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Intracellular Delivery of a Protein Antigen with an Endosomal-Releasing Polymer Enhances CD8 T-Cell Production and Prophylactic Vaccine Efficacy

Suzanne Foster[†], Craig L. Duvall[†], Emily F. Crownover, Allan S. Hoffman, and Patrick S. Stayton^{*}

Department of Bioengineering and the Center for Intracellular Delivery of Biologics, University of Washington, Seattle WA 98195

Abstract

Protein-based vaccines have significant potential as infectious disease and anticancer therapeutics, but clinical impact has been limited in some applications by their ability to generate a coordinated cellular immune response. Here, a pH-responsive carrier incorporating poly(propylacrylic acid) (PPAA) was evaluated to test whether improved cytosolic delivery of a protein antigen could enhance CD8⁺ cytotoxic lymphocyte generation and prophylactic tumor vaccine responses. PPAA was directly conjugated to the model ovalbumin antigen via reducible disulfide linkages and was also tested in a particulate formulation after condensation with the cationic poly(dimethylaminoethyl methacrylate) (PDMAEMA). Intracellular trafficking studies revealed that both PPAA-containing formulations were stably internalized compared to control conjugates and evaded exocytotic pathways, leading to increased intracellular accumulation and potential access to the cytosolic MHC-1 antigen presentation pathway. In an EG.7-OVA mouse tumor protection model, both PPAA-containing carriers robustly inhibited tumor growth and led to an approximately 3.5 fold increase in the longevity of tumor free survival relative to controls. Mechanistically this response was attributed to the 8-fold increase in production of ovalbumin-specific CD8⁺ T-lymphocytes and an 11-fold increase in production of anti-ovalbumin IgG. Significantly, this is one of the first demonstrated examples of *in vivo* immunotherapeutic efficacy using soluble protein-polymer conjugates. These results suggest that carriers enhancing cytosolic delivery of protein antigens could lead to more robust CD8⁺ T-cell response and demonstrate the potential of pH-responsive PPAA-based carriers for therapeutic vaccine applications.

Introduction

There is continuing effort to develop more effective vaccines for infectious disease and as a therapeutic modality for cancer. For protein-based vaccines that represent an outside-in delivery paradigm, a considerable challenge is the intracellular delivery of antigens into the cytosol of antigen presenting cells (APCs) where the class I antigen presentation pathway may be more efficiently accessed. This pathway leads to generation of CD8⁺ and cytotoxic T-lymphocytes (CTLs), which are capable of immunorecognition and direct apoptotic induction in cancer cells(1,2). A diversity of approaches has been explored for delivering

^{*}Corresponding Author: Patrick S. Stayton, Ph.D., University of Washington, Department of Bioengineering, Box 355061, Seattle, WA 98195, Tel: (206) 685.8148, stayton@u.washington.edu.

[†]Equally Contributing Co-First Authors

Supporting Information Available: Table S1: Particle sizing and zeta potential measurements on PPAA-Ova/PDMAEMA ionically complexed particles for a range of $-/+$ charge ratios.

antigens to optimally stimulate CD8⁺ T-cell responses. Incorporation of MHC-1 adjuvants, such as CpG-DNA(3) or toll-like receptor (TLR) ligands(4,5) into carrier formulations has been utilized to promote MHC-1 antigen presentation and induce production of antigen-specific CD8⁺ T-cells. Additionally, recombinant viral vectors have been employed as inside-out strategies for efficiently transfecting target cells(6,7), and fusion with bacterial toxins(8,9) has also been employed to enhance cytosolic delivery of vaccine antigens.

Synthetic carriers for protein antigen delivery have been intensively investigated to circumvent the safety concerns of viral vectors, but their efficiency is generally significantly lower. Liposomal systems (10–13), particles formed from poly(D, L-lactic-co-glycolic acid) (PLGA), and micelle carriers have been investigated for antigen delivery(14–22). There is substantial evidence from these studies that particle and micelle based systems yield a more efficient MHC-1 response relative to soluble conjugates due to uptake via phagocytotic pathways, as well as the possible adjuvancy of PLGA itself(14,15,18,23–25).

