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Polyanhydride microparticles enhance dendritic cell antigen presentation and activation

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Abstract

The present studies were designed to evaluate the adjuvant activity of polyanhydride microparticles prepared in the absence of additional stabilizers, excipients, or immune modulators. Microparticles composed of varying ratios of either 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and sebacic acid (SA) or 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and CPH were added to in vitro cultures of bone marrow-derived dendritic cells (DCs). Microparticles were efficiently and rapidly phagocytosed by DCs in the absence of opsonization and without centrifugation or agitation. Within 2 h, internalized particles were rapidly localized to an acidic, phagolysosomal compartment. By 48 h, only a minor reduction in microparticle size was observed in the phagolysosomal compartment, indicating minimal particle erosion consistent with being localized within an intracellular microenvironment favoring particle stability. Polyanhydride microparticles increased DC surface expression of MHC II, the co-stimulatory molecules CD86 and CD40, and the C-type lectin CIRE (murine DC-SIGN; CD209). In addition, microparticle stimulation of DCs also enhanced secretion of the cytokines IL-12p40 and IL-6, a phenomenon found to be dependent on polymer chemistry. DCs cultured with polyanhydride microparticles and ovalbumin induced polymer chemistry-dependent antigen-specific proliferation of both CD4⁺ OT-II and CD8⁺ OT-I T cells. These data indicate that polyanhydride particles can be tailored to take advantage of the potential plasticity of the immune response, resulting in the ability to induce immune protection against many types of pathogens.

Keywords

Polyanhydrides; Dendritic cells; Adjuvants; Vaccine delivery; Microparticles

Introduction

The World Health Organization (WHO) estimated that in 2002, 2.1 million deaths occurred worldwide that resulted from diseases that could have been prevented by routine vaccination

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[1]. In an effort to minimize the mortality associated with infectious diseases, the WHO, United Nations Children's Fund (UNICEF) and several other partnering organizations developed a Global Immunization Vision and Strategy (GIVS). The primary mission of GIVS is the introduction of new and efficacious vaccines and delivery technologies to combat diseases for which no treatment currently exists [2]. The rapid development of protein-based biopharmaceuticals suggests that many future vaccines will involve the delivery of peptide or protein subunits. Currently, such vaccines lack a suitable carrier, and, thus, there is an urgent need to develop new adjuvants for delivery of efficacious vaccines [3].

An adjuvant is a substance which when incorporated into a vaccine will enhance the immune response to the antigen [4]. Classically, adjuvants fulfill one of three roles: 1) act as a depot, preventing rapid clearance of the antigen; 2) direct the antigen to antigen presenting cells (APCs) for phagocytosis, processing, and presentation; and 3) induce co-stimulatory signals on APCs necessary for activation of naïve T cells [5,6]. Currently, the common adjuvants employed in human vaccines are aluminum-based salts (e.g., alum) and often require multiple doses to achieve protective immunity. The resulting immune response is primarily antibody-mediated with limited engagement of an effector T cell population [7]. Use of aluminum-based salts is often associated with the induction of adverse reactions at the site of injection. Alternatively, we and others have extensively studied polyanhydrides, a novel class of biodegradable polymers, as protein carriers and/or vaccine delivery systems with inherent adjuvant properties [8-20]. Currently approved by the FDA for use in humans, polyanhydrides are tissue compatible and degrade into non-mutagenic and non-cytotoxic products [8,21-25]. Another advantage of these polymers is their degradation by a surface erosion mechanism resulting in controlled release of the antigen with predictable degradation profiles that can vary from days to months depending on the polymer chemistry [8,9,16]. Moreover, this controlled release of antigen enhances the induction of an immune response and modulates the ensuing immune response with respect to Th1-Th2 bias, and may potentially improve patient compliance by requiring only a single injection [20,26]. Investigations have also shown that polyanhydrides may have intrinsic adjuvant properties, distinct from any antigen payload [20,27,28].

In this study, we have further investigated the intrinsic adjuvanticity of two polyanhydride chemistries based on the aliphatic sebacic anhydride (SA), the aromatic 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), and the amphiphilic 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Fig. 1A). In particular, 50:50 CPH:SA and 50:50 CPTEG:CPH copolymers were chosen based on previous studies indicating the ability of these materials to serve as vaccine adjuvants and protein carriers [19,20]. As adjuvants are thought to elicit many of their effects via directing antigen to and activation of APCs, mouse bone marrow-derived dendritic cells (DCs), the most potent APC involved in the induction of an immune response, were selected for use in these experiments. It was found that polyanhydride microparticles were efficiently phagocytosed by DCs and localized to phagolysosomal compartments. Dendritic cell surface expression of MHC II, CD86, CD40, and CIRE was significantly increased after exposure to microparticles, as was secretion of IL-12p40 and IL-6. Lastly, DCs incubated in the presence of polyanhydride microparticles and ovalbumin induced antigen-specific proliferation of both CD4⁺ OT-II and CD8⁺ OT-I T cells. The results of these studies demonstrate that polyanhydride microparticles are capable of activating DCs as well as inducing, in a chemistry-dependent manner, antigen-specific T cell responses in vitro.

