

A Comparative Study of the Antigen-Specific Immune Response Induced by Co-Delivery of CpG ODN and Antigen Using Fusion Molecules or Biodegradable Microparticles

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ABSTRACT: CpG ODN are toll-like receptor 9 (TLR9) agonists that can enhance antigen presentation by antigen presenting cells (APCs) such as dendritic cells (DCs). The most potent antigen-specific responses are seen when CpG ODN and the antigen are colocalized in the same APC. CpG ODN–antigen fusion molecules and biodegradable microparticles entrapping CpG ODN and antigen can ensure both components are delivered to the same APC. In this study, we compared the efficacy of the CpG–ODN fusion molecules against biodegradable microparticles entrapping antigen and CpG ODN. Microparticles were prepared using a double emulsion solvent evaporation methodology. CpG ODN–OVA fusion molecules were prepared by mixing maleimide-activated protein with thiolated CpG ODN. Both CpG ODN–OVA fusion molecules and microparticles co-entrapping CpG ODN and OVA generated stronger IgG2a and interferon-gamma (IFN- γ) responses than delivery of soluble CpG ODN and OVA. The microparticles generated stronger IgG2a and IFN- γ immune responses than did CpG ODN-antigen fusion molecules. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci*

Keywords: CpG ODN; CpG-antigen fusion molecule; biodegradable microparticle; immunotherapeutic; vaccines; poly (lactic-co-glycolic acid)

INTRODUCTION

Vaccines based on antigens alone lack the ability to optimally activate dendritic cells (DCs) and are notoriously poor at stimulating strong protective immune responses.¹ Delivering antigens with an adjuvant is an effective way to enhance the

immune response.^{2,3} The only FDA-approved adjuvant, alum, is a poor stimulant of T helper-type 1 (T_H1) immune responses which are essential against intracellular pathogens. Alternative adjuvants such as Freund's adjuvant will never reach clinical use because of the severe inflammation and toxicity they induce. CpG ODN is a new class of adjuvant that includes unmethylated CpG dinucleotides similar to those found more commonly in bacterial DNA than in vertebral DNA.^{4–7} CpG ODN specifically targets toll-like receptor 9 (TLR9), which is found within phagoendosomes of antigen presenting cells (APCs) such as dendritic cells (DCs).^{8,9} CpG

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ODN triggers activation and maturation of DCs and helps to increase expression of the antigens. CpG ODN is also known to induce polarized T_H1-type immune responses.^{8,10}

We have previously shown that when CpG motifs in plasmid DNA were co-delivered with an antigen on multi-component nanorods, a stronger CD8 immune response was generated than antigen delivery alone.² In a number of recent studies, it has been shown that CpG ODN must be co-localized with the antigen in the same APC in order to induce a potent antigen-specific immune response.^{9,11–13} For example, in pigs, it has been shown that administration of CpG ODN and HBsAg vaccine in separate sites of the same muscle did not show an enhanced antibody response compared to administration of the HBsAg vaccine alone. In contrast, administration of CpG ODN with the HBsAg vaccine significantly enhanced antibody responses.¹⁴

As a result of these observations, a number of approaches have been developed to co-deliver CpG ODN and antigens. In one approach, CpG ODN sequences were covalently conjugated to a protein antigen. This process resulted in enhanced uptake of the antigen and improved immunostimulatory responses in comparison to free CpG ODN.^{12,15–18} Whilst CpG ODN has demonstrated a desirable safety profile, high concentrations still have the potential to induce inflammation and systemic septic shock-like systems.¹⁶ The advantage of a fused CpG ODN-antigen protein molecule is that it demonstrates much higher efficacy than mixtures of antigen and CpG ODN at equivalent concentrations.