The majority of synthetic vaccine delivery vehicles have been designed to explore or exploit the effects of particle size on immune response, but relatively little attention has been paid to other strategies for directing intracellular trafficking of antigens within APCs. Previous studies have employed pH-responsive degradable elements based on poly(γ -glutamic acid)-poly(L-phenylalanine ethyl ester)(26,27), and acid labile particles and microgels that degrade at endosomal pH(28–31). However, pH-responsive polymeric carriers that actively alter vesicular trafficking pathways and promote endosomal escape of protein or peptide antigens have not been previously explored. This study aimed to test whether an endosomal-releasing carrier would increase generation of antigen-specific CD8⁺ T-cells and provide a prophylactic effect in the ovalbumin vaccine model. Poly(propylacrylic acid) (PPAA)-based carriers have been investigated for intracellular delivery of biologic drugs including proteins, peptides, and siRNA(32–40). A PPAA carrier has previously been shown to enhance MHC-1 presentation and specific T-cell activation in an *in vitro* ovalbumin cell culture model (41). The present study further explored the mechanism of PPAA as a protein vaccine carrier and evaluated its efficacy in an *in vivo* mouse tumor protection model that allowed quantitation of the ovalbumin-specific CD8⁺ T-cell response. Both soluble conjugate and particulate PPAA-based formulations were tested and importantly both carriers were able to stimulate specific CD8⁺ T-cell development, antibody production, and significant tumor protection responses *in vivo*. These data show the potential of PPAA-based systems for vaccine delivery, and also show how modulating intracellular trafficking might enhance the effectiveness of induced immune responses.

Experimental Procedures

Polymer Synthesis and Formulation with Ovalbumin

The synthesis and characterization of PPAA and poly(methacrylic acid) (PMAA) polymers and ovalbumin conjugates has been described previously(41). Briefly, PPAA was prepared by bulk free radical polymerization at 60°C using the initiator azobisisobutyronitrile (AIBN). PMAA was prepared as a control polymer with similar structure to PPAA except that, due to its lower pK_a and decreased hydrophobicity, does not enable effective endosomal escape(41). PMAA was prepared by reversible addition fragmentation chain transfer (RAFT) polymerization at 40°C using the chain transfer agent 4-cyanopentanoic acid dithiobenzoate(41) (CPAD) and the initiator 2,2'-Azobis (2,4-dimethyl valeronitrile) (V65) in the absence of oxygen. A Pyridyl disulfide functional acrylate monomer (PDSA) (37) was incorporated into these polymers at 3–5 mol % to provide protein conjugation sites. The polymers were purified by 3 rounds of precipitation from dimethyl formamide into diethyl ether. Characterization was performed by ¹H-NMR and by spectrophotometric quantification of pyridine 2-thione release following reduction with dithiothreitol (DTT).

Polymer molecular weight and polydispersity were determined by gel permeation chromatography (GPC) (Viscotek VE2001 sample module, VE3580 RI Detector, Waters Corp. ultrahydrogel columns) in 0.1 M sodium phosphate buffer, pH 8 using poly(ethylene oxide) (PEO) standards (Polysciences, Inc., Warrington, PA).

Cationic poly(dimethylaminoethyl methacrylate) (PDMAEMA) was synthesized for nanoparticle formation driven by ionic complexation with anionic PPAA ovalbumin conjugates. DMAEMA monomer (Sigma-Aldrich) was distilled and polymerized using RAFT in the absence of oxygen at 33 weight % monomer in DMF at 30°C for 16 hrs. 4-cyano-4-(ethylsulfanylthiocarbonyl)sulfanyl pentanoic acid (ECT) served as the chain transfer agent (CTA) and 2, 2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) was the radical initiator at an [ECT]/[V-70] molar ratio of 10. PDMAEMA was isolated by precipitation 3 times in 50% diethyl ether 50% pentane. Molecular weights were determined using GPC ran with a DMF 0.1 weight % LiBr mobile phase at 60 °C and based on elution times of poly(methyl methacrylate) (PMMA) standards.

Ovalbumin (Ova) was conjugated to PPAA and PMAA via disulfide exchange reactions. Thiol groups were incorporated into ovalbumin using 2-iminothiolane (Traut's Reagent) as previously described(41). Thiolated proteins were subsequently reacted with the pendant pyridyl disulfide groups on the PPAA and PMAA polymers. Disulfide exchange reactions were carried out with a 2.5:1 molar ratio (polymer:ovalbumin) in 0.1 M phosphate buffer, pH 7.8, 0.15M NaCl, 5mM EDTA. Conjugation efficiency was quantified by measuring absorbance at 343 nm of the pyridine-2-thione, which is released upon disulfide exchange. PPAA-ovalbumin (PPAA-Ova) and PMAA-ovalbumin (PMAA-Ova) conjugates were purified using PD-10 desalting columns (GE Healthcare).

