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Activation of innate immune responses in a pathogen-mimicking manner by amphiphilic polyanhydride nanoparticle adjuvants

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ABSTRACT

Techniques in materials design, immunophenotyping, and informatics can be valuable tools for using a molecular based approach to design vaccine adjuvants capable of inducing protective immunity that mimics a natural infection but without the toxic side effects. This work describes the molecular design of amphiphilic polyanhydride nanoparticles that activate antigen presenting cells in a pathogen-mimicking manner. Biodegradable polyanhydrides are well suited as vaccine delivery vehicles due to their adjuvant-like ability to: 1) enhance the immune response, 2) preserve protein structure, and 3) control protein release. The results of these studies indicate that amphiphilic nanoparticles possess pathogen-mimicking properties as evidenced by their ability to activate dendritic cells similarly to LPS. Specific molecular descriptors responsible for this behavior were identified using informatics analyses, including the number of backbone oxygen moieties, percent of hydroxyl end groups, polymer hydrophobicity, and number of alkyl ethers. Additional findings from this work suggest that the molecular characteristics mediating APC activation are not limited to hydrophobicity but vary in complexity (e.g., presentation of oxygen-rich molecular patterns to cells) and elicit unique patterns of cellular activation. The approach outlined herein demonstrates the ability to rationally design pathogen-mimicking nanoparticle adjuvants for use in next-generation vaccines against emerging and re-emerging diseases.

1. Introduction

Successful vaccines induce protective immune responses that mimic those induced by a natural infection but do not elicit the negative effects associated with disease [1]. To do so, they must signal to the innate immune system using mechanisms similar to those employed by pathogens. Upon infection, the innate immune system is activated by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) found on the surface of gram negative bacteria, which interact with Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) on the surface of antigen presenting cells (APCs), including dendritic cells (DCs) [2,3]. PAMPs contain repetitive molecular patterns that are recognized as “danger signals” by the host [3]. Moreover, PAMPs are often comprised of insoluble, hydrophobic moieties and are presumed to interact with PRRs, thereby providing signals that activate the innate immune system [4,5]. In contrast, the soluble antigens and adjuvants found in current vaccine formulations cannot provide the same degree of continued stimulation. An opportunity exists to exploit the material properties of biodegradable polymers in order to rationally design vaccine adjuvants that mimic the behavior of pathogens, including prolonged *in vivo* residence times capable of providing extended immune activation and continued stimulation of APCs, without inducing the deleterious effects of disease.

Polyanhydrides are biodegradable materials that have been well documented to provide sustained delivery of proteins and stabilization of vaccine antigens [6–12]. Copolymers based upon sebacic acid (SA), 1,6-bis-(*p*-carboxyphenoxy)hexane (CPH), and 1,8-bis-(*p*-carboxyphenoxy)-3,6-dioxaocatane (CPTEG) have been studied as antigen carriers and adjuvants. These copolymers

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degrade into non-toxic, non-mutagenic degradation products and have demonstrated biocompatibility both *in vivo* and *in vitro* at concentrations expected for human use [13–16]. Some amphiphilic polyanhydrides have also been reported to exhibit adjuvant characteristics capable of enhancing the adaptive immune response [13,16].

When designing vaccine adjuvants, it is integral to understand the molecular properties of the adjuvant responsible for immune cell activation that would facilitate the induction of long-lived, protective immunity. In this work, we investigated the molecular properties of polyanhydride nanoparticles responsible for their robust stimulation of DCs. A simultaneous investigation of nanoparticle internalization and activation of DCs was complemented by informatics analysis to identify important polymer descriptors responsible for mimicking the adjuvant capabilities of the PAMP, LPS. While many previous studies have investigated the expression of cell surface markers and production of cytokines caused by stimulation with polymeric adjuvants [12,17,18], this study reports on the complex relationship between nanoparticle internalization, DC activation, and polymer chemistry (i.e., through polymer descriptors). This study also presents a direct comparison of cellular activation between DCs that have engulfed the nanoparticles and those that have not in the same culture.