Materials and Methods

Materials

Chemicals used for synthesis of CPH and CPTEG monomers included: 4-*p*-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, and tri-ethylene glycol. All these chemicals and sebacic acid (99%) were purchased from Sigma Aldrich (St Louis, MO); 4-*p*-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ).

Polymer synthesis and characterization

CPH:SA and CPTEG:CPH copolymers were synthesized by melt polycondensation as described previously [15,17]. The purity and degree of polymerization of the polymers was analyzed using ^1H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer. Detailed analysis of ^1H NMR characteristic spectra of polyanhydrides can be found elsewhere [15,16]. The molecular weight and molecular weight distribution of the copolymers was analyzed by gel permeation chromatography (GPC) as previously reported [17]. The number average molecular weight of 50:50 CPTEG:CPH was 8,000 Da (PDI = 1.7) and that of 50:50 CPH:SA was 17,000 Da (PDI = 2.1).

Microparticle fabrication by cryogenic atomization

To prevent microbial contamination, all glassware and equipment was soaked in 70% ethanol prior to fabricating microparticles. The procedure used to fabricate microparticles was modified from previously reported studies [29,30]. Briefly, polymer dissolved in methylene chloride was pumped through an 8700-1200 MS ultrasonic atomizing nozzle (Sono Tek Corporation, Milton, NY) into 200 mL of ultra-cold ethanol overlaid with ~100 mL of liquid nitrogen. This procedure was performed at 4°C for 50:50 CPH:SA and CPTEG:CPH microparticles in order to maintain the temperature below the glass transition temperature of the polymers during microparticle preparation. After atomization, microparticles were stored at -80 °C for three days to allow the methylene chloride to be extracted into the ethanol. Microparticles were then collected by filtration and dried under vacuum overnight. The microparticle morphology was characterized by scanning electron microscopy (SEM). Particle size distribution was obtained from SEM images (150-250x) by using an image analysis software package (analySIS®, Soft Imaging System Corp, Lakewood, CO). An average of 800 particles per image was analyzed.

Endotoxin assay

To ensure that the DC activation observed was due to the polymers and not endotoxin contamination, endotoxin levels of polyanhydride microparticles were tested with the *Limulus* Amebocyte Lysate (LAL) QCL-1000 test kit (Cambrex, Walkersville, MD). Solutions of CPTEG:CPH and CPH:SA microparticles (5 mg/mL) fabricated as described above were prepared using endotoxin-free, sterile water and incubated overnight at 37°C while shaking. After centrifugation of microparticles, the LAL test was performed on the supernatant according to manufacturer's procedure. All the polyanhydride microparticles 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 [31]. Solutions of ovalbumin were prepared in endotoxin-free water and also tested for endotoxin content according to manufacturer's instructions. Ovalbumin, as purchased, was found to contain high levels of endotoxin, which was removed using AffinityPak Detoxi-Gel endotoxin removing gel columns (Thermo Scientific, Rockford, IL) according to

manufacturer's instructions. The resulting ovalbumin contained less than 10 EU/mg, which is equivalent to ~1 ng of endotoxin per mg of protein. Endotoxin-free ovalbumin was lyophilized and stored at -20°C until use.

Mice

C3H/HeNHsd and C57BL/6 mice were purchased from Harlan Sprague Dawley. Male and female C57BL/6 Tg(TcraTrab)1100Mjb/J (OT-I) and C57BL/6 Tg(TcraTrab)425Cbn/J (OT-II) TCR transgenic mice were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were housed under specific pathogen-free conditions and all bedding, caging, water, and feed were sterilized prior to use. Animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

Isolation and culture of DCs

Bone marrow-derived dendritic cells (DCs) were isolated from the femurs and tibia of C3H/HeNHsd or C57BL/6 mice and cultured *in vitro* in the presence of 10 ng/mL murine GM-CSF (PeproTech, Rocky Hill, NJ) according to a previously published protocol with minor modifications [32]. At day 6 of culture, ~90% of DCs were positive for the DC marker CD11c and veiled projections were visible on the cells. Six-day-old DCs were harvested and transferred to 24-well plates (2.5×10^6 cells/well).

