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Development and Evaluation of Biodegradable Particles Co-loaded with Antigen and the Toll-like Receptor Agonist, Pentaerythritol Lipid A, as a Cancer Vaccine

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Abstract

Immune adjuvants are important components of current and prospective cancer vaccines. In this study we aimed at evaluating the use of a synthetic lipid A derivative, pentaerythritol lipid A (PET lipid A), loaded into poly(lactic-co-glycolic acid) (PLGA) particles, as a potential cancer vaccine adjuvant. PLGA particles (size range: 250 – 600 nm) were successfully formulated to include PET lipid A and/or the model tumor antigen, chicken ovalbumin (OVA). It was shown that particulated PET lipid A had a distinct advantage at promoting secretion of the immune potentiating cytokine, IL-12p70, and upregulating key costimulatory surface proteins, CD86 and CD40 in murine dendritic cells in vitro. In a murine tumor model, involving prophylactic vaccination with various permutations of soluble versus particulated formulations of OVA with or without PET lipid A, modest benefit was observed in terms of OVA-specific cell-mediated immune responses when PET lipid A was delivered in particles. These findings translated into a corresponding trend toward increased survival of mice challenged with OVA-expressing tumor cells (E.G7). In terms of translation of safe adjuvants into the clinic, these results promote the concept of delivering Toll-like receptor-4 (TLR-4) agonists in particles since doing so improves their adjuvant properties, whilst decreasing the chances of adverse effects due to off-target uptake by nonphagocytic cells.

Keywords

vaccine adjuvants; cancer vaccine; PET lipid A

1. Introduction

Immunostimulatory adjuvants are an important inherent or added component of many vaccines whether developed for infectious disease or cancer [1]. To date in the United States only two adjuvants are currently FDA-approved and being actively used in vaccines: alum and monophosphoryl lipid A (MPLA). Alum, whilst being a well-established adjuvant for

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the generation of antibody responses, is a poor stimulator of the cellular arm of the immune system, or T helper type-1 (Th1) responses, which are considered vital for the generation of effective anti-tumor immune responses [2–5]. In contrast, MPLA, a less toxic derivative of lipid A, is capable of stimulating strong Th1 responses depending on the antigen and route of administration used [6]. More potent and even safer versions of lipid A have been sought as potential vaccine adjuvants of which PET lipid A is a strong candidate [7]. PET lipid A has advantages over MPLA in terms of quality control and batch to batch consistency since it is fully synthetic, whilst MPLA is a natural derivative of gram negative bacteria such as *Salmonella Minnesota* [8, 9].

Lipid A's and their derivatives exert their adjuvant activity through TLR-4. This receptor, expressed on the surface of dendritic cells (DCs) in association with auxiliary proteins and MD-2 (a co-receptor), can induce the production of a range of immune-related genes (e.g. IL-6, IL-12, TNF-alpha and IL-10) upon being triggered by a TLR-4 agonist [10, 11]. Lipid A itself is the lipid component of lipopolysaccharide (LPS) and accounts for most of the adjuvant activity as well as toxicity of LPS [12, 13]. However, lipid A and LPS, despite promoting strong inflammatory responses, are considered too toxic for clinical use and thus modified versions of lipid A have been sought [14]. One of the first LPS derivatives to have been developed, MPLA, has potent adjuvant capacity yet is orders of magnitude less toxic than lipid A due to the removal of an *O*-1-position phosphate group [15, 16]. There are many other modified or synthetic lipid A derivatives that have been developed and tested for their vaccine adjuvant potential, examples of which are glucopyranosyl lipid adjuvant (GLA) and 7-acyl lipid A, with the primary goal being to create structurally altered versions of lipid A that have lost their toxicity while retaining their important immune stimulating properties [7, 17–20]. PET lipid A is a fully synthetic TLR-4 agonist and a simplified method for its synthesis was described by Jiang *et. al* [8]. Structurally PET lipid A contains an amine-substituted pentaerythritol instead of a glucosamine unit found in natural lipid A, and, like MPLA, retains only one phosphoryl group [8]. PET lipid A also retains the hexaacylated chain structure, which is required for binding to the TLR-4 signaling complex via MD-2. To date, there has been little investigation into PET lipid A as a potential cancer vaccine adjuvant, and thus, here, an immunogenic murine tumor model was used to explore this possibility.