A second approach to co-delivery and localization of antigens and CpG ODN is to load them into nano or microparticles prepared from a biodegradable polymer.^{9,19,20} CpG ODN and PLGA microparticles are two distinct types of vaccine adjuvant.^{20,21} While CpG ODN interacts with immune cells expressing TLR9, stimulating them to secrete pro-inflammatory and T_H1 cytokines and chemokines, PLGA microparticles once taken and processed by APCs, promote induction of humoral and cytotoxic T-lymphocyte responses.^{22–26} Such microparticles are efficiently internalized by APCs thus ensuring that they co-deliver antigens and CpG to the same cell as the polymer matrix degrades. For example, we have recently demonstrated that the microparticles upregulate markers for activation of DCs, are efficiently taken up by DCs, and stimulate stronger IgG2a responses than CpG ODN and

antigen in solution.¹¹ In the present study, we compare the efficacy of the CpG–ODN fusion molecules against biodegradable particles entrapping antigen and CpG ODN to determine the comparative IgG and IFN- γ immune responses stimulated. These responses are also compared against the only clinically available adjuvant, alum.

MATERIALS AND METHODS

Materials

CpG ODN 1826: 5'TCCATGAC**CG**TTTCCTGAC**CG**-TT 3 was obtained from Coley Pharmaceutical Group (Wellesley, MA). (CpG motif is in **bold**). The phosphothioated sulfhydryl-modified ODN was purchased from TriLink Biotechnologies (La Jolla, CA). Ovalbumin (OVA, chicken egg albumin, grade VI) was purchased from Sigma (St. Louis, MO). Poly (lactic-co-glycolic acid) (Fig. 1, PLGA) with a lactide:glycolide ratio of 75:25 was purchased from Absorbable Polymers, Pelham AL. The PLGA had an inherent viscosity of 0.47 dL/g in CHCl₃ @30°C and Mw of 56500.

Mice and Cell lines

C57BL/6 female mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN). All mice were housed at the animal care unit of the University of Iowa and used at 8–10 weeks of age. Naïve C57BL/6 OT-1 mice that are transgenic for the TCR, which recognizes MHC class I H-2Kb-restricted OVA257-264 (SIINFEKL) in H-2Kb were purchased from Jackson Labs (Bar Harbor, ME).

Preparation of Microparticles for Immunization Studies

PLGA microparticles were prepared using a double emulsion solvent evaporation methodology. Briefly, 200 mg of 75:25 PLGA (MW = 56500) was dissolved in 2 mL dichloromethane (DCM). A

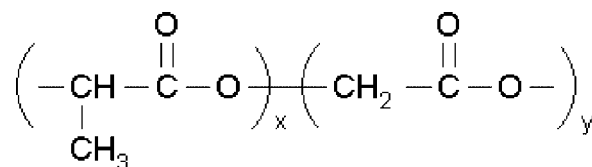


Figure 1. Structure of poly(lactic-co-glycolic acid) (PLGA).

solution with OVA at a concentration of 100 $\mu\text{g}/\mu\text{L}$ and CpG ODN at 50 $\mu\text{g}/\mu\text{L}$ was prepared in 0.5% (w/v) PVA (87–89% hydrolyzed, Avg MW = 13000–23000; Sigma) in deionized water. Using a microtip probe sonicator set at level 2 (Sonic Dismembrator Model 100, Fisher Scientific, Pittsburgh, PA), 500 μL of the protein/oligonucleotide solution containing 10 mg OVA and 5 mg CpG was mixed with the PLGA/DCM solution for 20 s (10 s pulses) to form the first emulsion. This emulsion was then rapidly added to 100 mL of 1% (w/v) PVA in deionized water with stirring at 6400 rpm. The mixture was stirred overnight during which time the DCM solvent evaporated. The microparticles were then washed six times with 50 mL of sterile filtered distilled/deionized water, collecting between washes by centrifugation and resuspension in deionized water. Microparticles were then lyophilized (Labconco freezezone 4.5, Kansas City, MO). After resuspending, the microparticles in sterile filtered deionized water, they were placed under ultraviolet light in a laminar flow hood to sterilize for 1 h before injection.

