PMMA particle-mediated DNA vaccine for cervical cancer

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Abstract: DNA vaccination is a novel immunization strategy that possesses many potential advantages over other vaccine strategies. One of the major difficulties hindering the clinical application of DNA vaccination is the relative poor immunogenicity of DNA vaccines. Poly(methyl methacrylate) (PMMA) is a synthetic polymer approved by the Food and Drug Administration for certain human clinical applications such as the bone cement. *In vivo*, PMMA particles are phagocytosable and have the potential to initiate strong immune responses by stimulating the production of inflammatory cytokines. In this study, we synthesized a series of PMMA particles (PMMA 1–5) with different particle sizes and surface charges to test the feasibility of implementing such polymer particles for DNA vaccination. To our knowledge, this is the first report to show that the gene gun can deliver DNA vaccine by propelling PMMA particles mixed with plasmid DNA for cervical cancer. It was found that PMMA 4 particles (particle size: 460 ± 160 nm, surface charge: +11.5 ± 1.8 mV) stimulated the highest level of TNF-α production by macrophages *in vitro* and yielded the best result of antitumor protection *in vivo*. Therefore, our results possess the potential for translation and implementation of polymer particles in gene gun delivering DNA vaccination. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 88A: 849–857, 2009

Key words: poly(methyl methacrylate) (PMMA) particles; DNA vaccine; gene gun; cervical cancer

INTRODUCTION

Cancer vaccine targeting tumor antigens has attracted much attention in recent years because of its higher specificity and less toxicity than traditional modalities such as radiotherapy and chemotherapy.[1] Among the various vaccine strategies currently being investigated, DNA vaccination possesses many potential advantages over other vaccine strategies and its efficacy has been successfully observed in murine settings.[2] DNA vaccines employ genes encoding proteins of interest, rather than using the proteins themselves and represent a novel means of expressing antigens *in vivo* for producing both humoral and cellular immune responses.[3,4] Several measures have been used to deliver DNA vaccines, including the intramuscular and the intradermal routes. The former uses conventional needle/syringe for immunization, whereas the latter employs a gene gun to propel gold particles coated with DNA into the epidermis.[5] Generally, gold particle-mediated epidermal delivery of DNA vaccines is based on the acceleration of DNA-coated gold directly into the cytoplasm of antigen-presenting cells (APCs) in the epidermis, resulting in antigen presentation via direct transfection and cross-priming mechanisms.[5] Only a low DNA dose and a small number of cells are needed for transfection to elicit humoral, cellular, and memory responses.[5]

Despite extensive studies of DNA vaccination using gold particles, it is unclear whether we can use the gene gun to propel polymer particles instead of gold particles to deliver DNA vaccine. Therefore, the purpose of this study is to assess the feasibility of using the gene gun to propel poly(methyl methacrylate) (PMMA) particles to deliver DNA vaccine

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for cervical cancer. PMMA, one of the few synthetic polymers approved by the Food and Drug Administration (FDA) for human clinical applications, has been used successfully in bone cements to fix total joint prostheses for a number of years. In cemented implants, wear debris particles have been isolated from periprosthetic tissues during revision surgery and found to be less than 3 μm.[6] A number of studies have investigated the cytokine secretion by macrophages when PMMA particles are phagocytosed.[7–9] *In vivo*, these particles have the potential to initiate strong immune responses by stimulating the production of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), by macrophages.[10–12] Local cellular and tissue response to the wear cement particles have thought to play an important role in determining the survival of orthopedic implants.[10–12]

Although the efficacy of DNA vaccine has been successfully observed in murine settings, DNA vaccination within human systems has achieved little success because of the inability of DNA vaccines to elicit robust immune responses during the course of early clinical trials.[13,14] PMMA particles from orthopedic joint implants have been postulated to initiate cellular and tissue responses[7–12]; thus, it is reasonable to assume these events are advantageous for PMMA particles to mediate the host immune responses of DNA vaccine. To test this hypothesis, we performed an *in vivo* tumor protection experiment using a previously characterized cervical cancer animal model and vaccination strategy.[15] PMMA particles were coupled with a DNA vaccine encoding calreticulin and HPV-16 E7 (CRT-E7) to test the tumor protection effects against TC-1 cells that express the HPV-16 E7 oncoprotein.[15] Furthermore, the murine macrophage cell line J774A.1 was used to examine the effects of PMMA particles alone or with CRT-E7 DNA, for the production of inflammatory cytokines. Our results showed that different sizes and surface charges of PMMA particles have different capabilities to stimulate the production of inflammatory cytokines. When coupled with the CRT-E7 DNA, PMMA 4 (particle size: 460 ± 160 nm; surface charge: +11.5 ± 1.8 mV) particles stimulated the highest level of TNF-α production and yielded the best results of tumor protection.