Besides the importance of the inherent immunostimulatory properties of an adjuvant, the vehicle in which it is delivered can also affect the net immune response. Adjuvants delivered in soluble form are considered less efficient and require higher doses than if delivered in discrete packages. In addition, since TLR4 agonists, under certain circumstances, can promote tumor progression [11, 21], packaging them into submicron sized particles will preferentially target them to phagocytic cells, such as DCs and macrophages, thus potentially rendering them both safer and more efficient than their soluble counterparts. Polymeric particles have been proven to be a valuable means of delivering antigen and/or adjuvant for vaccine applications [22, 23]. The use of polymeric particles in cancer vaccine offers several advantages, examples of which are: 1) protecting the antigen and/or the adjuvant from premature degradation, 2) providing sustained release of the loaded cargo, 3) improving the induced immune responses in comparison to soluble antigen and/or adjuvant

[24], and 4) reducing the antigen and or adjuvant dose required [25–28]. Using PLGA polymers in fabricating particles offers additional advantages in that they are biodegradable, biocompatible and FDA approved [29]. This study investigates polymer-based particle formulations loaded with PET lipid A for cancer vaccine applications. In vitro activation of DCs, in vivo induction of antigen-specific antibody and cell-mediated responses, as well as tumor protection studies, were performed to evaluate the potential of PET lipid A-loaded PLGA particles as cancer vaccine adjuvants.

2. Materials and Methods

2.1 Particle preparation and characterization

PLGA particles co-loaded with OVA and PET lipid A (PLGA (OVA+PET lipid A)) or loaded with OVA alone (PLGA (OVA)) or PET lipid A alone (PLGA (PET lipid A)) were prepared using a double emulsion-solvent evaporation method [7, 30]. In brief, for PLGA(OVA+PET lipid A) particles, 2 mg of purified endotoxin-free OVA was dissolved in 50 μ l 1% w/v poly(vinyl alcohol) (PVA) (Mowio 8-88IR; Sigma, Allentown, PA) as water phase 1 and was emulsified in 800 μ l of the organic phase. The organic phase was composed of 100 μ l 1:40 methanol:chloroform solution containing 1.2 mg PET lipid A (generously provided by Oncothyreon) and 700 μ l chloroform in which 100 mg PLGA (Resomer RG 503) was dissolved. The emulsion was performed using a sonic dismembrator equipped with an ultrasonic converter probe operated at 40% amplitude for 30 seconds. This primary emulsion was then emulsified into 4 ml of 9% w/v PVA in phosphate buffer saline (PBS, pH 7.4) solution at 60% amplitude for 2 minutes. The secondary emulsion was added to 16 ml of 5% w/v PVA in PBS and stirred in a fume hood for 1 hour. Chloroform was then completely removed by evaporation under reduced pressure (46 mbar) over a period of 3 hours using a rotary evaporator. Particles were collected by sequential centrifugation as previously described [31]. To ensure narrow size distribution, particle suspensions were first centrifuged at 800 $\times$ g for 3 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged at 10,000 $\times$ g for 10 minutes at 4°C to collect the pelleted particles used in this study. Collected particles were washed twice with sterile deionized water, frozen at –80°C, and lyophilized overnight (at –54°C, 0.08 mBar). Dry particles were stored at –20°C. PLGA(OVA) particles were prepared by the same method with the organic phase composed of 100 mg PLGA in 800 μ l chloroform. PLGA(PET lipid A) particles were prepared by using 1% w/v PVA as water phase 1 and 0.6 mg PET lipid A in the organic phase.

Particle size and zeta potential were determined from measurements made on particles suspended in deionized water using Zetasizer Nano ZS (Malvern, Southborough MA). Particle morphology was examined using scanning electron microscopy (SEM). A suspension of lyophilized particles in deionized water was placed onto a silicon wafer mounted on an aluminum stub and left to dry in a fume hood. Samples were coated with gold-palladium using Argon beam K550 sputter coater and imaged at 1.5 Kv accelerating voltage using a Hitachi S- 4800 SEM.