Microparticle internalization by DCs using fluorescence microscopy

To visualize cellular interactions with microparticles, the particles (250 $\mu\text{g/mL}$) were incubated with DCs for 30 min and then washed briefly to remove extracellular and non-adherent particles. Cultures continued to incubate with fresh medium for either 2 or 48 h. Dendritic cell monolayers incubated with microparticles were fixed at either 2 h or 48 h post-internalization with 4% paraformaldehyde (PFA) for 10 min at room temperature. Acidic vesicles and lipid rafts were labeled by incubating cells for 20 min prior to fixation with either LysoTracker at 1:2000 dilution (DND-99) (acidic vesicles) or Alexa555 conjugated cholera toxin B-subunit (CTx) at 1:150 dilution (lipid rafts) (Molecular Probes-Invitrogen, Carlsbad, CA) [27]. Intracellular structures were visualized by immunofluorescence microscopy by incubating fixed cover slips with primary and secondary antibodies in PBS containing albumin and 0.1% saponin (BSP) [33]. Stained cover slips were washed and mounted on glass slides (Pro-Long with Dapi; Molecular Probes-Invitrogen). Wide field, epifluorescence images were captured using a DP-70 CCD camera, with a 40x LWDPlanFluor objective mounted on an IX-61 inverted microscope (Olympus, USA). Detailed high resolution imaging in X, Y, and Z dimensions was subsequently performed with a Leica NTS laser scanning confocal microscopy (LSCM) with Ar, Kr and HeNe laser lines. The step size for Z-stack image data was maintained at 0.24 μm while total stack volume varied to match cell thickness for each field. Epifluorescence and confocal image data sets were exported to ImageJ v1.36b for cell and particle analysis (epifluorescence) and constructing Z-projection images (confocal) [34].

DC stimulation using flow cytometry

For flow cytometric analysis of surface molecule expression, bone marrow-derived dendritic cells (DCs) were isolated and cultured as described above. Polyanhydride microparticles were suspended in complete culture medium, sonicated briefly, and added to the DC cultures at day nine at a concentration 250 $\mu\text{g/mL}$, corresponding to a microparticle:DC ratio of 12:1. Unstimulated DCs and DCs stimulated with LPS (200 ng/mL) were used as negative and positive controls, respectively. Cultures were incubated for 48 h (37°C , 5% CO_2). DCs were harvested after 48 h of stimulation and washed in fluorescence-activated cell sorting buffer (FACS, 0.1% sodium azide and 0.1% bovine serum albumin in phosphate buffer saline). Fc

receptors were blocked with 10 $\mu\text{g}/\text{mL}$ purified rat anti-mouse CD16/CD32 antibody (eBioscience, San Diego, CA), 0.5% homologous mouse serum and 50 $\mu\text{g}/\text{mL}$ rat IgG (Sigma) for 1 h at 4°C to prevent nonspecific binding. DCs were then incubated with the appropriate antibody or isotype control for 1 h on ice. Antibodies used included Alexa Fluor® 700 anti-mouse CD11c (clone N418), FITC conjugated anti-mouse/rat MHC Class II (I-E^k, clone 14-4-4S), PE/Cy7 anti-mouse CD86 (clone GL-1), allophycocyanin (APC) anti-mouse CD40 (clone 1C10) and PE conjugated anti-mouse CIRE (DC-SIGN CD209; clone 5H10) antibodies. Isotype-specific control antibodies included Alexa Fluor® 700 conjugated Armenian hamster IgG (clone eBio299Arm), FITC IgG2a κ (clone eBM2a), PE/Cy7 conjugated rat IgG2b (clone KLH/G2b-1-2), APC rat IgG2a κ (clone eBR2a) and PE-conjugated rat IgG2a (clone eBR2a). Anti-CD86 was purchased from BD Biosciences (San Jose, CA) and all other antibodies and isotype controls were purchased from eBioscience (San Diego, CA). Propidium iodide (PI) was used to establish the live/dead cell gate. Following staining, cells were washed in FACS buffer and fixed in 1% paraformaldehyde and stored at 4°C until analysis. Analysis was performed on a BD FACScanto flow cytometer (Becton Dickinson, San Jose, CA), and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). No significant differences in surface marker expression were observed between DCs co-cultured with polyanhydride particles plus free ovalbumin as compared to particles alone or between DCs co-cultured with free ovalbumin as compared to unstimulated DCs (data not shown).

Cytokine analysis

Polyanhydride microparticles were suspended in complete culture medium, sonicated briefly, and added to the DC cultures at day nine at a concentration of 250 $\mu\text{g}/\text{mL}$, corresponding to a microparticle:DC ratio of 12:1. Unstimulated DCs and DCs stimulated with LPS (200 ng/mL) were used as negative and positive controls, respectively. Cell-free supernatants were collected from DCs cultured for 48 h in the presence of microparticles and stored at -20°C until analysis. Cytokines (TNF- α , IL-4, IL-6, IL-10, and IL-12p40) were measured using a multiplex bead assay and a Luminex® 100 system (Austin, TX). Multiple experiments were analyzed individually.

OT-I and OT-II T cell stimulation

Dendritic cells were cultured and harvested as described above and incubated with 250 $\mu\text{g}/\text{mL}$ microparticles and 100 $\mu\text{g}/\text{mL}$ ovalbumin for 12 h. Lymph nodes were removed from either OT-I or OT-II mice and single cell suspensions were prepared. Lymph node cells were washed by centrifugation and resuspended in complete culture medium. To assess in vitro immune stimulation, 2.5×10^5 lymphocytes from OT-I or OT-II mice were combined with 0.5×10^5 DCs in 96-well round bottom culture plates. Plates were incubated at 37 °C in humidified 5% CO₂. After 72 h of co-culture, 0.5 μCi of methyl-[³H]-thymidine (specific activity of 6.7 Ci mmole⁻¹, Amersham Life Science, Arlington Heights, IL) was added to each well and cells were incubated for additional 18 h. Well contents were harvested onto glass fiber filters and the incorporated radioactivity was measured using a liquid scintillation counter. Assays were performed in triplicate and data are presented as mean counts per minute (cpm) of triplicate wells.