MATERIALS AND METHODS

Preparation and characterization of PMMA particles

In this work, five PMMA particles were prepared by changing the emulsion process, identified by the numbers 1–5. PMMA 1 and 2 particles were prepared by emulsion polymerization of methyl methacrylate (MMA, Acros), using sodium dodecyl sulfate (SDS, Wako) as an emulsifier. The recipe for PMMA 1 and 2 particles was 5.1 g of MMA, 0.14 g of initiator, 10 g of SDS, and 100 g of water. PMMA 3 and 4 particles were prepared in the absence of emulsifier. The recipe for PMMA 3 and 4 particles was 10.9 g of MMA, 0.08 g of initiator, and 100 g of water. First, the reaction mixture without initiator was heated to 60°C under nitrogen atmosphere, stirred at a speed of 300 rpm. Subsequently, the initiator, anionic potassium persulfate (Aldrich) for PMMA 1 and 3 particles, and cationic 2, 2-azobis (2-methyl propionamide) dihydrochloride (Acros) for PMMA 2 and 4 particles was added. Polymerization was allowed to proceed for 1 h at 60°C, stirred continuously at 300 rpm with nitrogen gas passed continuously through the reactor. These particles were purified by dialysis using Spectra/Pro molecular porous membrane tubing (molecular weight cutoff: 8000, Spectrum Laboratories) for 1 week. PMMA 5 particles were prepared by dropwise addition of 0.01 mg/mL PMMA (ChiMei CM-211) solution in dimethyl sulfoxide (DMSO, Acros) into water, stirred at a speed of 300 rpm. After 10 min of stirring, larger particles with the micron-size range was obtained by free precipitation.

The morphology of the produced PMMA particles was observed by using a JOEL-JEM 1230 transmission electron microscope (TEM). The average size of particles was obtained from measurements using a Coulter counter N4-PLUS. The surface charge of particles was determined by the measurement of zeta potential using a Zetasizer 3000 HS (Malvern Instruments) based on the laser-Doppler microelectrophoresis.

Gel retardation assays

The generation of pcDNA3-CRT-E7 has been described previously.[15] Plasmid constructs were confirmed by DNA sequencing.

PMMA/CRT-E7 DNA complexes were prepared in Hank's buffered saline (HBS) by mixing 0.675 mg of PMMA particles with 2 μg of CRT-E7 DNA. After 30-min incubation at room temperature for complex formation, the samples were electrophoresed on a 0.8% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL in the gel) and analyzed with an UV illuminator.

Cell culture

The murine macrophage cell line J774A.1 (ATCC, Rockville, MD) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco-RBL Life Technologies, Paisley, UK) and antibiotic/antimycotic (penicillin G sodium 100 U/mL, streptomycin 100 μg/mL, amphotericin B 0.25 μg/mL, Gibco-BRL Life Technologies, Paisley, UK). J774A.1 cells were trypsinized using 0.2% trypsin and 0.2% ethylenediaminetetraacetic acid for 5 min, centrifuged at 1500 rpm for 5 min, and resuspended in the medium.

The metabolic activity of cells was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium

bromide, Sigma] colorimetric assay.[16] To enhance the response of J774A.1 cells to PMMA particles and plasmid DNA, 2×10^4 J774A.1 cells were exposed to PMMA particles (3.375 mg) or PMMA (3.375 mg)/CRT-E7 DNA (10 μg) complexes in 24-well tissue culture polystyrene plates (Costar, USA). Untreated cells were used as a control group. After culturing in a humidified atmosphere with 5% CO_2 at 37°C for 7 days, the culture medium was removed and the cells were incubated with 0.1 mL of MTT solution (2 mg/mL in phosphate-buffered saline) for 3 h at 37°C. Subsequently, the MTT solution was aspirated and the formazan reaction products were dissolved in DMSO and shaken for 20 min. The optical density of the formazan solution was read on an ELISA plate reader (ELx 800, BIO-TEK) at 570 nm.