Particles containing PPAA-Ova soluble conjugates were formed by ionic complexation of cationic PDMAEMA with the anionic PPAA-Ova conjugate (PPAA-Ova/PDMAEMA). To form particles, PPAA-Ova conjugates were dissolved in PBS, pH 7.4 and PDMAEMA was added at $-/+$ charge ratios ranging from 1:1 to 60:1. The particle size and zeta potential were measured using a Brookhaven BI90Plus instrument, and particle sizes are reported as the number average. Particle size standard deviations were calculated from the reported polydispersity as previously described (41), and particle formation conditions were identified for subsequent testing based on production of nanoparticles in a desirable size range.

In vitro Analyses on PPAA-Ova Conjugates and PPAA-Ova/PDMAEMA Particles

Cytotoxicity of Polymer-Ovalbumin Conjugates and Particles—The cytotoxicity of PPAA-Ova soluble conjugate and PPAA-Ova/PDMAEMA particulate delivery systems was tested in RAW macrophages using the Alamar Blue cytotoxicity assay. RAW macrophages were plated at 25,000 cells per well in 150 μ l media (DMEM supplemented with 10% fetal bovine serum (FBS)) in a 96-well plate and incubated for 30 min at 37°C. Alamar Blue reagent (20 μ l) was added to each well, and PPAA-Ova conjugates or PPAA-Ova/PDMAEMA particles were added to the cells at polymer concentrations of 100 μ g/ml, 200 μ g/ml, 500 μ g/ml, and 750 μ g/ml. Samples were incubated for 24 hrs, and fluorescence was measured using a fluorescence plate reader. Percent viability compared to untreated cells was calculated for each sample.

Intracellular Uptake and Exocytotic Trafficking of PPAA-¹⁴C-Ova and PPAA-¹⁴C-Ova/PDMAEMA in Macrophages—Radiolabeled conjugates were prepared for uptake and exocytosis studies as described previously(41). Briefly, Traut's reagent was reacted with ovalbumin at a 20-fold excess, followed by a 3-fold molar excess of ¹⁴C-iodoacetamide (MP Biomedical). Polymer-Ova conjugation and particle formulation was

subsequently completed as described above. To assess intracellular uptake and accumulation, RAW macrophages were plated in a 48-well plate at 75,000 cells/well and allowed to grow overnight. Then, ^{14}C -Ova, PPAA- ^{14}C -Ova, PMAA- ^{14}C -Ova, or PPAA- ^{14}C -Ova/PDMAEMA was added to the cells at a concentration of 50 $\mu\text{g}/\text{ml}$ ovalbumin ($n \geq 3$ per treatment). Samples were incubated for 1 min, 15 min, 30 min, 1 hr, or 2 hrs. The cells were then washed twice with PBS and lysed using 1% Triton X-100 in water. Radioactivity in the cell media, PBS wash, and cell lysate was measured using a liquid scintillation counter. Percent uptake of ^{14}C -ovalbumin was calculated based on the percent of the total radioactivity delivered that was present in the cell lysate.

Exocytosis studies were completed to gauge the ability of each of the Ova formulations to escape vesicular entrapment and endocytosis/exocytosis trafficking pathways in order to access the cytosolic MHC-1 presentation pathway. In these studies, ^{14}C -Ova, PMAA- ^{14}C -Ova, PPAA- ^{14}C -Ova or PPAA- ^{14}C -Ova/PDMAEMA was added to RAW cells at a concentration of 50 $\mu\text{g}/\text{ml}$ ovalbumin ($n \geq 3$ per treatment). Samples were incubated for 15 min, media containing non-internalized ovalbumin was aspirated, and cells were washed twice with media. In a subset of samples, the percentage of the total ^{14}C -ovalbumin delivered that was internalized during the initial 15 minute pulse was determined using cell lysates gathered in 1% Triton X. Parallel samples were then incubated with new media, which was then collected and replaced with fresh media at 5 min, 10 min, 20 min, 30 min, 1 hr, 2 hrs, and 4 hrs. After 4 hrs, the cells were treated with 1% Triton X and the cell lysates were collected. Recycling of ^{14}C -ovalbumin into the extracellular media at each time point and the quantity remaining in the cell lysates after 4 hrs was determined as a percentage of the quantity of ^{14}C -ovalbumin internalized during the initial 15 minute pulse.