Statistical analysis

Differences among group means were tested by one-way analysis of variance (ANOVA) F-test. If the F-test was significant, post-hoc Tukey's t-tests were performed for pairwise comparisons of group means. Significance was defined as $p < 0.05$.

Results

Polymer synthesis and microparticle fabrication

The chemical structures of SA, CPH, and CPTEG are shown in Fig. 1A. NMR spectroscopy was used to confirm that the desired compositions of the copolymers (i.e., 50:50 CPH:SA and 50:50 CPTEG:CPH) were synthesized. Next, microparticles were fabricated by cryogenic atomization and typical yields for both chemistries were ~60%, which is consistent with previous work [29]. The morphology of the microparticles was analyzed with SEM and microparticles of both chemistries exhibited a relatively smooth surface (Fig. 1B), which is consistent with previously published observations [19,35,42]. An analysis of particle size distribution revealed that more than 50% of the polyanhydride microparticles used in this study were below 10 μm in diameter (data not shown). This particle size has been reported to be readily phagocytosed by APCs [36]. More specifically, the mean diameter of the 50:50 CPH:SA microparticles was $10 \pm 5 \mu\text{m}$ and that of the 50:50 CPTEG:CPH microparticles was $13 \pm 8 \mu\text{m}$.

Efficient uptake and intracellular delivery of microparticles to DC lysosomes

After ensuring that polyanhydride microparticles were not contaminated with endotoxin, initial experiments were performed to determine the best microparticle-DC incubation conditions to accurately visualize intracellular microparticles. These studies revealed that incubating DCs with microparticles for 30 min at 37 $^{\circ}\text{C}$ without agitation, centrifugation, or opsonization of particles introduced the fewest staining artifacts and allowed for detailed microscopic analysis of particle uptake and intracellular localization. Non-adherent and extracellular microparticles were effectively removed by PBS washes at 30 min to establish the time zero for “post-internalization” analysis of the microparticles. At 2 h, large phagosomes positive for the lysosomal membrane protein Lamp1 contained individual microparticles of 50:50 CPH:SA or 50:50 CPTEG:CPH chemistries (Fig. 2A). Staining of replicate samples revealed that the Lamp1⁺ vesicles harboring the particles were also acidic (data not shown). At 2 h, approximately 25% of DCs had engulfed microparticles for each of the chemistries (data not shown). Although no difference in the percentage of DCs positive for microparticles was observed between the two chemistries, the number of microparticles internalized was significantly different. Specifically, the number of 50:50 CPTEG:CPH microparticles internalized was higher than that for 50:50 CPH:SA microparticles. This result is consistent with a previous study from our laboratories in which we demonstrated that the hydrophobicity of particles influenced their uptake by monocytes [27].

Intracellular microparticles were also observed at 48 h and the majority of the microparticles remained inside a Lamp1⁺ phagolysosomal membrane (Fig. 2B). DCs appeared healthy and shared a similar morphology with cells that had not phagocytosed microparticles and with cells not cultured in the presence of microparticles (data not shown). Within 48 h, the shape of phagocytosed microparticles changed in a chemistry-dependent manner without appreciable loss of particle fluorescence. Initially observed by epifluorescence and confirmed by confocal microscopy, the surface-eroding 50:50 CPH:SA microparticles retained uniform shapes while 50:50 CPTEG:CPH microparticles appeared more irregular in shape, while remaining sufficiently intact to be readily visible as a microparticle within cells (Fig. 2B). These results are consistent with the degradation profiles of both polymers; our previous work has shown that 50:50 CPH:SA copolymers were largely intact after 48 h [16], whereas 50:50 CPTEG:CPH copolymers exhibited a significant molecular weight loss in aqueous media for over the same incubation period [15]. During confocal imaging, the central portion of microparticles frequently exhibited either partial or complete absence of FITC fluorescence, despite the fact that the surfaces of all of these particles were highly fluorescent (Fig. 2). This phenomenon was observed for both chemistries and observed at

both 2 and 48 h post-internalization. Based on confocal images, surface fluorescence typically extended from 0.5 to 1 μm into the intact microparticle. In separate experiments, these results were also seen with microparticles not incubated with cells (data not shown).