DNA vaccination and *in vivo* tumor protection experiments

PMMA/CRT-E7 DNA complexes were prepared by incubating 0.675 mg of PMMA particles with 2 μg of plasmid DNA in HBS for 30 min at room temperature. Subsequently, the complexes of PMMA particles and plasmid DNA were delivered to the shaved abdominal region of C57BL/6 mice (five per group) using a helium-driven gene gun (BioWare Technologies, Taipei, Taiwan) as described previously.[15] One week later, mice were boosted with the same regimen as the second vaccination. One week after the last vaccination, mice were subcutaneously challenged with 5×10^4 TC-1 cells/mouse in the right leg. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed at day 60.

Cytokine assay

For determination of cytokine release of J774A.1 cells after exposure to PMMA particles and CRT-E7 DNA, 1 mL medium of cell suspension (2×10^4 cells) plus PMMA particles (3.375 mg) or PMMA (3.375 mg)/CRT-E7 (10 μg) complexes were cultured in 24-well tissue culture polystyrene plates. Untreated cells were used as a control group. After incubation for 1 day, the supernatant was harvested and the cytokines (IL-6 and TNF-α) released by cells into the media were measured by ELISA following the manufacturer's protocol (Endogen, Boston, MA).

Cell transfection

J774A.1 and HeLa cells were transfected using PMMA particles or polyethyleneimine (PEI) reagent (ExGen500, Fermentas) as the DNA vector. Plasmid pEGFP-N1 (Clontech), containing the cDNA for enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus promoter, was used for the tests of transfection. Briefly, cells were seeded onto 24-well tissue culture polystyrene plates at 5×10^4 cells/well and incubated at 37°C in 5% CO_2 for 24 h. Subsequently, EGFP DNA (1 μg) mixed with the PMMA particles (0.337 mg) or PEI reagent (3.3 μL) was added into the culture wells for transfection, which were incubated at 37°C in 5% CO_2 for 24 h. The EGFP expression was assessed using a fluorescent microscope (Axiovert 100TV, ZEISS, USA) equipped with green fluorescent filter set, and cell counts were manually taken (100–200 cells for each treatment group) to determine the percentage of cells displaying EGFP expression.

Statistical analysis

The data of MTT and cytokine assay presented as the mean ± standard deviation are representative of at least four independent experiments. Statistical significance was calculated using one-way analysis of variance followed by Student's t test. In the tumor protection experiment, the principal outcome of interest was time to development of tumor. The event time distributions for different mice were compared by Kaplan and Meier and by log-rank analyses.

RESULTS

Particle size and surface potential

Figure 1 illustrates the morphology of synthesized PMMA particles from TEM. Excluding PMMA 5, PMMA 1–4 were all quite globular. Table I summarizes the particle sizes and surface charges of different PMMA particles. For PMMA 1 and 2, which were obtained by the emulsion polymerization with SDS surfactant, the average size was about 50 and 150 nm, respectively. On the other hand, emulsifier-free emulsion polymerization yielded a diameter of about 340 and 460 nm for PMMA 3 and 4, respectively. Clearly, the diameter of PMMA particles was significantly reduced from >300 nm without a surfactant to <200 nm upon the addition of SDS during the polymerization. For PMMA 5, which was prepared by dropwise addition of PMMA solution into water, the particle size was in the micron range. Table I also shows the surface charge, determined by the measurement of zeta potential, of prepared PMMA particles. Reasonably, PMMA 1, 3, and 5, prepared from anionic initiators, exhibited a negative zeta potential, whereas PMMA 2 and 4, prepared from cationic initiators, exhibited a positive zeta potential.