Characterization of PLGA Microparticles

Particle Size and Surface Morphology Analysis

Microparticle size analysis was conducted using the Zetasizer Nano ZS (Malvern, Southborough, MA). The cumulants analysis provides two values, a mean value for the size and a width parameter, known as Polydispersity that reflects the broadness of the microparticle size distribution. The cumulants analysis is a fit of the polynomial to the log of the G1 correlation function, where $\text{Ln}[G1] = a + bt + ct^2$. The co-efficient of the squared term, c , when scaled as $2c/b^2$ is the polydispersity. Decreasing polydispersity values represent narrower particle size distributions. Microparticle morphology was assessed by scanning electron microscopy (SEM, Hitachi S-4000). Briefly, air-dried microparticles were placed on adhesive carbon tabs mounted on SEM specimen stubs. The specimen stubs were coated with approximately 5 nm of gold by ion beam evaporation prior to examination in the SEM operated at 5 kV accelerating voltage.

CpG ODN and OVA Loading Estimation

OVA loading in PLGA microparticles was determined by dissolving 200 mg of lyophilized micro-

particles in 2 mL of acetonitrile. The suspension of dissolved polymer and precipitated OVA was centrifuged at 6000g for 7 min and the polymer containing supernatant was then discarded. The pellet consisting of the precipitated OVA was redispersed in 0.1 N NaOH. The protein content of the neutralized alkaline solution was estimated using a microBCA protein assay kit (Pierce, Rockford, IL). Empty microparticles in acetonitrile were spiked with a known amount of OVA. Following the procedure, recovery of the extracted OVA was found to be complete. Microparticles containing CpG ODN were also dissolved in acetonitrile and the CpG was extracted in TE buffer (10 mM, pH 8.3). The amount of CpG was determined based on absorbance at 260 nm. Empty microparticles in acetonitrile were spiked with a known amount of CpG. Following the procedure, recovery of the extracted CpG was found to be complete.

Synthesis of CpG–OVA Fusion Molecule

The phosphothioated sulfhydryl-modified ODN (TriLink Biotechnologies, La Jolla, CA) used in this synthesis consisted of 20 bases and contained CpG motifs (1826 5'-S-TCCATGACGTTTCCT-GACGTT-3'). OVA was activated with a 20-fold molar excess of m-maleimidobenzoyl-N-hydroxy-sulfo-succinimide ester (sulfo-MBS, Pierce) in a 5-mM EDTA-PBS buffer pH 7.0 for 2.5 h at room temperature. The amino groups of L-lysine residues on the OVA were modified with maleimide groups. Unbound sulfo-MBS was removed chromatographically on a Bio-Econo P6 gel column (Bio-Rad, Munich, Germany). The sulfhydryl-modified ODN was then reduced in a 50 mM 1,4-dithiothreitol-PBS solution at room temperature for 2 h and residual reagents removed by chromatography on a Bio-Econo P6 gel column. The resulting 5'-S-ODN was then incubated with modified OVA at a molar ratio of 5:1 for 3 h at room temperature and L-cysteine was then added to quench reactive maleimide groups. Free ODN was removed by dialysis against PBS (MWCO10000, Pierce). The dialyzed product was desalted by chromatography on a PD-10 desalting column, followed by lyophilization. Conjugates were analyzed on a 6–20% gradient SDS–PAGE (silver stain) and a 4–15% gradient non-denaturing, non-reducing PAGE (ethidium bromide). Protein concentration was determined using the Lowry method (Pierce).

Immunization Experiments

C57BL/6 mice of 8–10 weeks of age were used for immunization. Mice were immunized intraperitoneally on day 1 (week 0) and day 14 (week 2) with OVA and CpG ODN in the following combinations: (Group I) microparticles containing OVA (68 μg) + CpG (68 μg), (Group II) OVA–CpG fusion molecule (100 μg), (Group III) OVA and alum (100 μg), (Group IV) OVA (100 μg) + CpG (100 μg), and (Group V) Baseline.

ELISA Determination of Anti-OVA Levels

Serum obtained from mice at week 4 and week 22 time-points following immunization was obtained by retro-orbital puncture from mice. Microtiter plates were coated with 10 $\mu\text{g}/\text{mL}$ OVA antigen and incubated overnight at 4°C. The wells were then blocked with 5% milk and serial dilutions of serum were added. Plates were washed, and heavy chain-specific goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates, Birmingham, AL) added followed by the colorimetric substrate *p*-nitrophenylphosphate. Serum from naïve mice served as a standard. The negative control was pre-treatment mouse serum. Plates were evaluated using a microplate reader and curves established for each sample. Test curves were compared with standard curves to determine the concentration of anti-OVA. Values were considered valid only if the standard curves and the sample curves had the same shape. The area under the curve (AUC) was measured for each sample using the trapezoidal rule.