Microparticles enhance DC expression of MHC II and co-stimulatory molecules

To assess activation of CD11c⁺ DCs by 50:50 CPH:SA or 50:50 CPTEG:CPH endotoxin-free microparticles, flow cytometry was used to measure levels of cell surface expression of the major histocompatibility complex molecule class II (MHC II), the co-stimulatory molecules CD86 and CD40, and the C-type lectin CIRE (the murine homologue of human DC-SIGN). A significant upregulation was observed in the MHC II mean fluorescence intensity (MFI) on DCs cultured with microparticles as compared to unstimulated DCs (Fig. 3A). Moreover, DCs cultured with 50:50 CPTEG:CPH microparticles expressed significantly greater levels of MHC II than those stimulated with 50:50 CPH:SA microparticles. Both polymer chemistries equally enhanced the surface expression of the co-stimulatory molecules CD86 and CD40 (Fig. 3B and 3C). In contrast, surface expression of CIRE, a molecule important for pathogen uptake and DC migration, was only significantly increased over the unstimulated cultures by the less hydrophobic 50:50 CPTEG:CPH microparticles. Taken together, these results indicate that both 50:50 CPH:SA and 50:50 CPTEG:CPH microparticles were able to significantly enhance the expression of key surface markers that are involved in DC maturation and antigen presentation.

Polyanhydride microparticles induce DC cytokine secretion

In addition to antigen presentation and T cell activation, DCs also modulate immune responses via the cytokines they secrete. After a 48 h culture with microparticles, only 50:50 CPH:SA microparticles enhanced DC secretion of IL-12p40 and IL-6 in comparison to the unstimulated DCs (Fig. 4). The presence of the cytokines TNF- α , IL-10, and IL-4 in culture supernatants were below the limits of detection when DCs were stimulated with either formulation of polyanhydride microparticles, but were detectable when DCs were stimulated with LPS, lipotechoic acid, or monophosphoryl lipid A (data not shown) as previously reported in other studies [37]. In contrast to the data obtained for surface expression of MHC II and DC co-stimulatory molecules, hydrophobicity of the microparticle formulations significantly influenced DC cytokine production.

Polyanhydride chemistries differentially impact T cell proliferation

To assess the effect of polymer chemistry on the ability of DCs to prime naïve lymphocytes, unfractionated lymphocytes from OT-I and OT-II transgenic mice were co-cultured with ovalbumin-pulsed DCs either stimulated with microparticles and ovalbumin or with ovalbumin only. Approximately 90% of the CD8⁺ T cells from OT-I mice express a clonotypic T cell receptor that recognizes residues 257-264 of ovalbumin while approximately 90% of the CD4⁺ T cells from OT-II mice recognize residues 323-339 of ovalbumin [38,39]. A significantly enhanced antigen-specific proliferative response was observed when OT-I lymphocytes (CD8⁺ T cells) were incubated with DCs stimulated with 50:50 CPTEG:CPH microparticles plus ovalbumin as compared to ovalbumin alone or 50:50 CPH:SA microparticles plus ovalbumin (Fig. 5A). In contrast, antigen-specific OT-II lymphocyte (CD4⁺ T cell) proliferation was significantly increased in the presence of DCs stimulated with 50:50 CPH:SA microparticles plus ovalbumin but not 50:50 CPTEG:CPH microparticles plus ovalbumin (Fig. 5B). These data clearly demonstrate a role for particle chemistry in enhancing both antigen processing and presentation in the context of antigen-specific T cell responses.

Discussion

Adjuvants provide much-needed immunostimulatory properties to vaccine preparations by either acting as a depot to prevent rapid antigen clearance, by delivering the vaccine antigen to APCs for uptake, processing, and presentation, and/or by inducing APC expression of the co-stimulatory molecules necessary for T cell activation [4-6]. The data presented herein demonstrate that polyanhydride microparticles were efficiently phagocytosed by DCs, localized within the phagolysosomal compartment, and subsequently activated DCs *in vitro*. The combination of a rapid and efficient intracellular delivery vehicle along with strong DC activation potential supports the concept that these materials have intrinsic adjuvant properties and excellent potential for vaccine delivery.

The microparticles were efficiently phagocytosed by immature DCs within 30 min after addition to the culture without the addition of opsonins, culture centrifugation, or agitation to enhance uptake (Fig. 2A). DC internalization of microparticles is clearly a phagocytic event. We observed large amounts of polymerized actin at the surface of the cell, immediately juxtaposed to the microparticle contacting the plasma membrane (data not shown). The exact mechanism of internalization is the subject of ongoing research. The microparticles were observed to persist intracellularly for at least 48 h within a phagolysosomal compartment. During this time, erosion of the particles was evident by the shift in average particle size from 2 to 48 h. Related to particle erosion was the apparent caging/uncaging phenomenon of FITC fluorescence observed with both the chemistries. It was frequently noted using confocal microscopy that the center portion of FITC positive microparticles did not emit fluorescence. While FITC-dextran was uniformly dispersed throughout the particle regardless of chemistry, the CPH:SA microparticles appeared hollow when observed under fluorescence microscopy (Fig. 2). Microparticles composed of both chemistries produced images showing that there was brighter fluorescence at the surface of the particle than its core. The enhanced surface fluorescence of FITC suggests that the surface of the particle presents a unique microenvironment that changes the fluorescence spectra of FITC to one characterized by either ablation of excitation or emission of photons of light. The phenomenon of spatial variation of fluorescence characteristics in microparticles is mostly likely related to “molecular caging.”