Gel retardation of PMMA/CRT-E7 DNA complexes

Two micrograms of CRT-E7 was incubated with equivalent weight of various PMMA particles and the formation of stable PMMA/plasmid DNA complexes was checked by the gel retardation assay. As shown in Figure 2, PMMA 4 could retard the

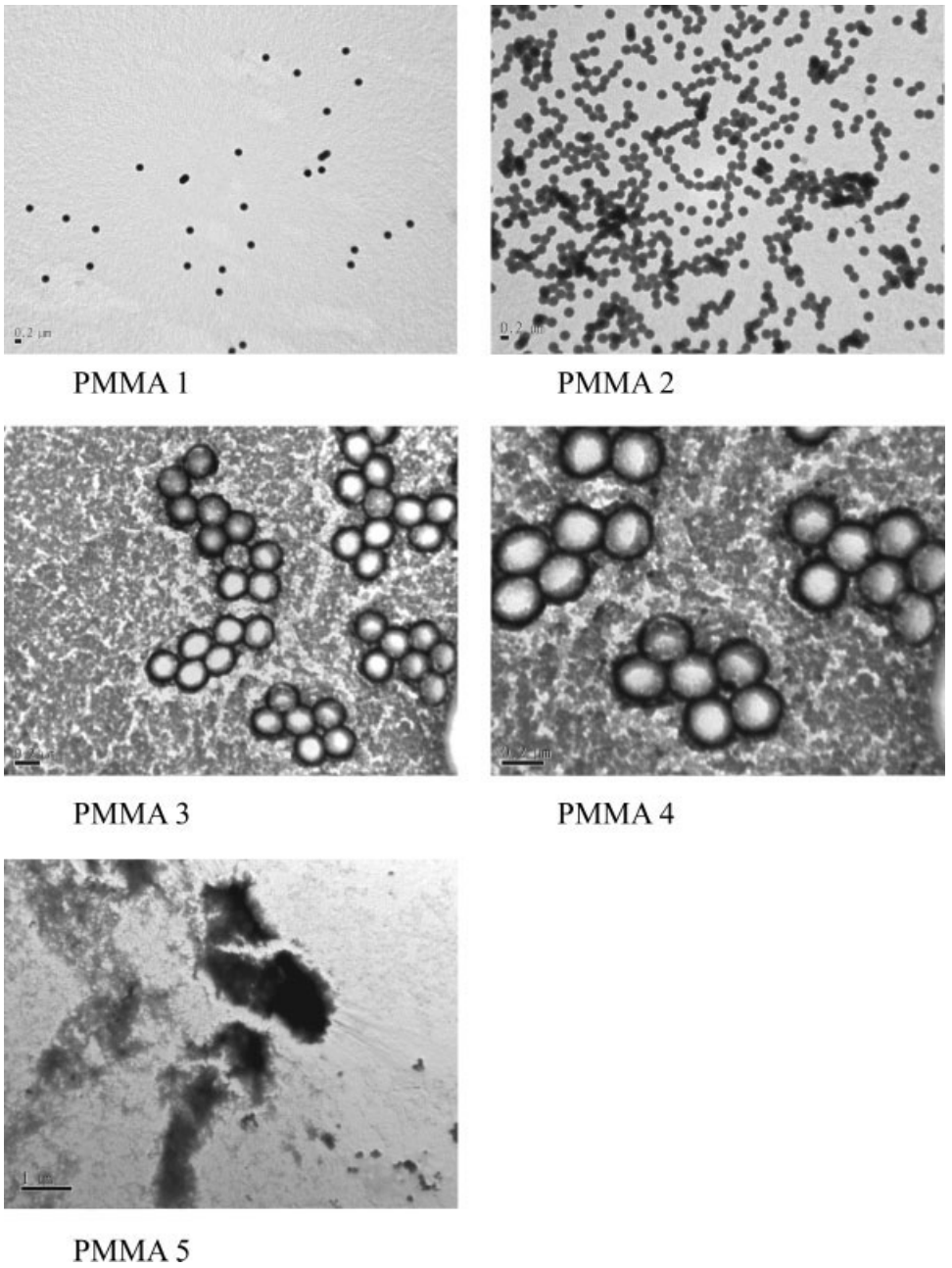

Figure 1. TEM photographs of PMMA particles: PMMA 1, PMMA 2, PMMA 3, PMMA 4, and PMMA 5.

migration of CRT-E7 DNA completely whereas other PMMA particles failed to do so. This result indicates that the PMMA 4 may form the most stable complex with plasmid DNA when compared with the other PMMA particles under such experimental conditions.