In Vivo Tumor Protection Against Ovalbumin Expressing Tumors

All animal studies were performed in compliance with the University of Washington Animal Care and Use Committee. Female C57Bl/6 mice 7–8 weeks old were purchased from Jackson Labs. E.G7-OVA thymoma cells stably transfected with the ovalbumin gene leading to cell surface expression of ovalbumin epitopes were obtained from ATCC and cultured per the supplier's instructions. Mice were anesthetized with isofluorane and injected subcutaneously on the dorsum with 150 μl of PBS, Ova, PMAA-Ova, PPAA-Ova, or PPAA-Ova/PDMAEMA, with an equivalent of 100 μg ovalbumin delivered to each mouse ($n \geq 4$ per group). Seven days after vaccination, mice were anesthetized and the hair on the left flank was removed using depilatory cream. The E.G7-OVA tumor cells (1×10^6 cells per mouse) were injected in a volume of 100 μl PBS subcutaneously on the left flank. Mice were monitored every 2–3 days and tumor length and width were measured using digital calipers. Tumor volume was estimated using the commonly employed formula that is based on an ellipsoid geometry (30,42).

Flow Cytometric Tetramer Detection of Ovalbumin-specific CD8+ T-cell Response In Vivo

In order to assess the ability of the PPAA carrier to induce CD8+ T-cell production, a flow cytometric assay using phycoerythrin (PE)-conjugated iTagTM MHC-1 tetramers (Immunomics/Beckman Coulter) was used. MHC-1 tetramers have been employed to evaluate CD8+ responses in vaccine strategies using ovalbumin(42–44), as well as other antigens(45,46). Here, the fluorescently labeled tetramers (PE-MHC-1/SIINFEKL) are complexes of MHC molecules and a specific ovalbumin epitope (SIINFEKL) that allows for the evaluation of the percentage of CD8+ T-cells from spleens of immunized mice that can recognize this specific ovalbumin fragment presented via an MHC-1 mechanism. For this experiment, 6–8 week old female C57Bl/6 mice (4 per group) were immunized subcutaneously with 150 μl of PBS, Ova, PMAA-Ova, PPAA-Ova, or PPAA-Ova/PDMAEMA, with an equivalent of 100 μg ovalbumin delivered to each mouse. Seven days

later mice were euthanized and their spleens harvested. Spleens were forced through a 100 μ m cell strainer into a petri dish containing DMEM culture medium. Cells were counted and resuspended at 8×10^6 cells/ml. They were then stained according to the manufacturer's protocol, in addition to simultaneous staining with a FITC-labeled rat anti-mouse CD8a antibody to allow for identification of CD8+ T-cells within the heterogeneous cell population. The cells were analyzed using flow cytometry, and the percent of CD8a+ cells that stained positive for PE-MHC-1/SIINFEKL was determined.

ELISA Measurement of In Vivo Antibody Response

To evaluate the humoral immune response to the administered vaccines, the production of anti-ovalbumin IgG antibody was measured for the mice in the tumor study. Plasma was collected from blood harvested retro-orbitally and assayed for anti-ovalbumin IgG using a modification of the ELISA procedure described by Chung *et. al*(47). Nunc Maxisorp 96-well plates were coated overnight with 5 μ g/ml ovalbumin at 4°C and then blocked with 1% bovine serum albumin (BSA) solution in PBS for 1.5 hrs at room temperature. Plasma samples diluted 1:5000 in PBS and anti-ovalbumin standards (3–200 ng/ml clone OVA-14 IgG1, Sigma) were added in triplicate for 3 hrs at room temperature. The plate was washed 3 times with PBS-Tween and treated for 2 hrs with goat anti-mouse IgG conjugated to peroxidase (Sigma) at 1:3000 in 0.1% BSA PBS-Tween. The plate was washed 3 times with PBS-Tween, and SureBlue Reserve TMB peroxidase substrate (KPL Inc.) was used to quantify anti-ovalbumin antibody concentration in plasma samples relative to the standards by measuring absorbance at 450 nm.

Statistical Analysis

ANOVA was utilized to test for treatment effects, and Tukey's test was employed for post-hoc comparisons. Significant differences were defined as $p < 0.05$.