Polyanhydride microparticles may also function as adjuvants by enhancing APC expression of key molecules involved in antigen presentation and T cell co-stimulation. Indeed, these particles enhanced DC surface expression of MHC II (Fig. 3A), a molecule critical for presentation of antigen to and the activation of naïve CD4 helper T cells. Both polymer chemistries studied similarly enhanced DC expression of the T cell co-stimulatory molecules CD86 and CD40 (Figs 3B and 3C). CD86, a member of the B7 family of co-stimulatory molecules, is upregulated following activation of DCs by microbial stimuli and promotes T cell survival and proliferation [40]. The co-stimulatory molecule CD40 binds to CD154 expressed predominantly on T cells and promotes bidirectional signaling that amplifies the activation status of both the antigen presenting cell and the lymphocyte [41]. In addition, polyanhydride microparticles enhanced the T cell priming capacity of DCs, as evidenced by increased antigen-specific CD4⁺ and CD8⁺ T cell proliferation (Fig. 5).

Differential effects of particle chemistry were observed throughout these studies. Only stimulation with 50:50 CPH:SA microparticles enhanced DC secretion of the cytokines IL-12p40 and IL-6 (Figs. 3 and 4). Particle hydrophobicity also influenced T cell responses. Amphiphilic 50:50 CPTEG:CPH microparticles induced a greater antigen-specific CD8⁺ T cell proliferative response while the more hydrophobic 50:50 CPH:SA microparticles enhanced antigen-specific CD4⁺ T cell proliferation *in vitro*. With these observations, it is tempting to speculate that 50:50 CPH:SA and 50:50 CPTEG:CPH are recognized by

different DC receptors and/or trigger unique signaling pathways resulting in differential DC phenotypes that preferentially present antigenic peptides in the context of MHC I or MHC II. This leads to the hypothesis that polyanhydride microparticles are internalized by immature DCs after being recognized by distinct pathogen-recognition receptors on their surface. Taken together, these results indicate that polyanhydride particles possess intrinsic adjuvant properties, and that immune modulation can be achieved by altering the chemistry of the polymer adjuvants.

Other work from our laboratory has shown that polyanhydride chemistry also affects the release kinetics and stability of encapsulated immunogens [42-44]. In those studies, amphiphilic particles rich in CPTEG provided an environment that favored enhanced antigen stability. This observation is consistent with previous reports in which carriers containing both hydrophobic and hydrophilic domains provided superior conditions for protein stabilization [45,46]. The amphiphilic nature of these CPTEG-containing materials results in the presence of a combination of surface (as seen with the 50:50 CPH:SA copolymers) and bulk (like that of PLGA) erosion.

Relative to previously published studies, an important advantage of biodegradable polyanhydride microparticles over other microparticle adjuvants (e.g., poly(lactic-co-glycolic acid) or PLGA) lies in the lower amount of copolymer required to significantly enhance DC activation [47-49]. Specifically, the ratio of polyanhydride microparticles to DCs (1:12) used in our studies was markedly lower (by as much as 50X) compared to the amounts of polyester microparticles used in other published studies to elicit DC activation [48]. In other studies, the addition of the adjuvant MPLA (a known TLR4 ligand) was required in order for PLGA microparticles to facilitate the activation of immune cells [47]. The ability to use a low dose of polyanhydride microparticles without the addition of stabilizers, excipients, or immune modulators is particularly favorable when considering its use in vaccine design, as the induction of more robust immune responses while using a minimum amount of adjuvant would be desirable.

The data presented herein strongly support the concept that polyanhydrides particles are potential vaccine adjuvants. Only a small number of activated DCs are sufficient to induce strong immune responses [50]. Small amounts of materials derived from either pathogens or damaged cells, are capable of triggering the activation of innate immune cells through Toll-like receptors, C-type lectin receptors and other pattern recognition receptors [51]. Matzinger and colleagues have proposed that hydrophobic molecules may also trigger signal transduction pathways via interactions with pattern recognition receptors [51]. Polyanhydride particles are relatively hydrophobic, especially when compared to sugars and lipids, and it is likely that DCs were activated by the hydrophobic characteristics of the polymers. Moreover, this study demonstrated that the extent of activation is further influenced by the individual chemistries. We suggest that an optimal vaccine formulation might consist of a cocktail of microparticles of different compositions to balance antigen release kinetics [19,20], antigen stability [9,29,42,44], and DC activation phenotype in order to facilitate the development of a robust immune response tailored to a specific immunogen, pathogen, or disease.

Conclusions

Currently, aluminum salts and monophosphoryl lipid A are two of the adjuvants approved for use in human vaccines and provide only a one-size-fits-all approach to enhancing immunogenicity. These compounds are not tunable and, as many pathogens have evolved to evade the host immune response, using such approaches may not induce adequate protective immunity. The next generation of efficacious vaccine adjuvants must possess the ability to

be tailored to generate the optimal type of immune response to and provide protection against a particular pathogen. The results of this study demonstrate that polyanhydride microparticles activate DCs, supporting the concept that these materials have intrinsic adjuvant properties. Moreover, DC cytokine secretion and the priming of specific T cell subsets were further influenced by individual microparticle chemistries. These data indicate that polyanhydride particles can be tailored to take advantage of the potential plasticity of the immune response, resulting in the ability to induce immune protection against many types of pathogens.