MTT assay

To investigate if PMMA particles and PMMA/CRT-E7 DNA complexes affect the survival of J774A.1 cells, 3.375 mg of PMMA particles without or with 10 μg of CRT-E7 DNA were incubated with 2×10^4 J774A.1 cells for 7 days and then assayed the cells' metabolic activity by the MTT test. Control

TABLE I
Size and Surface Charge of Synthesized PMMA Particles

	Particle Size (nm)	Surface Charge (mV)
PMMA 1	50 ± 14	−30.6 ± 4.5
PMMA 2	150 ± 15	+10.5 ± 1.9
PMMA 3	340 ± 80	−37.3 ± 2.2
PMMA 4	460 ± 160	+11.5 ± 1.8
PMMA 5	1000 ± 250	−28.7 ± 3.1

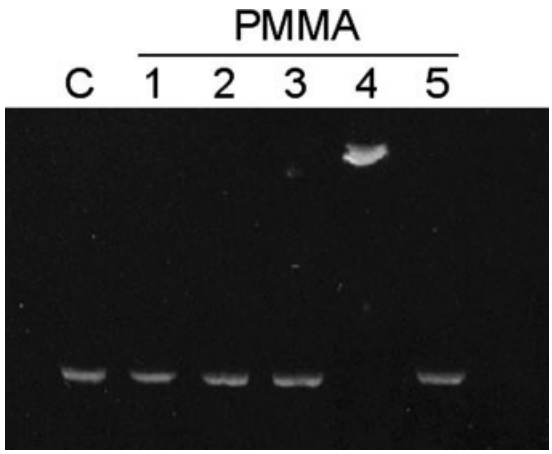

Figure 2. Gel retardation of PMMA/CRT-E7 DNA complexes. C: plasmid DNA only.

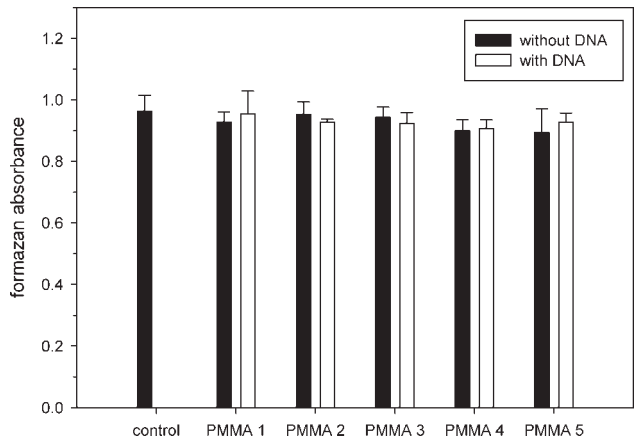

Figure 3. The metabolic activity, determined by the MTT assay, of J774A.1 cells exposed to PMMA particles without and with CRT-E7 DNA after incubation for 7 days. Data are presented as mean ± standard deviation ($n = 6$). Statistical significance was calculated using one way analysis of variance (ANOVA) followed by Student's t test ($p < 0.05$ was considered significant).

cells were cultivated without adding PMMA particles and DNA. As shown in Figure 3, the mean formazan absorbance obtained was comparable for all prepared PMMA particles and was not significantly different from the control cell population, regardless of the presence or absence of CRT-E7 DNA. When MTT assay was measured for 1 and 3 days of incubation, it also showed the similar result (not shown here). This indicated that the PMMA particles, even in the presence of CRT-E7 DNA, did not affect the survival of J774A.1 cells.

In vivo tumor protection

To assess the feasibility of using the gene gun to propel prepared PMMA particles to deliver DNA vaccine, we performed an *in vivo* tumor protection experiment using a previously characterized tumor model and vaccination strategy.[15] All of the mice without treatment developed tumors within 10 days after tumor challenge (data not shown). Similarly, mice only receiving PMMA particles also developed tumors within 10 days after tumor challenge, indicating PMMA itself did not show an antitumor effect (data not shown). Figure 4 shows that 20% of the mice receiving CRT-E7 DNA alone or PMMA 5/CRT-E7 DNA complex vaccination, and 40% of the mice receiving either PMMA 1/, PMMA 2/, or PMMA 3/CRT-E7 DNA complex vaccination remained tumor-free 60 days after TC-1 challenge. The best result was observed in mice vaccinated with the PMMA 4/CRT-E7 DNA complex. Sixty percent of the mice were still tumor-free at the end of this study, which was significantly better than the control group ($p = 0.0027$).[15] The operating parameters used in the gene gun of this study were for the delivery of gold particles. Therefore, better tumor protection effects are anticipated if appropriate operating parameters for PMMA particles are developed. As shown in Figure 4, the protection rate can be up to 100% by using gold particle-mediated DNA vaccination, which is consistent with the result of the previously characterized cervical cancer animal model.[15]