IFN- γ Measurement

DCs were generated from the bone marrow of wild type C57BL/6 mice by flushing the pelvis, femurs, and tibias and culturing the resulting cells in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 10 U/mL penicillin, 10 $\mu\text{g}/\text{mL}$ glutamine supplemented with 1000 U/mL murine GM-CSF (R&D Systems, Minneapolis, MN) and 1000 U/mL murine IL-4 (Peprotech, Rocky Hill, NJ). Media was changed at 4 or 5 days, and DCs were harvested and used on day 7. DC phenotype and morphology was confirmed by flow cytometry and scanning electron microscopy. The cells were pulsed for 24 h with CpG-ODN–OVA fusion molecules, microparticles containing CpG and OVA, OVA/alum, or OVA and CpG ODN in

solution. Splenocytes were harvested from naïve C57BL/6 OT-1 mice transgenic for the T cell receptor (TCR), which recognizes MHC class I H-2 K_b restricted OVA 257–264 (SIINFEKL) in H-2K_b. Red blood cells were lysed using 0.83% ammonium chloride solution. Purified CD8⁺ cells were positively selected by magnetic bead separation of T-cells according to the manufacturers specifications (MiniMACS; Miltenyi Biotec, Auburn, CA). CD8(+) OT-1 T cells were then added to DCs for 24 h at a T-cell:DC ratio of 100:1. Supernatant from OT-1 T cell cultures with DCs were analyzed for the presence of IFN- γ by ELISA using commercially available kits (eBioscience, San Diego, CA). All samples were tested in triplicate.

Statistical Analysis

Group data were reported as mean \pm SEM. Differences between groups were analyzed by one-way analysis of variance with a Tukey post-test analysis. Levels of significance were accepted at the $p < 0.05$ level. Statistical analyses were performed using Prism 3.02 software (Graphpad Software, Inc., San Diego, CA.)

RESULTS AND DISCUSSION

We have previously observed that microparticles upregulate CD80 and CD86 markers for activation of DCs, are efficiently internalized by a pure DC2.4 cell line, and stimulate stronger IgG2a responses than CpG ODN and antigen in solution. In addition, a number of previous studies have shown that delivery of CpG ODN in a particulate form can significantly enhance its adjuvancy efficacy.^{9,11,13,19,20,27} CpG-antigen fusion molecules have also been demonstrated to stimulate potent T_H1-type immune responses.^{12,15–18} In both cases, the capacity of the delivery system to ensure that both CpG ODN and the antigen enters the same APC results in significantly stronger immune responses than CpG ODN and antigen in solution at equivalent doses. Thus, our first objective was to prepare microparticles entrapping CpG ODN and antigen and to prepare antigen-CpG ODN fusion molecules. Microparticles were prepared using a double emulsion solvent evaporation methodology. The average diameter of microparticles were 2.3 $\mu\text{m} \pm \text{SE}$ 0.27. The polydispersity was 0.39 $\pm \text{SE}$ 0.06. The

average loading of OVA was on average 9.5 $\mu\text{g}/\text{mg}$ of microparticles with entrapment efficiencies ranging from 18 to 23%. For CpG ODN, the average loading was 8.1 $\mu\text{g}/\text{mg}$ of microparticles with entrapment efficiencies ranging from 32–34%. Scanning electron microscopy analysis showed that microparticles had a smooth morphology and spherical shape (Fig. 2). In previous studies on the release profiles of CpG and OVA measured using UV spectrophotometry at 260 nm and the micro-BCA protein assay respectively, we observed that the release of both CpG and OVA showed a burst release up to 4 h followed by a more sustained cumulative release profile.¹¹ The release of CpG ODN was faster than OVA. This is most likely due to the fact that CpG ODN has an Mw of 6363 Da, which is a much smaller molecule than OVA (44.3 kDa).