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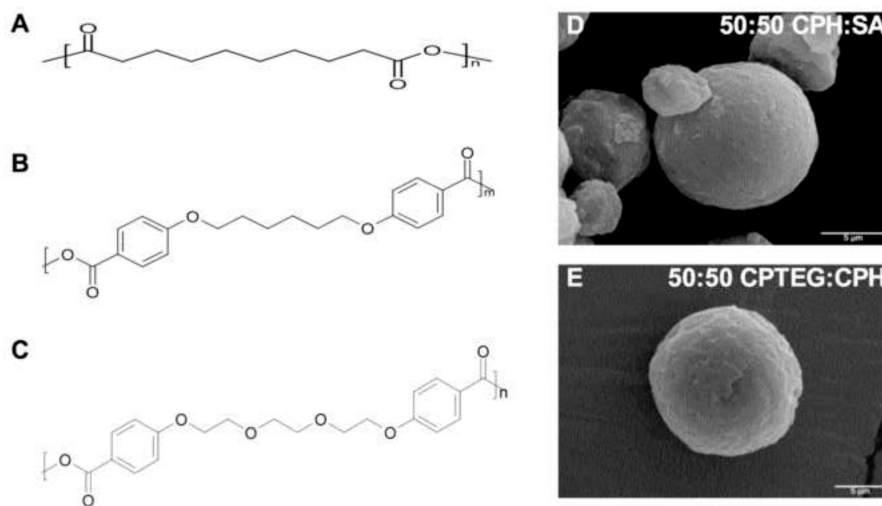


Figure 1. (A) Chemical structures of (i) poly(SA), (ii) poly(CPH), and (iii) poly CPTEG. Here, n and m represent degree of polymerization. (B) Scanning electron photomicrographs of 50:50 CPH:SA and 50:50 CPTEG:CPH microparticles. Scale bar: 5 μ m.

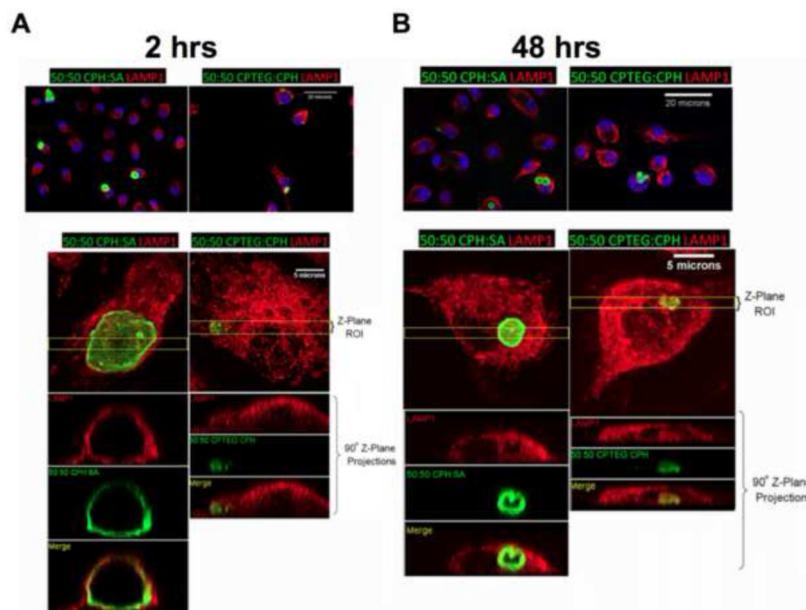


Figure 2. Efficient uptake and intracellular delivery of microparticles to DC lysosomes. Laser scanning confocal microscopy of DCs (blue nuclei) at (A) 2 and (B) 48 h post-internalization demonstrated that 50:50 CPH:SA and 50:50 CPTEG:CPH microparticles (green) are localized within phagosomes positive for the lysosomal membrane protein Lamp1 (red). Representative wide field views of monolayers are shown in the top panels to allow for relative comparisons of cell health and morphology and changes observed over time (scale bar = 20 μm). Confocal image data sets were collected to construct 0 6 (top-down view) and 90 6 Z-plane projections (side-view) for each sample (scale bar = 5 μm). A 2 μm wide slice of the image data set (yellow box) was used to generate the 90 6 Z-plane projections. Both wide field and Z-plane images shown are representative of five fields of view analyzed for each chemistry and consistently observed over three independent experiments. ROI = region of interest.