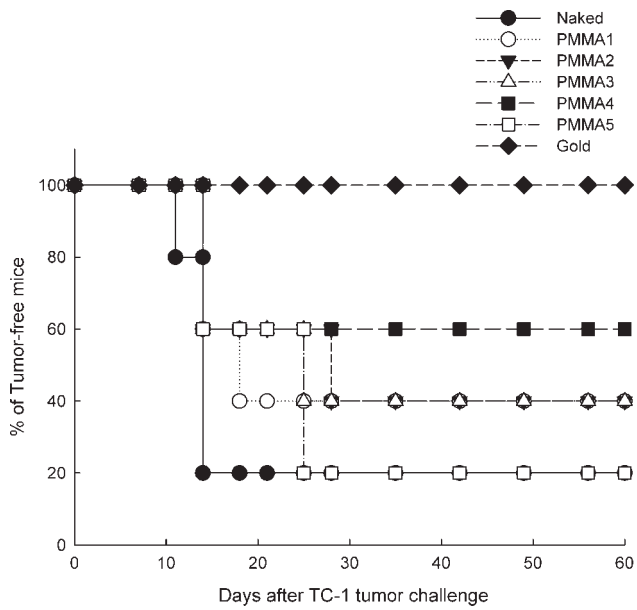

Figure 4. *In vivo* tumor protection experiments in mice vaccinated with various DNA vaccines. Mice were immunized with naked DNA alone, PMMA 1–5/plasmid DNA, and gold/plasmid DNA (five per group) as indicated and challenged as described in the "Materials and Methods" section to assess the antitumor effect generated by each DNA vaccine. The Kaplan–Meier product-limit method for survival was assessed for significance using the log-rank test.

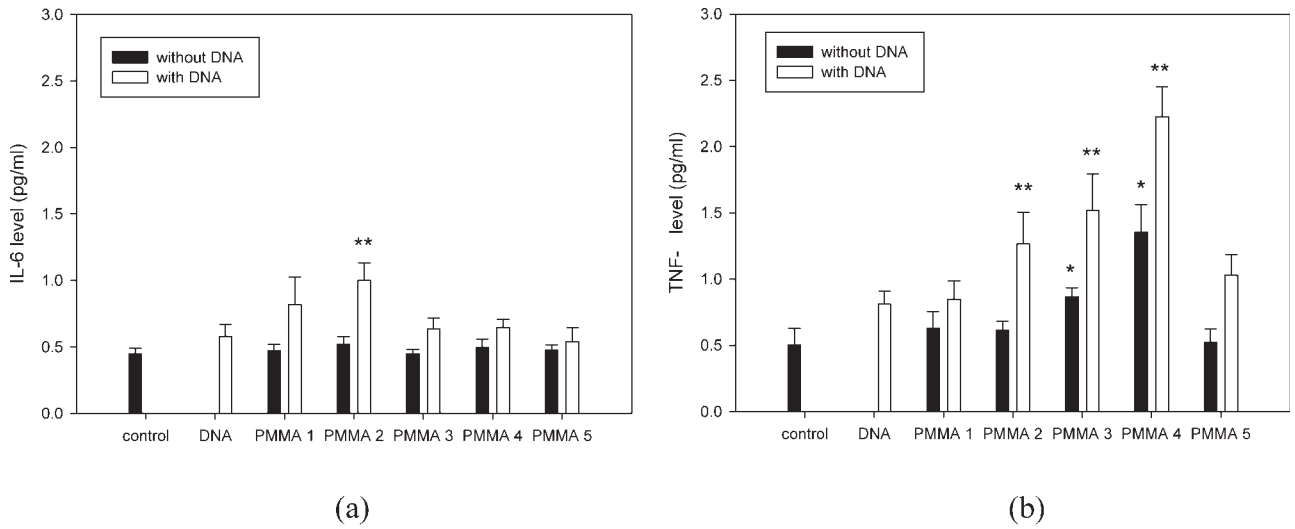

Figure 5. The level of IL-6 (a) and TNF-α (b) released by J774A.1 cells exposed to PMMA particles without and with CRT-E7 DNA after incubation for 1 day. Data are presented as mean ± standard deviation ($n = 4$). Asterisk and double asterisks denote significant differences of $p < 0.05$ and $p < 0.01$ of the cytokine release compared with control cells, respectively, which were calculated using one-way analysis of variance (ANOVA) followed by Student's t test.