2.2 Loading estimations

5–10 mg particles were degraded in 1 ml 0.3N NaOH until a clear solution was obtained. This solution was neutralized with 1 N HCl. Loading was then calculated using equation 1.

$$\text{loading } (\mu\text{g}/\text{mg particles}) = \frac{\text{concentration } (\mu\text{g}/\text{mL}) \times \text{sample volume (mL)}}{\text{particles weight (mg)}} \quad \text{Eq. 1}$$

To estimate OVA loading in prepared particles, OVA concentration in the neutralized solution was measured using a micro-BCA assay kit according to the manufacturer's instructions. Blank unloaded particles were used as a control for OVA loaded particles. The possible interference of lipids with the protein measurements of samples of PLGA(OVA +PET lipid A) particles was eliminated by the use of 2% sodium dodecyl sulfate (SDS) [32]. To estimate PET lipid A loading in the prepared particles, PET lipid A concentration in the neutralized solution was measured using Limulus Amebocyte Lysate LAL endotoxin kit (Kinetic-QCL 192 test kit, LONZA) according to the manufacturer's instructions [33]. In parallel, aqueous PET lipid A solution in endotoxin free water, at serially diluted concentrations, was assayed to establish a calibration curve. Blank (unloaded) particles and PLGA(OVA) particles were used as controls for PLGA(PET lipid A) and PLGA(OVA+PET lipid A) particles, respectively.

2.3. In vitro dendritic cell stimulation

bone marrow derived dendritic cells (BMDCs) were generated as described previously [34]. BMDCs were maintained in RPMI media supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 1 mM sodium pyruvate, 0.1 mM minimal essential medium non-essential amino acids, and 2 mM GlutaMAX, 50 nM 2- mercaptoethanol, and 50 ng/ml gentamicin sulfate. Upon addition to cells, the media was also supplemented with granulocyte macrophage-colony stimulating factor, GM-CSF, at a final concentration of 20 ng/ml. On day 9 of culture, BMDCs were harvested and plated in a 12-well moat cell culture tray at a density of 3.5×10^5 cells/well. PET lipid A was added at 1 or 5 μg per well or in PLGA particles delivering a total dose of 1 μg per well. Blank controls involved incubating BMDCs with the same amount of unloaded PLGA particles as used for the PET lipid A loaded particles. BMDCs incubated with culture media only were also used as a control. Following 24 hours incubation with the designated treatment, cell culture supernatant was collected and analyzed for IL-12, specifically IL-12p70, and IL-10 levels using an enzyme linked immunosorbent assay (ELISA) kit according to product instructions (eBioscience) with a standard curve range of 13–2000 pg/ml and 32–4000 pg/ml for IL-12 and IL-10, respectively. All samples were run in triplicate. Cell viability following incubation was determined using trypan blue dye and was found to be > 95% in all samples.

For CD86 and CD40 upregulation, BMDCs, set-up in triplicate for each treatment, were flushed from the wells and stained with anti-CD11c-FITC and either anti- CD86-PE or anti-CD40-PE (eBioscience) using a standard direct immunofluorescence procedure. Controls involved staining BMDCs with FITC or PE isotype antibodies. Samples were measured using a FACScan flow cytometer (Becton Dickinson, NJ) and CD11c positive cells

(dendritic cells) were analyzed for levels of expression of CD40 and CD86 using FlowJo software (TreeStar, OR).