As a comparison to the biodegradable microparticle method of co-delivering CpG ODN and antigen to the same cell, CpG-OVA fusion molecules were prepared (Fig. 3). It has been previously shown that CpG covalently attached to antigens created a strong immune response by shifting antigen-specific antibody responses from $T_{\text{H}2}$ -type responses to $T_{\text{H}1}$ -type responses.¹² Similar to the microparticles, CpG ODN-antigen fusion molecules do not directly stimulate T cells, but instead activate cells of the innate immune system such as DCs. Previous studies have shown that DCs internalize CpG-OVA fusion molecules more efficiently than CpG ODN and OVA mixed in

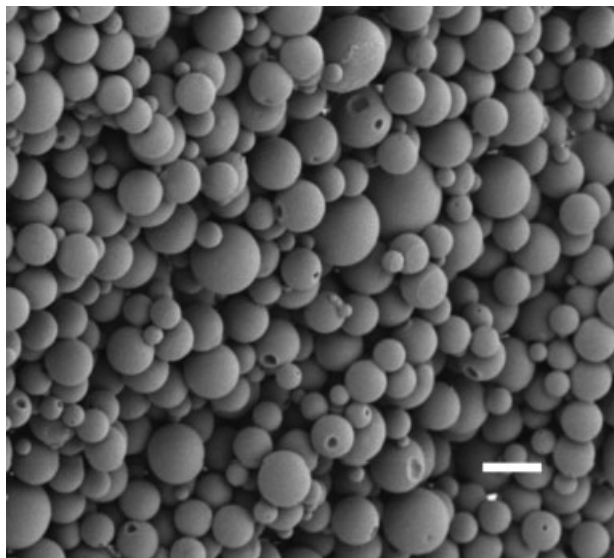


Figure 2. Scanning electron microscopy image of biodegradable microparticles shows smooth and spherical appearance. Scale bar = 5 μm .

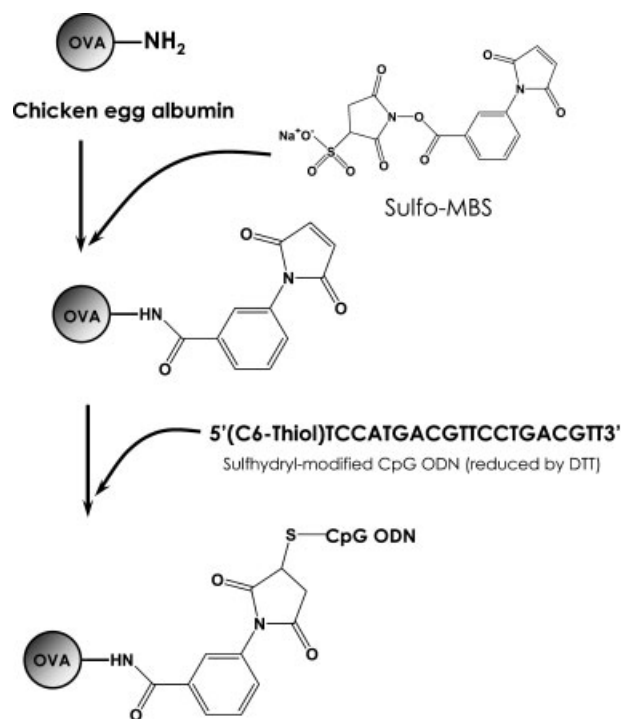


Figure 3. Schematic of CpG-OVA synthesis.