Cytokine production

We next investigated the release of inflammatory cytokines TNF-α and IL-6 by J774A.1 cells to gain further insights as to how PMMA particles and PMMA/CRT-E7 DNA complexes were involved in regulating the immune responses. We still used 3.375 mg of PMMA particles and 10 μg of plasmid DNA, five times the concentrations of *in vivo* tumor protection tests were added, to enhance the response of J774A.1 cells. As shown in Figure 5(a), all PMMA particles tested in this study did not induce significant IL-6 release by J774A.1 cells in the absence of CRT-E7 DNA. However, in the presence of CRT-E7 DNA, PMMA 2 significantly increased the level of IL-6 secretion by J774A.1 cells when compared with control cells ($p < 0.01$). On the contrary, Figure 5(b) shows PMMA 3 and PMMA 4 significantly induced the TNF-α secretion by J774A.1 cells even in the absence of CRT-E7 DNA, compared with control cells ($p < 0.05$). After coupling with CRT-E7 DNA, the levels of TNF-α in the culture supernatants of PMMA 2/, PMMA 3/, and PMMA 4/CRT-E7 DNA complexes treated cells were significantly higher than the control cells ($p < 0.01$). In this study, PMMA 4/CRT-E7 DNA complex was the most potent inducer of TNF-α secretion by J774A.1 cells.

Transfection of macrophages

Taken together, our data showed that the combination of PMMA 4 particles and CRT-E7 DNA had the best tumor protection effect to augment the host immune responses, but we did not know whether PMMA 4/plasmid DNA complexes could be taken up into macrophages and expressed. To assess this possibility, we used another plasmid DNA encoding EGFP to complex with PMMA 4 particles to transfect J774A.1 macrophages. For comparison, another nonviral vector PEI with superior transfection performance and another cell type HeLa cells with good transfection activity were used in this study. Figure 6 shows J774A.1 macrophages were very resistant to transfection that could be ascribed to EGFP expression, regardless of using PMMA 4 particle or PEI reagent to complex with EGFP DNA. After 24 h of transfection, we could not observe any cells showing

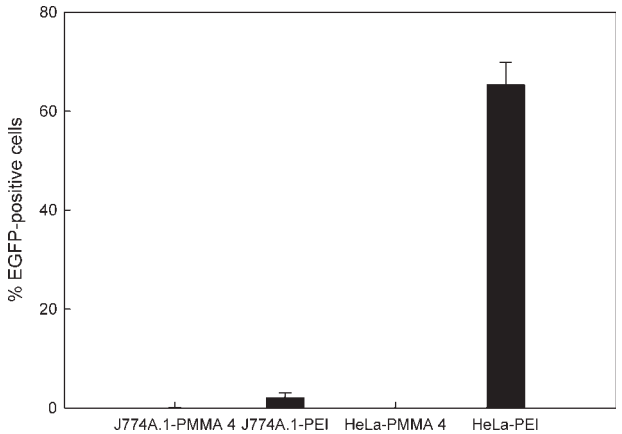

Figure 6. Transfection of J774A.1 macrophages and HeLa cells with EGFP DNA using PMMA 4 particles and PEI reagent as the DNA vector. The assessment of EGFP expression using a fluorescent microscope equipped with green fluorescent filter set, and cell counts were manually taken (100–200 cells) from three independent wells to determine the percentage of cells displaying EGFP expression.

EGFP fluorescence using PMMA 4 particles and only a small fraction (<3%) of transfected cells were found using PEI. In contrast, we could observe obvious EGFP fluorescence of HeLa cells using PEI reagent. However, PMMA 4 still had no detectable gene transfer activity in HeLa cells. These results suggested that PMMA 4 particles were not a potent DNA delivery vehicle in gene transfection. In addition, compared with that HeLa cells could be transfected using PEI reagent, J774A.1 macrophages exhibited very low transfection efficiency, which was in agreement with previous reports.[17,18]