2.4 Animal vaccination and tumor protection study

Eight to twelve week-old female wild type C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) (n = 5 per group) were assigned to one of the eight groups involved in the study: 1. Naive (no treatment), 2. OVA solution (OVA s), 3. OVA solution + PET lipid A solution (OVA s + PET lipid A s), 4. OVA solution + PLGA(PET lipid A) particles (OVA s + PET lipid A p), 5. PLGA(OVA) particles (OVA p), 6. PLGA(OVA) particles + PET lipid A solution (OVA p + PET lipid A s), 7. PLGA(OVA) particles + PLGA(PET lipid A) particles (OVA p + PET lipid A p), 8. PLGA(OVA+PET lipid A) particles (OVA/PET lipid A p). Designated treatments were administered subcutaneously in 100 μ l sterile PBS in the right flank. For mice in groups 3, 4, and 6, OVA and/or PET lipid A solutions and the designated particles were admixed immediately prior to injection. Each mouse was vaccinated on both day 0 and day 7 with the designated formulation. When applicable, OVA was administered at a dose of 60 μ g and PET lipid A was administered at a dose of 18 μ g. On day 14 post-prime, the frequency of OVA-specific T lymphocytes in peripheral blood was measured (see below). On day 28 post-prime, OVA-specific IgG_{2c} and IgG₁ antibody titers were measured in serum harvested by submandibular bleeds (see below). On day 36 post-prime, mice in selected treatment groups were subcutaneously challenged with 2×10^6 OVA-expressing E.G7 cells (American Type Culture Collection, Manassas, VA) and tumor volumes were monitored over time using a caliper. Tumor volumes were determined using the following formula: $W \times L \times H \times \pi / 6$. Animal handling and use was performed according to the University of Iowa Institutional Animal Care and Use Committee guidelines. When needed, animals were anesthetized by injecting, intraperitoneally, 87.5mg/kg ketamine and 12.5mg/kg xylazine.

2.5. Levels of OVA-specific CD3+ CD8+ T lymphocytes in peripheral blood

Blood samples were collected via submandibular bleeding on day 14 post-prime. The frequency of OVA-specific CD3+ CD8+ T lymphocytes was determined by tetramer staining and direct immunofluorescence, as previously described [35]. The tetramer used was the H-2Kb SIINFEKL Class I iTagTM MHC Tetramer (Kb-OVA257) labeled with PE (MBLI, Woburn, MA). Surface CD8 and CD3 were stained with FITC-labeled rat anti-mouse CD8 (eBioscience) and PE-Cy5-labeled hamster anti-mouse CD3 (eBioscience) antibodies, respectively. Samples were acquired using a FACScan flow cytometer and analyzed with FlowJo software. Results are expressed as percentage of total CD3+ CD8+ T lymphocytes in peripheral blood that were positive for tetramer staining.

2.6. Estimation of anti-OVA antibody titers in sera using an ELISA

OVA-specific IgG₁ and IgG_{2c} antibody titers in sera derived from the peripheral blood of variously vaccinated mice were quantified using ELISA [31]. Briefly, serum samples were collected via submandibular bleeding and serially diluted in ImmulonR 2HB 96-well high binding polystyrene microtiter plates (Thermo, Milford, MA) that had been previously coated with 5 μ g/mL OVA solution in PBS. Following overnight incubation, plates were washed with 0.05% tween in PBS and incubated with the appropriate alkaline phosphatase

conjugated anti-mouse IgG antibodies for 3 hours. Excess antibody was washed away followed by the addition of p-nitrophenylphosphate and then a short incubation (approximately 15 minutes) in the dark. Absorbance at 405 nm was recorded using a SpectraMaxR Plus384 microplate reader when the least diluted sample reached an absorbance value of 1.2 – 1.8.

2.7. Statistical analysis

data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test unless otherwise stated. Differences were considered significant at p-values < 0.05. Survival data were analyzed using the log-rank test. Statistical analyses were performed using GraphPad Prism 5 software.

3. Results

3.1 Particle preparation and characterization

Particles were prepared using a double emulsion solvent evaporation method as described in the methods section. All prepared particles had a spherical shape and smooth surface as revealed by SEM images (Figure 1). Particles possessed sizes ranging from 250 to 600 nm in diameter and comparable zeta potentials for the different formulations (Table 1). OVA and PET lipid A were co-loaded successfully to provide a sufficient vaccine dose (Table 1).