solution although the presence of the CpG motif in the ODN was not specifically necessary for this enhanced uptake.^{16,28} The CpG-OVA fusion molecule has subsequently shown strong promise in single dose protection of lethal doses of *Listeria monocytogenes*.¹⁵ In this study, we linked the antigen to CpG ODN by mixing maleimide-activated protein with a thiolated CpG ODN which generates fusion molecules that are linked through maleimide-sulfur thioether linkages. Alternative approaches to conjugation of CpG ODN to antigens not explored in this study include the conjugation of biotinylated antigens with biotinylated CpG ODN via a tetrameric avidin bridge.²⁹ CpG conjugation to OVA was confirmed by using ethidium bromide staining native PAGE. In OVA controls and CpG ODN controls, no staining was observed in the 50.4-kDa region corresponding to the OVA stains in the ethidium bromide-stained native PAGE. The molar ratio of bound CpG ODN per OVA molecule was found to be approximately 1.3:1 as determined by the Lowry protein assay. In addition, alum was utilized as an additional control to evaluate the microparticles performance in comparison to the only FDA-approved adjuvant for clinical human use. An additional proposed advantage of biodegradable microparticles is their

ability to provide sustained release of antigens and adjuvants, thus providing stronger longer-term immunity.²¹ Previous studies have shown that a single dose of PLGA microparticles entrapping OVA via the subcutaneous and oral delivery routes stimulates similar serum antibody levels to two doses.³⁰ Other studies have shown that vaccinating with two doses significantly enhances the antigen-specific immune response in comparison to a single inoculation but that further vaccinations had no additional benefit.³¹ Consistent with these observations, we have observed that a two dose schedule via the IP route significantly enhances the IgG response in comparison to single doses.¹¹ Therefore, this dosing schedule was selected for our vaccinations. IgG responses were measured at week 4 and week 22 (Fig. 4). Microparticles entrapping CpG and OVA stimulated significantly higher IgG responses than the CpG–OVA fusion molecules at both week 4 ($p < 0.001$) and week 22 ($p < 0.001$). Mean AUC measurements determined using the trapezoidal rule showed an increase from 2.16 for CpG–OVA fusion molecules to 4.44 for microparticles entrapping CpG and OVA at week 4. This increase was found to be consistent at week 22 with an AUC value of 2.68 for CpG–OVA fusion molecules in comparison to 4.65 for microparticles entrapping CpG and OVA.

In mice, measurement of IgG of the IgG2a isotype is considered a reflection of the T_H1 -type response, while total IgG and IgG1 without an increase in IgG2a is seen with a strong T_H2 -type response. At week 4, OVA and alum produced the highest IgG1 responses (AUC 4.91) followed by microparticles entrapping CpG and OVA (AUC 3.38) but by week 22, there was no significant difference between the two groups ($p > 0.05$) suggesting that the microparticles are more effective at stimulating IgG1 responses over a longer time-period. Alum is known to enhance largely a T_H2 -type response. In addition to the negligible IgG2a responses generated by alum, there is a need for the development of alternative adjuvants to alum because of increasing evidence that alum can stimulate autoimmune responses.³² The CpG–OVA fusion molecules stimulated little to no IgG1 responses at either 4 or 22 weeks (AUC 1.36 and AUC 1.25 respectively), which is consistent with previous work that has shown that the CpG ODN-antigen fusion molecules produce a polarized T_H1 -type response by translocating to lysosomal-associated membrane protein 1 (Lamp-1)-positive endosomal-lysosomal

compartments.¹⁵ By week 22, CpG ODN–OVA fusion molecules stimulated stronger IgG2a responses (AUC 2.02) than OVA/alum (AUC 1.75) and OVA/CpG ODN in solution (AUC 1.77). However, the strongest IgG2a response was seen with the PLGA microparticles entrapping CpG ODN and OVA (AUC 2.34). The IgG2a responses were consistent with the IFN- γ responses. Supernatants from co-cultures containing OT-1 T cells and DCs incubated with the microparticles or the CpG ODN–OVA fusion molecules were analyzed for the presence of IFN- γ by ELISA. The presence of IFN- γ indicated a T_H1 -type response. CpG ODN–OVA fusion molecules and microparticles entrapping CpG ODN and OVA generated 16-fold and 18-fold higher IFN- γ production (957 pg/mL and 862 pg/mL respectively) than OT-1 T cells co-cultured with DCs pulsed with CpG ODN and OVA in solution (53 pg/mL) ($p < 0.001$) (Tab. 1). Consistent with our previous observations, no T-cell activation was seen when the T-cells were cultured with DCs pulsed with OVA-containing microparticles that lacked CpG ODN.¹¹ DCs alone did not generate IFN- γ under any circumstances.