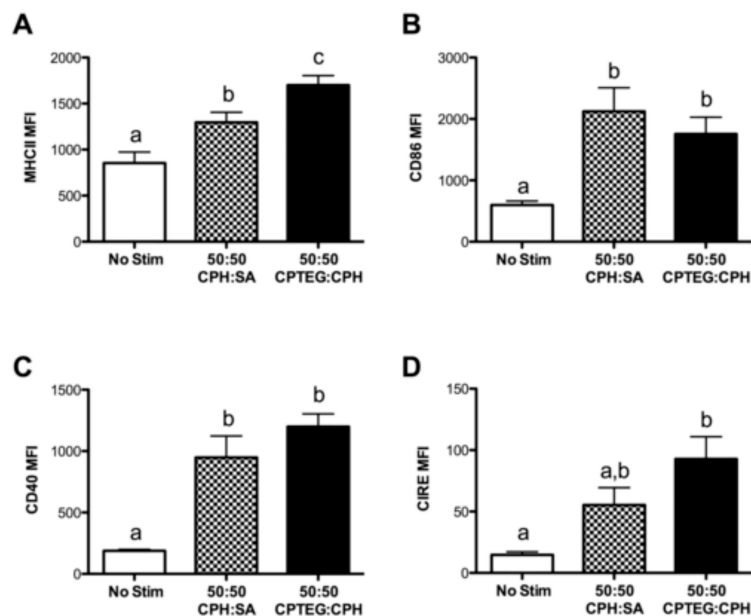


Figure 3. Microparticles enhance DC expression of MHC II and co-stimulatory molecules. DCs were stimulated with either 50:50 CPH:SA or 50:50 CPTEG:CPH microparticles or LPS or left unstimulated for 48 h. DCs were harvested and analyzed via FACS for surface expression of (A) MHC II, (B) CD86, (C) CD40, or (D) CIRE. Data are expressed as the mean \pm the SEM of three independent experiments. Treatments with different letters are significantly different from one another at $p < 0.05$. MFI = mean fluorescence intensity. Mean MFI for DCs stimulated with LPS (200 ng/mL): MHC II = $2,009 \pm 303$; CD86 = $4,120 \pm 1,235$; CD40 = $6,739 \pm 1,552$; CIRE = 27 ± 10 .

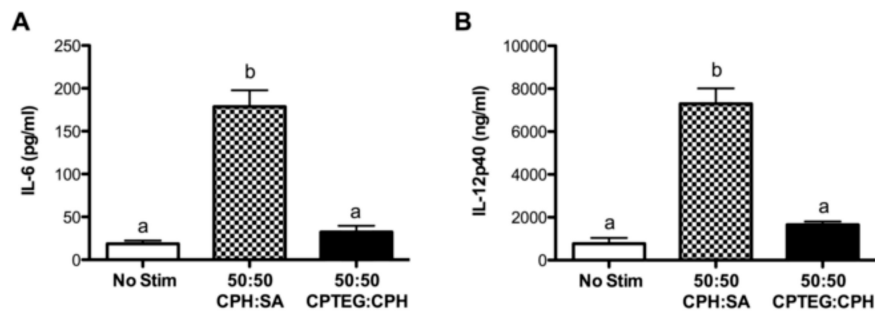


Figure 4.

Polyanhydride microparticles induce DC cytokine secretion. DCs were stimulated with either 50:50 CPH:SA or 50:50 CPTEG:CPH microparticles or LPS or left unstimulated for 48 h. Supernatants were harvested and assayed for (A) IL-6 and (B) IL-12p40. Data are expressed as the mean \pm the SEM of four independent experiments. Treatments with different letters are significantly different from one another at $p < 0.05$. Mean cytokine production for DCs stimulated with LPS (200 ng/mL): IL-6 = $14,387 \pm 4,928$ pg/ml; IL-12p40 = $33,321 \pm 8,851$ pg/ml.

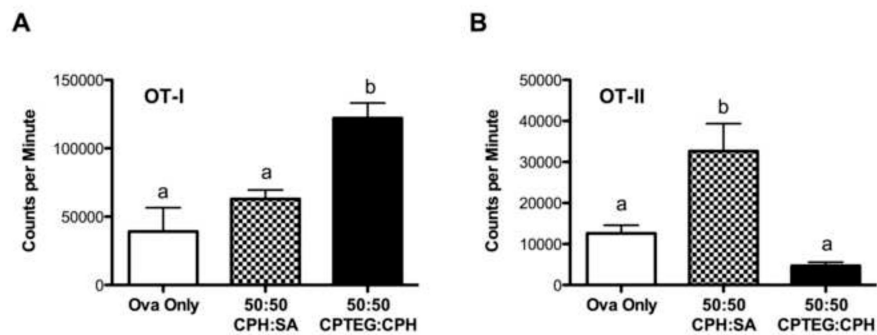


Figure 5. Polyanhydride chemistries differentially impact antigen-specific T cell proliferation. Unfractionated lymph node cells were isolated from either (A) OT-I (CD4⁺ T cells) or (B) OT-II (CD8⁺ T cells) mice and co-cultured with DCs either stimulated with 50:50 CPH:SA or 50:50 CPTEG:CPH microparticles plus ovalbumin or with ovalbumin (Ova) only for 72 h. Proliferation was assessed via ³H-thymidine incorporation. Data from one representative experiment of three independent experiments is shown and is expressed as the mean ± the SEM of replicate wells. Treatments with different letters are significantly different from one another at p < 0.05.