2. Materials and methods

2.1. Materials

The chemicals utilized in the monomer synthesis include: 1,6-dibromohexane, tri-ethylene glycol, 4-hydroxybenzoic acid, 1-methyl-2-pyrrolidinone, 4-*p*- and 1,6-dibromohexane; these were purchased from Sigma Aldrich (St. Louis, MO); 4-*p*-fluorobenzonitrile was purchased from Apollo Scientific (Cheshire, UK); and sulfuric acid, acetonitrile, dimethyl formamide (DMF), toluene, and potassium carbonate were obtained from Fisher Scientific (Fairlawn, NJ). Chemicals for the polymerization and nanoparticle fabrication include petroleum ether, pentane, acetic anhydride, chloroform, and methylene chloride; all were purchased from Fisher Scientific. Deuterated chemicals for NMR analysis include chloroform and dimethyl sulfoxide (DMSO; Cambridge Isotope Laboratories, Andover, MA). β-Mercaptoethanol, *Escherichia coli* lipopolysaccharide (LPS), and rat immunoglobulin (rat IgG) were purchased from Sigma Aldrich and brefeldin A from eBioscience (San Diego, CA). The materials required for the DC culture medium include: granulocyte macrophage colony stimulating factor (GM-CSF), purchased from PeproTech (Rocky Hill, NJ); HEPES buffer, RPMI 1640, penicillin–streptomycin, and L-glutamine, purchased from Mediatech (Herndon, VA); and heat inactivated fetal calf serum, purchased from Atlanta Biologicals (Atlanta, GA). Materials used for flow cytometry included: permeabilization buffer (10×) and intracellular (IC) fixation buffer, purchased from eBioscience (San Diego, CA); unlabeled anti-CD16/32 FcγR, purchased from Southern Biotech (Birmingham, AL); unlabeled hamster IgG, peridinin–chlorophyll proteins–Cy5.5 (PerCP/Cy5.5)-conjugated anti-mouse IL-12/23 p40 (clone C17.8), phycoerythrin (PE)-conjugated anti-mouse IL-6 (clone MP5-20F3), biotin-conjugated anti-mouse MHC II (I-Eκ) (clone 14-4-4S), phycoerythrin–Cy5 (PE/Cy5)-conjugated anti-mouse CD11c (clone N418), fluorescein isothiocyanate (FITC)-conjugated anti-mouse/rat MHC Class II (I-Eκ) (clone 14-4-4S), PE-conjugated anti-mouse MHC Class I ($H\text{-}2K^d/H\text{-}2D^d$) (clone 34-1-2S), allophycocyanin (APC) anti-mouse CD40 (clone 1C10), phycoerythrin–Cy7 (PE/Cy7) anti-mouse CD86 (clone GL-1), Alexa Fluor® 700 anti-mouse CD11c (clone N418), and biotin-conjugated anti-mouse CIRE (DC-SIGN or CIRE) (clone 5H10); and corresponding isotypes: PerCP/Cy5.5-conjugated rat IgG2a κ, PE-conjugated rat IgG2 κ, biotin-conjugated mouse IgG2a κ, PE/Cy5 rat IgG2a κ (clone eBR2a), PE-conjugated rat IgG2a (clone eBR2a), APC rat IgG2a κ (clone eBR2a), PE/Cy7-conjugated rat IgG2b (clone KLH/G2b-1-2), Alexa Fluor® 700-conjugated Armenian hamster IgG (clone eBio299Arm), and biotin-conjugated rat IgG2a (clone eBR2a); and APC–Cy7-conjugated streptavidin. All these reagents were purchased from eBioscience. Cadmium selenide quantum dots (QDs) (emission at 630 nm) were a gift from Dr. Aaron Clapp at Iowa State University.

2.2. Polymer synthesis, nanoparticle fabrication, and characterization

SA monomer was purchased from Sigma Aldrich. 1,6-Bis(*p*-carboxyphenoxy) hexane (CPH) monomer, 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaocatane (CPTEG), and conventional CPTEG:CPH and CPH:SA polymers were synthesized as described previously [19–22]. Combinatorial discrete libraries of CPTEG:CPH and CPH:SA copolymers were synthesized at high throughput as described previously [9,10,17,18]. Following synthesis, the polymers were dissolved in a solvent (methylene chloride), QDs added to the dissolved polymer (nothing was added at this step for blank particles), dispersed by sonication at 40 Hz for 30 s, and then rapidly precipitated into a non-solvent (pentane) for the formation of nanoparticle libraries [9,10,18]. These libraries were dried under vacuum drying or via rapid filtration and stored under dry conditions until use in the cellular assays. Conventional nanoparticles were fabricated with previously described methods [16,23]. The polymer libraries were characterized by ^1H nuclear magnetic resonance (NMR) spectroscopy. The nanoparticle libraries were imaged using scanning electron microscopy (SEM) to assess size and morphology.