Results

Polymer Synthesis and Formulation with Ovalbumin

The PPAA copolymer had a $M_n = 22$ kD, $M_w/M_n = 2.0$, with 3 mol% PDSA, and the PMAA copolymer had a $M_n = 27$ kD, $M_w/M_n = 2.1$, with 2 mol% PDSA. The PDMAEMA used to form the PPAA-Ova/PDMAEMA ionic complexes had a $M_n = 7.8$ kD, $M_w/M_n = 1.2$. The PPAA-Ova and PMAA-Ova conjugates used in this study were similar to those characterized in our previous *in vitro* experiments(41). Fifteen percent of the total available lysines in ovalbumin were thiolated, which equates to an average of three thiols per protein. The BCA assay revealed that both the PPAA-Ova and PMAA-Ova conjugates contained 38% ovalbumin by weight. GPC analysis revealed that $M_n = 43$ kD, $M_w/M_n = 2.9$ for the PPAA-Ova conjugate and $M_n = 54$ kD, $M_w/M_n = 2.4$ for the PMAA-Ova conjugate. The ^{14}C -labeled conjugates used for the cellular internalization studies were similar in size with $M_n = 40$ kD, $M_w/M_n = 3.5$ for the PPAA- ^{14}C -Ova and $M_n = 50$ kD, $M_w/M_n = 2.4$ for the PMAA- ^{14}C -Ova. The degree of thiolation of the ovalbumin used to form these radiolabeled conjugates was determined to be 30% and the final conjugates were found to be 34% and 40% ovalbumin by weight for the PPAA- ^{14}C -Ova and PMAA- ^{14}C -Ova conjugates, respectively.

The particle formulation resulted in PPAA-Ova/PDMAEMA ionic particles that were characterized using dynamic light scattering (DLS) and zeta potential measurements (Supplementary Data Table 1). For subsequent *in vitro* and *in vivo* experiments, the focus was the formulation with a $-/+$ charge ratio of 20:1 due to the formation of particles in a desirable size range using this composition. The 20:1 $-/+$ formulation had a hydrodynamic radius of 178 ± 45 nm (reported as number average), in PBS at pH 7.4, and the zeta potential

was found to be -32 mV, which is consistent with the excess of negatively charged PPAA used in particle formation.

Mechanistic In vitro Analyses of PPAA-Ova Conjugates and PPAA-Ova/PDMAEMA Particles

Cytotoxicity of Polymer-Ovalbumin Conjugates and Particles—After incubating RAW macrophages for 24 hours with the polymer conjugates or PPAA-Ova/PDMAEMA particles, cell viability was evaluated using the Alamar Blue assay. Previous results (41) that found PPAA-Ova and PMAA-Ova conjugates to be nontoxic were corroborated, and it was confirmed that the particles also demonstrate cytocompatibility despite their larger size, different architecture, and inclusion of cationic PDMAEMA. For example, cells treated with PPAA-Ova/PDMAEMA particles maintained $>98\%$ viability at polymer concentrations up to 750 $\mu\text{g/ml}$, which is five times the concentration utilized for subsequent cellular uptake and exocytosis assays.

Intracellular Uptake, Cytosolic Accumulation, and Exocytotic Trafficking of PPAA- ^{14}C -Ova and PPAA- ^{14}C -Ova/PDMAEMA in Macrophages—The uptake and intracellular accumulation was evaluated for ^{14}C -Ova, PMAA- ^{14}C -Ova, PPAA- ^{14}C -Ova, and PPAA- ^{14}C -Ova/PDMAEMA in the RAW macrophages. The PMAA- ^{14}C -Ova control is analogous in structure to PPAA- ^{14}C -Ova, but PMAA has a shorter alkyl tail and its carboxylic acid has a lower pKa relative to PPAA. PMAA serves as a control polymer with related chemistry to PPAA but with significantly reduced endosomolytic activity. Initial cellular uptake was found to be statistically equivalent for all groups at the 1 minute timepoint (Figure 1A) indicating that significant early differences were not present between the different ^{14}C -Ova formulations. However, on longer timescales when intracellular quantities reflect a composite of uptake and exocytotic recycling, both the PPAA- ^{14}C -Ova and PPAA- ^{14}C -Ova/PDMAEMA formulations accumulated intracellularly to a significantly greater extent than the control ^{14}C -Ova and PMAA- ^{14}C -Ova (Figure 1B). After normalizing to the quantity of ovalbumin initially internalized in this 15 minute “pulse”, exocytosis of PPAA- ^{14}C -Ova and PPAA- ^{14}C -Ova/PDMAEMA particles was significantly reduced compared to the protein and PMAA control conjugate (Figure 1B) as seen previously with the soluble conjugate (41). At the conclusion of the study at 4 hours, less than 50% of the PPAA-containing formulations had been exocytosed. In contrast, ^{14}C -Ova and PMAA- ^{14}C -Ova were rapidly exocytosed, with over 75% of the free Ova recycled back into the extracellular environment within 30 minutes. The rapid recycling rate observed for ^{14}C -Ova was in accordance with previous studies(41, 48–50). The intracellular quantity of ^{14}C -labeled formulations was also evaluated and accurately complemented the exocytosis data, with the PPAA-containing formulations having significantly extended residence within the cells (Supplementary Figure 1). Taken together these data indicate that the pH-responsive, PPAA-containing formulations may be evading exocytosis pathways by aiding in endosomal escape and cytosolic delivery where protein cargo can potentially access the MHC-1 presentation pathway.