CpG-DNA is a pathogen-associated molecular pattern (PAMP) that is sequence specifically recognized by the pattern recognition receptor TLR9. TLR9 is expressed in endosomal compartments and not the cell membrane. Because both microparticles and CpG ODN–OVA fusion molecules have been reported to demonstrate enhanced uptake in DCs in comparison to a mixture of soluble OVA and CpG ODN, it seems logical that both systems will also generate a stronger IgG response than soluble CpG ODN and OVA. For example, conjugating CpG ODN to OVA shifts OVA uptake from inefficient fluid phase pinocytosis to efficient DNA receptor-mediated endocytosis. Even non-stimulatory ODNs linked to OVA enhanced uptake although the presence of the stimulatory CpG ODN was essential for triggering maturation of the DCs.²⁸ Conjugation of CpG ODN to OVA was shown to increase OVA uptake in B cells by 40-fold which in turn led to upregulation of co-stimulatory molecules and cytokines such as IL-12.¹⁵ PLGA particles also show enhanced uptake by professional APCs. When OVA was delivered to primary bone-marrow-derived dendritic cells by PLGA nanoparticles, it generated equivalent T-cell interleukin-2 secretion at 1000-fold lower concentrations than soluble OVA. In addition, the microparticles acted as an intracellular reservoir providing sustained MHC

