrated by enhanced IL-12 and IL-23 [5]. Recent work has shown that mice immunized with multiple TLR ligands were protected against lethal avian and swine influenza strains, and immunized rhesus macaques were protected against H1N1 influenza [6]. Further investigations demonstrated synergistic effects of ligands for TLR4, TLR7/8, and TLR9 in rhesus macaques, showing distinct activation patterns of local and systemic innate immunity [7].

A critical component in vaccine design is the delivery vehicle, the properties of which may influence the effects of delivered antigen and adjuvants [8]. Nanoparticles (NP) encapsulating

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antigen(s) are promising vaccines for numerous reasons, including clinical relevance [9], flexible and reproducible control of antigen and adjuvant configurations, and APC targeting [10,11]. Because NP-based vaccines are modular systems comprised of at least three key components: a carrier, antigen(s), and adjuvant(s), these variables, which are often well-characterized, can be reliably selected for or arranged in different ways to allow optimal immune responses to a specific pathogen. Variables such as the material and size of the core matrix, surface attachment of ligands that target DCs or epithelial cells, or ligands that protect the carrier during trafficking or facilitate drainage to lymphatics, can increase the overall efficacy of the vaccine response with lower overall quantities of antigens and adjuvants [12]. The distinguishing feature is the flexibility of these systems, such that NP can address critical issues to optimize the vaccine response, including targeting different DC subsets for tailored priming for antigen presentation [10], and delivery of complex mixtures of antigens to induce reactivity against multiple viral and tumor epitopes. Recently, NP encapsulating a pool of peptides derived from viruses and cancer oncogenes enabled antigen recognition by different HLA haplotypes and stimulation of antigen-specific immunity in response to multiple peptides [13]. NP enable the assembly of different combinations of recognition and antigen components to affect a broad-spectrum CD4 and CD8 vaccine response [14] [15]. APCs efficiently internalize NP, enabling synchronous high intracellular concentrations of antigen and adjuvant [16,17]. Furthermore, particulate encapsulation of vaccine components protects from non-specific immune activation and proteasomal degradation and maintains proximity of antigens and adjuvants; well-designed NP vaccines facilitate antigen-cross presentation to $CD4^+$ and $CD8^+$ T cells while stimulating antibody-mediated immunity [14,15].

An advantage of NP-based vaccine systems is the ability to assemble different combinations of PAMPs and antigens for optimal vaccine responses [13,18]. Recent work has established the importance of targeting TLRs through PAMPs on the surfaces or interiors of NP [19]. Further work has shown the importance of combining TLR ligands to enhance vaccine responses [20], inducing immunity against diseases such as influenza [21], cancer [22], hepatitis B [23], and West Nile Encephalitis [24].

In this work, we sought to construct a versatile, pathogen-mimicking NP vaccine system using a logical approach that can be easily adapted to the requirements of a particular vaccine. This platform displays LPS-derived Monophosphoryl Lipid A (MPLA), a TLR4 agonist and clinically approved Th1-skewing adjuvant that is 100–10,000 times less toxic than LPS [25–27] on NP constructed from biocompatible polyester poly(lactic-co-glycolic acid) (PLGA). The cores of NP were loaded with model antigen Ovalbumin (OVA) and another adjuvant, synthetic oligodeoxynucleotides rich in unmethylated CpG motifs (CpG) that ligate endosomal TLR9 [28], facilitating targeted release of antigen and CpG into intracellular compartments. Based on demonstrations of the necessity of adjuvant and antigen to be presented within the same particle for cross-priming and in vivo $CD8^+$ responses, NP contained both antigen and adjuvants [29,30].