3.2 In vitro BMDC activation and cytokine secretion

BMDCs were cultured in the presence of soluble PET lipid A (1 µg/well and 5 µg/well) or PLGA(PET lipid A) particles (1 µg/well of PET lipid A). After 24 hours, cells and supernatants were harvested to measure cell surface expression (CD86 and CD40) and cytokine (IL-12p70 and IL-10) secretion, respectively. Results of surface staining of BMDCs showed that PLGA(PET lipid A) particles promoted the upregulation of both CD86 and CD40 to a significantly greater degree than soluble PET lipid A used at the equivalent dose (Figure 2). When the soluble PET lipid A dose was increased to 5 µg/well an increased expression of the costimulatory molecules was noted compared to 1 µg/well of soluble PET lipid A, however, it was not as effective as 1 µg of PET lipid A delivered in particles. This was significantly so for CD86 (Figure 2(i)). Using ELISA, it was shown that PLGA(PET lipid A) particles (1 µg/well) were significantly more efficient at stimulating IL-12p70 secretion than soluble PET lipid A (1 µg/well or 5 µg/well) (Figure 3). PLGA(PET lipid A) particles (1 µg/well) also increased, albeit not significantly, the amounts of IL-10 secreted when compared to soluble PET lipid A (1 µg/well).

3.3 Effect of incorporating PET lipid A on the immunogenicity of OVA based vaccines

All particle formulations resulted in higher in vivo OVA-specific antibody titers compared to administering OVA and/or PET lipid A in solution as measured by OVA-specific IgG_{2c} and IgG₁ isotype levels in sera of vaccinated mice by ELISA (Figure 4). No statistically significant differences in levels of serum anti-OVA antibodies were observed between the different particle-based formulations for either isotype, however, certain trends were noted. For instance, co-loading of PET lipid A and OVA in the same particle resulted in a higher IgG_{2c}/IgG₁ ratio on day 28 compared to other particle-based formulations (Figure 4C). This

enhanced ratio was short-lived however, with all particle formulations generating similar IgG_{2c}/IgG₁ ratios (values in the range of 0.02 – 0.04) by day 35 (data not shown).

When the levels of OVA-specific CD8⁺ T lymphocytes in the peripheral blood of vaccinated mice were analyzed, it was found that vaccination with formulations involving the delivery of both OVA and PET lipid A in particulated form (co-loaded and independently loaded) stimulated the highest levels of OVA-specific CD8⁺ T lymphocytes in the peripheral blood of vaccinated mice at 14 days post prime, and were the only groups to generate statistically significant differences (compared to the soluble OVA treated group) (Figure 5). These levels were, however, still relatively low and did not differ significantly from the naive group. Although not statistically significant, the percent of OVA-specific CD8⁺ T lymphocytes was trending such that vaccination with particle-based formulations stimulated higher levels compared to soluble formulations.

3.4. Tumor protection study

All mice used in the vaccination studies described above were subsequently (28 days after the boost) challenged with live OVA-expressing E.G7 cells. Survival curves showed that vaccinating mice with formulations that contained both particulated OVA and particulated pet lipid A, whether co-loaded or independently loaded, resulted in marginally increased survival compared to other groups, however no significant differences were observed (Figure 6).

4. Discussion

The pool of potential candidate vaccine adjuvants being developed and investigated is continually increasing and those that trigger TLRs are particularly attractive due to their targeting of, and potent effect on, DCs [36, 37]. TLR-4, when triggered by an agonist, stimulates DCs to promote Th1-type immune responses, making such agonists attractive candidates as adjuvants in cancer vaccine formulations [38]. In this study, we examined the potential of using the TLR-4 agonist, PET lipid A, as a cancer vaccine adjuvant delivered in PLGA particles. The reasons for delivering PET lipid A in particles are to increase its stability and to favor phagocytosis by antigen presenting cells, such as, DCs, thus potentiating increased adjuvant efficiency whilst simultaneously reducing its impact on other off-target cell types that may express TLR-4. Polymeric particles have been proven to be a valuable vehicle for delivering antigen and/or adjuvants to DCs to initiate antigen-specific immune responses [39, 40]. Soluble PET lipid A has been shown previously to possess potentially potent adjuvant properties by stimulating CD40 and CD86 up-regulation by murine dendritic cells in a manner comparable to LPS [7]. This group also demonstrated the feasibility of loading PET lipid A into sub-micron sized PLGA particles (350 – 450 nm). In the study presented here we compared the potency of soluble PET lipid A versus PET lipid A-loaded PLGA particles with respect to murine dendritic cell activation in vitro. Both CD86 and CD40 are costimulatory proteins capable of facilitating T cell activation and whose upregulation is indicative of dendritic cell stimulation/maturation [41]. It was shown that, at equivalent doses, 1 µg/well, PET lipid A was significantly better at promoting CD86 and, to a lesser degree, CD40, when delivered in particles. The cytokines, IL-12 and IL-10,