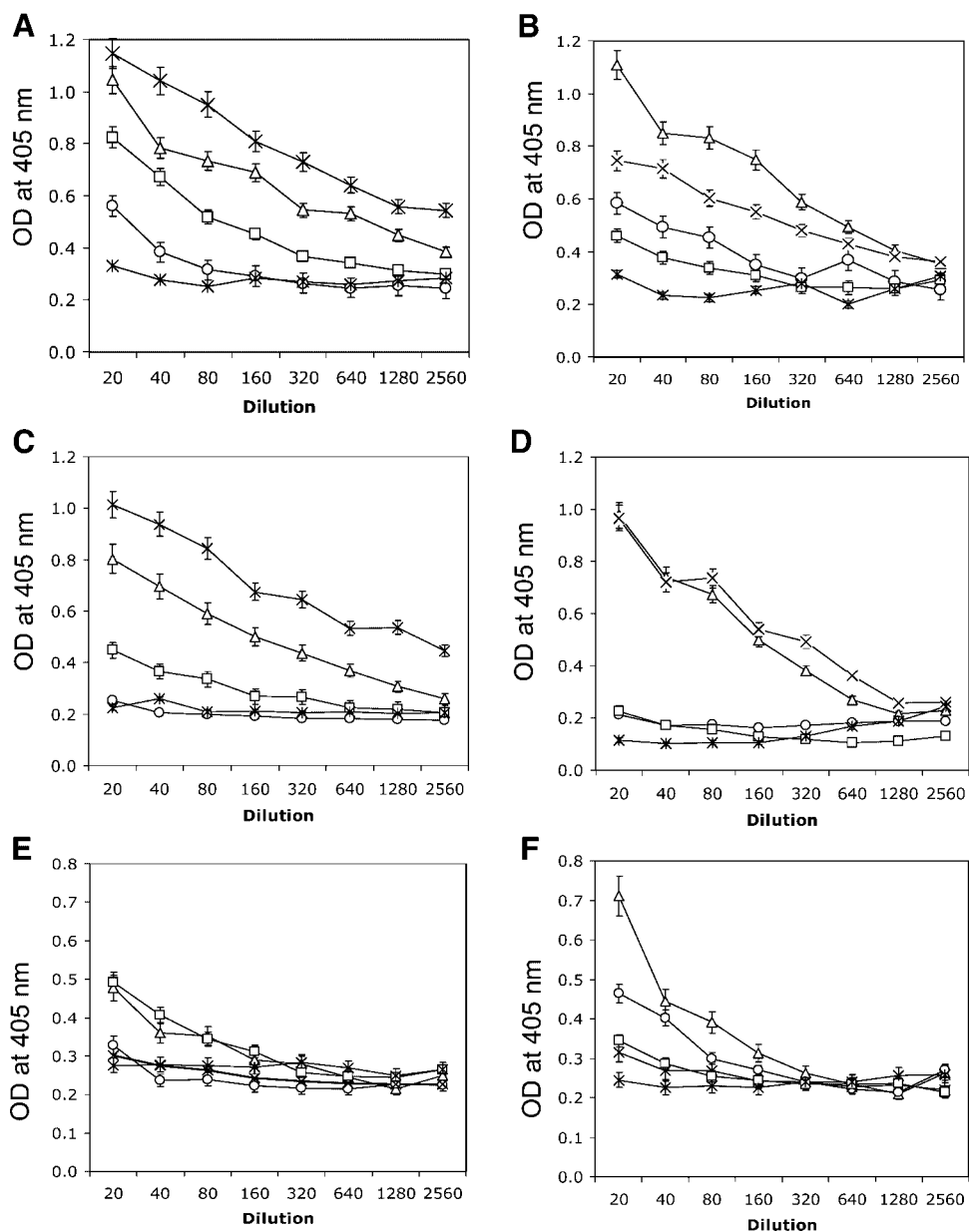


Figure 4. Normalized titration curves after inoculation with PLGA microparticles entrapping CpG and OVA, OVA-CpG fusion molecules, OVA/alum or Ova/CpG mixed in solution. (A) anti-OVA IgG in serum of mice after 4 weeks, (B) anti-OVA IgG in serum of mice after 22 weeks, (C) anti-OVA IgG1 in serum of mice after 4 weeks, (D) anti-OVA IgG1 in serum of mice after 22 weeks, (E) anti-OVA IgG2a in serum of mice after 4 weeks, (F) anti-OVA IgG2a in serum of mice after 22 weeks. Values are represented as mean \pm SEM with $n = 4$ for mice and all samples run in triplicate.

class I presentation of OVA for 96 h. At this same time-point, latex bead associated OVA and soluble OVA was not detected.³³ However, consistent with ours and others observations and similar to the CpG ODN-antigen fusion molecules, the presence of stimulatory CpG ODN is necessary for a T_H1 -type immune response.^{9,26} Alternative systems for

particulate delivery of CpG ODN and antigens that have promise include liposomal formulations. For example, cationic liposomes were used to co-deliver CpG ODN and the *Mycobacterium tuberculosis* protein ESAT-6 to mice. This resulted in significant protective immunity against aerosol challenge with virulent *M. tuberculosis* when

Table 1. IFN- γ Measurements.

DC + OT-1 Cells	IFN- γ \pm SE (pg/mL)
PLGA OVA CpG	957 \pm 61
OVA-CpG fusion	862 \pm 21
OVA and Alum	None detected
OVA + CpG	53 \pm 13
SIINFEKL	1763 \pm 108
Untreated DCs	None detected

compared with non-vaccinated control mice and mice vaccinated with ESAT-6 and a monophosphoryl lipid A adjuvant.³⁴ The enhanced response observed by both PLGA CpG ODN OVA microparticles and CpG ODN–OVA fusion molecules in comparison to soluble OVA and CpG ODN in this study is therefore likely due to significantly improved uptake by APCs and because they both co-localize CpG ODN and antigen to the same APCs.

In all of the formulations, mice tolerated the vaccines and remained in good health as determined by the Body Condition Scoring Technique.³⁵ The mice appeared well conditioned. The vertebrae and dorsal pelvis were not prominent but palpable with slight pressure indicating a state of good health in the mice. No adverse injection site reactions such as infection, redness, or wounding were observed.

In these studies, alum and OVA generated a strong IgG1 response. CpG ODN–OVA fusion molecules and microparticles co-entrapping CpG ODN and OVA generated a strong IgG2a response. The stronger IgG2a and IFN- γ responses stimulated by the microparticle vaccines developed in this study in comparison to CpG ODN-antigen fusion molecules and alum suggest significant potential in a wide variety of immunotherapeutic and prophylactic vaccine applications for diseases including anthrax,²⁰ tetanus,⁹ influenza,^{36,37} hepatitis,^{37,38} malaria,³⁹ and cancer.^{5,40}

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