Additionally, we sought to understand the influence of combinations and context of PAMP presentation, both in terms of spatial location and proximity, on the in vitro and in vivo antigen-specific immune response. We hypothesized that arranging PAMPs on or inside NP, which are in the size range of viruses and bacteria, created biomimetic platforms for efficient interactions with APCs that would outperform suspensions of equivalent doses of antigen and adjuvants. To verify, we compared NP to soluble antigen/adjuvant formulations to determine if maximal vaccine efficacy required a physiologically relevant arrangement of PAMPs, or if PAMP combinations would suffice. Furthermore, we compared the vaccine efficacy of artificial bacterial biomimetic NP, antigen-loaded NP containing both MPLA and CpG, tethering adjuvants, against a mixture of NP presenting either MPLA or CpG, in which each adjuvant remained in close proximity to antigen but could be distant from one another. Realizing that subsets of APCs express different Pattern-Recognition Receptors (PRRs) and perform complementary functions [31], we hypothesized that bundling PAMPs with antigen would maximize antigen-specific immune activation. Our approach is biomimetic in that vaccine NP approximate bacterial pathogens with protein antigen and DNA motifs inside and MPLA, a cell wall component, on the surface (Fig. 1A). We show that spatial and combinatorial presentation of PAMPs in or on NP influences the magnitude and direction of vaccine responses; artificial bacterial NP create a synergistic antibody-mediated and cellular antigen response in vitro and in vivo.

2. Materials and methods

2.1. Materials

Fully phosphorothioated Type B CpG 1826 ODN were synthesized by Yale Keck Facility (sequence: 5′ TCC ATG ACG TTC CTG ACG TT) and conjugated to biotin. Poly(vinyl alcohol) (PVA), palmitic acid N-hydroxy-succinimide ester, Atto 565-biotin, Coumarin 6, Ovalbumin, and chloroform were purchased from Sigma-Aldrich. Synthetic MPLA was purchased from Invivogen, avidin from Invitrogen, and research-grade PLGA (50:50, iv 0.55–0.75 dL/g) from Durect.

2.2. NP synthesis and characterization

PLGA NP were synthesized using a water/oil/water (w/o/w) double emulsion technique previously described [24,32]. Briefly, polymer was dissolved in chloroform at 50 mg/mL, OVA (100 mg/mL in PBS) added dropwise to polymer under vortex, then subjected to 30 s sonication using a Tekmar Sonic Distributor at 38% amplitude. Polymer/OVA was added dropwise to an aqueous solution of PVA (sometimes containing avidin-palmitate or MPLA) under vortex before further sonication then stirring in 0.2% PVA for NP hardening and solvent evaporation. NP were collected and rinsed by centrifugation at 12,000 rpm, flash frozen, lyophilized, and stored at −20 °C. Immediately before experiments, avidin-coated NP were suspended in PBS and incubated with biotinylated CpG to surface-attach the ODN.

Scanning Electron Microscopy was used to characterize NP morphology, and hydrodynamic diameters were measured using Dynamic Light Scattering (DLS). For DLS and zeta potential measurements, employing a Malvern Zetasizer, NP were suspended in deionized water at pH 6.5 and titrated from 100 μg/mL to 6.25 μg/mL, and average values taken across titrations. Dissolved NP were assayed for loading of OVA by Micro BCA protein assay and CpG by PicoGreen DNA Detection Assay. Blank, empty NP were used as negative controls.

A dye conjugation assay was developed to provide evidence that acyl chains of MPLA partition into PLGA during NP synthesis, resulting in NP surface-presenting bioactive MPLA carbohydrate structures. MPLA was reacted with N,N-Carbonyl Diimidazole (CDI) to convert the free hydroxyl group on MPLA to an imidazole carbamate reactive intermediate (termed 'MPLA-imidazole carbamate') (Supplementary Fig. 1A). NP were formulated, as previously described, using either MPLA-imidazole carbamate (resulting from CDI reaction) or non-reacted MPLA (with intact hydroxyl group). NP were collected, rinsed twice, and incubated with rhodamine that had been previously conjugated to polyamidoamine (PAMAM) dendrimer (termed 'rhodamine') to introduce functional amine