are known for being pro-inflammatory and anti-inflammatory, respectively. IL-12 promotes Th1-type immune responses through the induction of γ -IFN by T lymphocytes whilst IL-10 can block the promotion of Th1-type responses [42–44]. It was observed here that PET lipid A promoted an approximately 5-fold increase in IL-12p70 secretion by DCs when loaded into PLGA particles versus the soluble form ($***p < 0.001$). Whilst IL-10 also showed an increase when particulated versus soluble PET lipid A was compared, the difference was not significant. Also, IL-10 could not prevent the enhanced expression of CD86 and IL-12p70, which are capable of potentiating Th1 responses. The mechanism by which this enhanced benefit occurred in vitro was not determined but may be due to the particles providing sustained release of PET lipid A or that, by being provided in a phagocytosable form, and mimicking pathogenic infection, the particulated PET lipid A more readily stimulated the DCs [45]. Regardless, such a finding suggests the potential application for PET lipid A-loaded particles as an adjuvant in vaccine formulations requiring Th1-type immune responses.

We therefore investigated the ability of various soluble or PLGA-loaded formulations, involving OVA with or without PET lipid A, to stimulate OVA-specific antibody and T lymphocyte responses in vivo. The fact that PET lipid A, whether in soluble form or in particles, did not significantly enhance antibody responses to OVA-loaded particles was not entirely unexpected given that similar findings have been previously observed where PLGA particles co-loaded with hepatitis B core antigen and MPLA (also a TLR4 agonist) were not as effective at generating antigen-specific IgG responses compared to particles loaded with antigen alone or a mix of particles independently loaded with MPLA and antigen [30]. Another earlier study also reported that a liposomal vaccine involving the human tumor associated antigen, MUC-1, and MPLA failed to induce significant MUC-1-specific antibody responses in mice after subcutaneous vaccinations and yet cellular (Th1) immune responses were detected along with enhanced protection from subsequent challenge with MUC-1 expressing tumor cells [46]. Thus, the lack of induction of a strong antibody response is not necessarily an impediment for cancer vaccines. More promisingly, PET lipid A used in this current study, when loaded independently, or co-loaded, into particles with OVA, induced significantly greater OVA-specific CD8⁺ T lymphocyte responses than soluble OVA. These albeit marginal increases in levels of OVA-specific T lymphocytes (as a percent of total CD8⁺ T lymphocytes), which were detected in the peripheral blood, are likely to be further magnified in the tumor microenvironment as we have previously shown using the same tumor model [47], but a different vaccine strategy. Such a finding suggests the possibility of an enhanced antitumor immune status for mice vaccinated with OVA and PET lipid A loaded together or independently into PLGA particles. We therefore challenged all groups of mice used in the vaccination studies with a lethal dose of E.G7 28 days subsequent to the boost. The results from these studies implicated a trend toward improved survival as indicated by marginal, but not statistically significant, increases in areas under the curve, in the survival plots, for mice vaccinated with OVA and PET lipid A loaded together or independently into PLGA particles compared to other treatment groups. Based on our results it could be concluded that co-loading of antigen and adjuvant into particles had little effect on the immunogenicity of the vaccine compared to independently loaded antigen and adjuvant, however, it is possible that size differences in the particle preparations

contributed to a masking of the benefit of co-loaded particles. The co-loaded particles (~600 nm) were larger than the independently loaded particles (~400 nm for OVA-loaded; ~250 nm for PET Lipid A-loaded) and may have been taken up by DCs less efficiently since uptake has been reported to be optimal for particles < 500 nm [48].