In Vivo Tumor Protection Against Ovalbumin Expressing Tumors

The *in vivo* efficacy of PPAA as a vaccine carrier was evaluated using a murine ovalbumin expressing tumor protection model. This model allows the characterization of prophylactic vaccine tumor protection, and additionally it allows the quantitation of Ova-specific CD8+ induction to test whether the endosomal-releasing polymers enhance this presentation pathway. In this model, delivery of soluble PPAA-Ova and particulate PPAA-Ova/PDMAEMA vaccines dramatically prevented tumor growth compared to controls, as evidenced by the tumor growth curves (Figure 2A) and Kaplan-Meier survival plots (Figure 2B). Most PBS and Ova immunized control mice developed tumors within the 10 days of

tumor cell injection and had to be euthanized by day 19 when tumors exceeded 2000 mm³. PMAA-Ova delayed tumor growth to an extent compared to the saline controls, but statistical differences between tumor growth curves for free Ova and PMAA-Ova were not significant. Interestingly, PPAA-Ova conjugates and the PPAA-Ova/PDMAEMA particles both significantly delayed tumor growth, and there was no significant statistical difference between the soluble and particulate formulations in this study. As summarized in Table 1, PPAA-Ova and PPAA-Ova/PDMAEMA immunized mice averaged nearly 5 weeks before tumors developed following tumor cell inoculation. In terms of tumor-free survival following cell injection, PMAA-Ova immunized mice remained tumor free significantly longer than the PBS and Ova controls but developed palpable tumors significantly earlier than mice immunized with PPAA-containing vaccines. The larger size of the PMAA-Ova conjugates compared to Ova alone and potential inflammatory stimulation could explain the small effects of this control formulation.

Flow Cytometric Tetramer Detection of Ovalbumin-specific CD8⁺ T-cell Response In Vivo

To investigate whether enhanced CTL generation played a role in the prophylactic immune response observed for the PPAA-Ova and PPAA-Ova/PDMAEMA groups, MHC-1/SIINFEKL tetramers were used to measure the number of Ova-reactive CD8⁺ T cells in the spleen using flow cytometry. In splenocytes harvested from mice 7 days post-vaccination, delivery of free Ova or PMAA-Ova did not result in statistically significant CD8⁺ T-cell production compared to PBS. Vaccination with soluble PPAA-Ova and particulate PPAA-Ova/PDMAEMA resulted in enhanced CD8⁺ T-cell generation compared to all other treatment groups as evidenced by the increased number of CD8⁺/tetramer⁺ T-cells as depicted in Figure 3. Soluble and particulate PPAA-containing vehicles resulted in an approximately 8-fold increase in CD8⁺ T-cell development relative to delivery of free Ova. Surprisingly, the soluble PPAA-Ova conjugate was able to overcome any potential advantage of the PPAA-Ova/PDMAEMA particulate formulation in terms of increased uptake/presentation (15,18,23–25) and was found to lead to a similar level of ovalbumin SIINFEKL epitope recognition by CD8⁺ T-cells *in vivo*.

ELISA Measurement of In Vivo Antibody Response

To determine the role of the humoral component in the prophylactic immune response observed for PPAA-Ova and PPAA-Ova/PDMAEMA vaccinated mice, blood was drawn from all mice 20 days after vaccination and the anti-ovalbumin IgG titer was measured by ELISA. As shown in Figure 4, no appreciable antibody production was observed for mice vaccinated with PBS, Ova, or PMAA-Ova. Conversely, a significant increase in anti-ovalbumin IgG was detected for PPAA-Ova and PPAA-Ova/PDMAEMA vaccinated mice, indicating that an antibody-mediated response also contributed to the tumor protection observed in these groups. No differences were detected, however, in PPAA-containing soluble conjugate and particulate forms.

Discussion

In this study, PPAA-based protein vaccines effectively stimulated prophylactic immune responses with a single injection that led to a statistically significant delay in tumor development in the ovalbumin vaccine mouse model. Interestingly, both the soluble PPAA-Ova and particulate PPAA-Ova/PDMAEMA immunizations delayed tumor growth for nearly five weeks, while control mice injected with PBS and free ovalbumin developed tumors in less than 10 days. The robust anti-tumor response observed for both soluble and particulate PPAA-based carriers was mediated through an enhanced CD8⁺ T-cell response and a marked production of anti-ovalbumin antibodies. These data indicate that pH-responsive polymer protein conjugates may be able to access the MHC-1 pathway in the

cytosol by triggering endosome escape, and this is, to our knowledge, the first reported account of soluble protein-polymer conjugates eliciting comparable anti-tumor activity to particulate platforms.