2.3. Culture and stimulation of murine DCs

All experiments involving animals were carried out in accordance with procedures approved by the Iowa State University Institutional Animal Care and Use Committee. DCs were grown as described previously [12,18] and stimulated with either 200 ng/mL lipopolysaccharide (LPS; a positive control), 125 μg/mL of QD-loaded CPTEG:CPH copolymer nanoparticles of various molar ratios (60:40, 50:50, 40:60, 30:70, 25:75, 20:80, and 10:90), QD-loaded CPH:SA copolymer nanoparticles of various molar ratios (0:100, 13:87, 25:75, 37:63, 50:50, and 63:37) or left untreated (NS; non-simulated, negative control). Treatments were applied to the DCs on day nine post-harvest and incubated for 48 h. Flow cytometry was then performed to assess the phenotype of the DCs. In all cases, cells were >90% positive for the DC specific marker, CD11c (data not shown). To account for QD release due to particle degradation which would result in cells "false positive" for particles, a released QD control (background) was subtracted from each treatment group. In this control experiment, QD-loaded nanoparticles of all chemistries were allowed to incubate in DC culture medium for 48 h. After the incubation, the particles were centrifuged and the supernatants containing the released QDs were added to DCs for 48 h to account for any fluorescence caused by the uptake of released QDs as opposed to internalization of QD-loaded nanoparticles. For experiments investigating cytokine production via flow cytometry, brefeldin A was administered at the same time as the treatments to prevent secretion of the protein from the cells.

To ensure that the DC activation observed was due to the polymers and not endotoxin contamination, endotoxin levels of the particles were tested with the *Limulus* Amebocyte Lysate (LAL) QCL-1000 test kit (Cambrex, Walkersville, MD) as described previously [12]. All the polyanhydride nanoparticles exhibited an endotoxin content of less than 0.1 EU/mL, which is five times lower than the maximum level permitted by the United States FDA for new drugs tested by the LAL test.

2.4. Flow cytometry

After stimulation, DCs were assessed for the expression of MHC I, MHC II, CD40, CD86, and CIRE as described previously [12,18]. To detect the intracellular cytokines IL-12p40 and IL-6, cells were labeled according to the manufacturer's recommended protocol (eBioscience). No differences were observed in the expression of MHC I, MHC II, CD86, CD40 or CIRE when DCs were cultured with nanoparticles fabricated combinatorially or conventionally (Supplementary Fig. 1).

2.5. Statistical and informatics analyses

Statistical analysis was performed using JMP® statistical software. Two-group comparisons were made using a Student's *T*-test whereas multiple-group comparisons were made with Tukey's HSD in which case the data was log transformed.

Principal Component Analysis (PCA), a dimensionality reduction technique, was employed to provide a straightforward and parsimonious description of the covariance structure [24,25]. The principal components (PCs) are linear combinations of the original variables, and present new axes that represent the directions with maximum variance. The projection of the original multi-dimensional data to the two-dimensional space constructed by the first and second PCs provides a means of data visualization and interpretation and can uncover unknown relationships, thereby enabling new data interpretations [24,25]. In this work, PCA was used to uncover the latent features of the DC activation data and explains the relationships between polymer chemistry, cell marker expression, and cytokine production. The PCA for DC activation (Fig. 4F) used double positive populations based upon all combinations of MHC II, CD40, CD86, IL-6 and IL-12p40 data. Partial least squares (PLS) regression connected the descriptor data set with the results of the PC analyses. By projecting the data onto a high-dimensional hyperplane defined by the PCA of training data, the impact of the descriptors on the property was identified, while taking co-linearity into account [26].

3. Results

3.1. Importance of nanoparticle internalization for DC activation

Bacterial internalization by APCs is an important step in cellular activation and immune signaling [27–29]. The CPH:SA and CPTEG:CPH nanoparticles were, therefore, tested for their ability