Thus we show here for the first time that loading PET lipid A into PLGA particles improves its overall adjuvant properties. This was particularly noticeable with respect to the stimulation of DCs, the professional antigen presenting cells of the immune system. The modest enhancement in antigen-specific T lymphocyte responses and antitumor effects observed in vivo using an immunogenic tumor model suggest there is still room for improvement with regards to the vaccine formulation. Effects of varying particle type or size, or the potential of promoting adjuvant potency by combining PET lipid A with complementary adjuvants need to be investigated. Of particular importance is that PET lipid A adjuvant function was not inhibited by being loaded into particles, which implicates a potential improvement in safety when it comes to translation into the clinic since it reduces exposure of off-target cells (i.e. nonphagocytic cells) to soluble PET lipid A. This is an important consideration in the context of therapeutic cancer vaccines, particularly when tumor cells expressing TLR-4. All together, these results show the potential for PET lipid A to be used as a vaccine adjuvant when loaded into particles.

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Figure 1. Representative SEM images of prepared PLGA particles
A) PLGA(PET Lipid A) particles; B) PLGA(OVA) particles; C) PLGA(OVA+PET Lipid A) particles. Scale bar represents 3 μm .

Figure 2. Comparison of the stimulatory effect of soluble PET lipid A versus PET lipid A-loaded particles on BMDCs in vitro

BMDCs were cultured with indicated formulations for 24 hours and then harvested and stained for markers of DC activation/maturation, CD86 and CD40, using direct immunofluorescence and analyzed by flow cytometry (see methods) i: median fluorescent intensities (MFI) ii: representative histograms derived from flow cytometric analysis demonstrating the upregulation of CD86 and CD40 by BMDCs. Results are plotted as mean $\pm$ SD, $n=3$. *** $p < 0.001$, * $p < 0.05$.

Figure 3. Comparison of IL-12p70 and IL-10 secretion by BMDCs following stimulation with soluble PET lipid A versus PET lipid A-loaded particles
 BMDCs were cultured with indicated formulations for 24 hours and then the supernatant was harvested and analyzed for levels of IL- 12p70 or IL-10 using ELISA (see methods). No significant differences were found between particulated and soluble forms of PET lipid A for IL-10 secretion levels. Results are plotted as mean $\pm$ SD, $n = 3$. *** $p < 0.001$, ** $p < 0.01$.

Figure 4. Titers of IgG_{2c} and IgG₁ OVA-specific antibodies in serum of mice vaccinated with different combinations of OVA and/or PET lipid A formulations

On day 28 post-prime with indicated treatments, OVA-specific IgG₁ (A) and IgG_{2c} (B) titers were measured in sera using ELISA (as described in materials and methods section). Ratio of IgG₁ versus IgG_{2c} on day 28 (C). s = soluble, p = particles. Data are plotted as mean $\pm$ SEM, n=5.

Figure 5. Percent OVA-specific CD8+ T lymphocytes in serum of mice vaccinated with different combinations of OVA and/or PET lipid A formulations

On day 14 post-prime with indicated treatments, OVA-specific CD8+ T lymphocytes levels (expressed as a percentage of CD3+CD8+ cells derived from peripheral blood) were measured as described in the materials and methods section. s = soluble; p = particles. Data are plotted as mean $\pm$ SEM, n=5. * $p < 0.05$.

Figure 6. Efficiency of OVA/PET lipid A formulations in providing protection against tumor challenge

Mice were vaccinated with 60 μg OVA $\pm$ 18 μg PET lipid A in soluble (s) or particle (p) form. Mice were challenged with 2×10^6 E.G7 cells on day 35 post-prime.

Table 1**Particle characterization**

The PLGA particles were formulated using methods described in the materials and methods section.

Formulation	Size $\pm$ SD d, nm	PDI $\pm$ SD	Zetapotential $\pm$ SD, mV	Loading, μ g/mg	
				OVA	PET Lipid A
PLGA (PET Lipid A)	248.30 $\pm$ 46.05	0.54 $\pm$ 0.03	-14.70 $\pm$ 0.21	/	8.2 $\pm$ 0.8
PLGA (OVA+PET Lipid A)	604.70 $\pm$ 56.09	0.20 $\pm$ 0.06	-15.50 $\pm$ 0.32	10.7 $\pm$ 0.8	2.4 $\pm$ 1.2
PLGA (OVA)	389.50 $\pm$ 9.80	0.30 $\pm$ 0.02	-15.80 $\pm$ 0.90	11.2 $\pm$ 0.4	/