Mechanistically, both CD8⁺ T-cell generation and antibody production contributed to the robust anti-tumor response observed for the PPAA-based carriers. Enhanced CD8⁺ T-cell production of PPAA-based vaccines *in vivo* was in agreement with our previous *in vitro* observations that these carriers increase intracellular accumulation through pH-responsive endosomal escape and enhance MHC-1 presentation/T-cell activation (41). The anti-ovalbumin antibody production could be expected as well as previous studies have found concomitant CD8⁺ T-cell generation and antibody response(51). Since the PPAA-based carriers increase accumulation in APCs, it is logical that these carriers would also enter the MHC-2 antigen presentation pathway, which can be accessed through specialized endosomal vesicles. This would lead to CD4⁺ T-helper cell activation and increased antibody production following treatment with the PPAA-containing vaccines.

Interestingly, despite significant differences in RAW macrophage intracellular accumulation *in vitro*, both the soluble and particulate PPAA based carriers elicited comparable tumor protection and T-cell/antibody responses *in vivo*. As shown in Figure 1, both PPAA-Ova and PPAA-Ova/PDMAEMA had positive effects on protein evasion of exocytosis, but the particulate PPAA-Ova/PDMAEMA formulation resulted in slightly lower exocytosis in macrophages. The increase in stable internalization may be related to the presence of the cationic PDMAEMA component to neutralize a portion of the PPAA anionic charges and/or due to phagosomal uptake of particulate substances(52–56). It has not been clearly elucidated why the better *in vitro* performance of the PPAA-Ova/PDMAEMA particles relative to the PPAA-Ova conjugates was less evident in the *in vivo* studies. We hypothesize that diffusional limitations of the particles because of their larger size could hinder access to APCs and thus cell uptake *in vivo*. Unpackaging of the ionically complexed particles may also be a limiting step in antigen delivery to APCs for this formulation, and this step could lower this system's overall efficiency *in vivo*. Finally, it is possible that both the conjugate and the PPAA-Ova particles are saturating the *in vivo* response in this model, limiting our ability to discriminate differences due to the different formulations. It remains to be seen whether other types of particles incorporating PPAA may provide a better combination of particulate form and PPAA functionality.

It was also interesting to note that the PMAA-Ova conjugate provided an intermediate degree of tumor protection *in vivo* although this formulation did not enhance cytosolic accumulation *in vitro* or elicit measurable CD8⁺ T-cell or antibody production *in vivo*. This observation may be due to a CD4⁺ T-cell response, which is induced by MHC-2 presentation and does not require cytosolic entry of the antigen for activation. This pathway may have resulted in production of cytokines by CD4⁺ helper T-cells, leading to activation of cytotoxic macrophages and delayed tumor growth. Alternatively, there may have been a smaller or more transient effect from the PMAA-Ova treatment that was functionally significant for tumor protection although it was not detected at the specific timepoint analyzed in our CD8⁺ T-cell and antibody assays.

In the context of previous studies, the degree of tumor protection produced by the PPAA-based vaccines with a single dose and no adjuvant was substantial when compared to other vaccine formulations tested using the ovalbumin-based protein vaccine model. Discrepancies in the number of tumor cells injected, the number of rounds of immunization, and the inclusion of adjuvants hinders absolute comparison to previous studies, but some general comparisons demonstrate the effectiveness of the conjugate formulation. For example, the often used PGA nanoparticle vaccine delivery strategy prevented tumor

formation for approximately 16 days after tumor injection(27). Similarly, a particulate, acid-degradable carrier kept mice tumor free for just over 2 weeks(30). Liposomes, another extensively tested vaccine delivery vehicle, have been shown to prevent tumor formation for 20–25 days when co-delivered with the CpG-ODN adjuvant(57) and for 45 days when the carriers contain the cytokine IL-2(51).

Relative to these previous studies, one can conclude that both soluble PPAA-Ova conjugates (33 ± 3 days tumor protection) and PPAA-Ova/PDAEMA particulate vaccine carriers (34 ± 3 days tumor protection) meet or exceed the precedent established in previous reports. Furthermore, the current results represent the first evidence of a soluble polymer-protein conjugate performing as well as a particulate carrier, which has been the previous standard. The promise of PPAA-based vaccines can be further expanded when one considers the potential improvements that may further enhance their efficacy such as multiple immunization protocols, co-delivery with adjuvants, or inclusion of APC-specific targeting moieties. Examination of these avenues and evaluation of this technology against true therapeutic targets (human tumor xenografts, viral pathogens, etc.) are warranted for future investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Enhanced ^{14}C -Ova intracellular accumulation and decreased exocytosis for PPAA- ^{14}C -Ova conjugates and PPAA-Ova/PDMAEMA particles. (A) RAW macrophages incubated for 1 minute with samples at a concentration of $50\ \mu\text{g/ml}$ ^{14}C -ovalbumin showed that uptake of ^{14}C -ovalbumin was the same for all formulations during a timeframe that precludes the effects of exocytosis (no significant differences between treatment groups) (B) Following a 15 minute incubation with ^{14}C -ovalbumin formulations, the amount of ovalbumin exocytosed at each timepoint was determined. Significantly less exocytosis was seen with PPAA-containing carriers, indicating PPAA functionality in evading exocytotic fates. All experimental groups are statistically different in the exocytosis experiment.

Figure 2.

Enhanced tumor protection in mice immunized with PPAA-based vaccines. (A) C57Bl/6 mice were vaccinated with PBS, Ova, PMAA-Ova, PPAA-Ova, or PPAA-Ova/PDMAEMA at 100 μ g ovalbumin per mouse. After 1 week, mice were injected with E.G7-OVA tumor cells, and tumor size was measured until it exceeded 2000 mm³, at which point that animal was euthanized. The tumor size for euthanized mice was taken to be 2000 mm³ for the calculation of mean tumor size at all subsequent timepoints until all mice in that treatment group were euthanized. PPAA-Ova and PPAA-Ova/PDMAEMA showed significant tumor protection relative to all other treatment groups while PMAA-Ova had enhanced tumor protection relative to mice delivered the PBS control. Data are represented as mean $\pm$ standard error mean (SEM) and represent $n \geq 4$. * Significantly different from PBS and Ova. † significantly different from PMAA-Ova. ‡ significantly different from PBS. (B) Kaplan-Meier survival plots for this study display the probability of survival as the fraction of mice whose tumors have not exceeded 2000 mm³ on the indicated day. This plot illustrates the enhanced survival in mice vaccinated using PPAA-based carriers.

Figure 3.

Enhanced CD8⁺ spleen-derived T-cell production in mice immunized with PPAA-based vaccines. C57Bl/6 mice were vaccinated with PBS, Ova, PMAA-ova, PPAA-Ova, and PPAA-Ova/PDMAEMA (100 μ g ova per mouse). 7 days later spleens were collected and splenocytes were stained with FITC-anti-CD8 and PE-MHC-1/SIINFEKL tetramers. Flow cytometric evaluation showed a significantly enhanced anti-ovalbumin MHC-1 driven CD8⁺ T-cell response induced by the PPAA-Ova soluble and PPAA-Ova/PDMAEMA particulate vaccines. Data are presented as mean $\pm$ SEM. n=4 per group, and * indicates significantly different from PBS, Ova, PMAA-Ova.

Figure 4. PPAA-based vaccine triggered anti-ovalbumin IgG antibody production. Mice were vaccinated with PBS, Ova, PMAA-Ova, PPAA-Ova, PPAA-Ova/PDMAEMA (100 μg ovalbumin per mouse). 20 days after vaccine injection, blood was drawn and an ELISA was performed on plasma samples. The anti-ovalbumin antibody response induced by the PPAA soluble and particulate conjugates is significantly greater than that resulting from vaccination with Ova or PMAA-Ova. Data presented as mean $\pm$ SEM, and $n=4$ per group. * indicates significantly different than PBS, Ova, PMAA-Ova.

Table 1
Number of days of tumor protection was increased by PPAA-based vaccines

Tumor protection is reported as the number of days post tumor challenge that each mouse developed a measurable tumor larger than 200 mm³. For PPAA-Ova and PPAA-Ova/PDMAEMA groups, some mice were assigned a value of 35 days at the conclusion of the experiment even though they still did not present with tumors greater than 200 mm³ at that time.

Sample	Avg. Days Tumor Protection
PBS	9 ± 1
Ova	11 ± 3
PMAA-Ova	20 ± 7 [*]
PPAA-Ova	33 ± 3 ^{*,†}
PPAA-Ova/PDMAEMA	34 ± 3 ^{*,†}

^{*} Significantly different from PBS and Ova.

[†] Significantly different from PMAA-Ova.