DISCUSSION

Currently, DNA vaccines have been safely administered in several human studies. Intramuscular injection and transdermal particle bombardment are the two routes commonly used for DNA vaccination. Intramuscular injection results in plasmid DNA uptake by myocytes, which present antigen through MHC-I pathways.[19] However, the efficiency of transfection is probably low, because the requirement for cellular uptake of DNA and myocytes do not promote costimulatory molecules to function as efficient APCs. Unlike vaccination by intramuscular injection of DNA, vaccination by particle bombardment transfects cells of the epidermis and dermis by direct deposition of DNA-coated particles. The skin is a rich source of somatic cells such as keratinocytes and fibroblasts as well as potent bone-marrow-derived APCs such as Langerhans cells, the resident "professional APCs" of the skin. These APCs are directly transfected and then migrate to the draining lymph nodes.[20] It has been proposed that this mechanism is the predominant means of priming cytotoxic T-lymphocytes of using gene gun to deliver DNA vaccine into the epidermis and dermis.[21]

Gold particles are the most extensively used in gene gun to accelerate DNA vaccines into the cytoplasm of cells, facilitating DNA delivery and gene expression. In this study, we tried to use gene gun to propel PMMA particles to deliver DNA vaccine. A series of PMMA particles with different particle size and surface charge were prepared. The concentrations of PMMA particles used in our *in vitro* studies were not toxic to mouse J774A.1 cells. Although such particle concentrations were five times higher than the concentrations used in our *in vivo* studies, we still were not sure if the particle concentrations in our *in vivo* studies would have any detrimental effects to the mice. Nevertheless, PMMA is a biomaterial that has already been widely used in orthopedic implant devices. Furthermore, PMMA nanoparticles have been used for DNA vaccination by the route of intramuscular injection and already been shown to be nontoxic for the cells and well tolerated *in vivo*.[22–28] It might be argued that PMMA particle preparation was not a new technique, but, to our knowledge, this is the first report to show that the gene gun can deliver DNA vaccine by propelling PMMA particles mixed with DNA. The best result was PMMA 4 particles with the size of 460 ± 160 nm and surface charge of 11.5 ± 1.8 mV. Sixty percent of the vaccinated mice were still tumor-free 60 days after the tumor challenge. It is noted that the protection rate can be up to 100% by using gold particle-mediated DNA vaccination. As mentioned earlier, the operating parameters for the delivery of PMMA particles used in the gene gun were for gold particles. Therefore, better tumor protection effects are anticipated if appropriate operating parameters for PMMA particles are developed.

Because of the relatively poor immunogenicity of DNA in humans, several strategies have been employed to improve the immunogenicity of DNA vaccines. Among them, the use of adjuvants (e.g., aluminum salts, cytokines, lipopolysaccharide) alongside DNA immunization is effective in enhancing the level of immune response to a target antigen.[1,5] Adjuvants serve to activate innate immune cell subsets in order to better promote adaptive immunity through T-cell interaction. In this study, we took advantage of the proinflammatory cytokine inducing effects of PMMA particles and test if this property of PMMA could serve it as an effective adjuvant in DNA vaccination. As shown in Figure 5(b), TNF-α production by macrophages was significantly increased when the cells were treated with PMMA 3 and PMMA 4 particles. Coupling with CRT-E7 DNA seemed to increase the TNF-α-stimulating effects of all PMMA particles, although in PMMA 1 and PMMA 5 the difference did not reach statistical significance. PMMA 4/CRT-E7 DNA complexes induced the highest level of TNF-α production by mouse macrophage cells [Fig. 5(b)] and the best tumor protection effects *in vivo* (Fig. 4). It is known that TNF-α can promote antibody-dependent cytotoxicity and upregulate the expression of MHC class II molecules.[29] Thus, in this study, PMMA particles serve as a potent adjuvant to enhance host immune responses. In addition to the TNF-α, IL-6 is another potent proinflammatory cytokine that has been involved in a wide spectrum of biological events. However, overproduction of IL-6 may have detrimental effects to DNA vaccination because IL-6 suppresses MHC-II expression on dendritic cells and attenuates T-cell activation.[30] Our results showed that, except for the PMMA 2/CRT-E7 DNA complexes, PMMA particles and other PMMA/CRT-E7 DNA complexes did not induce significant IL-6 production by mouse macrophage cells [Fig. 5(a)]. This

might be another potential advantage of using PMMA particles as adjuvants for DNA vaccination. In fact, PMMA, in the form of nanoparticles, has recently been shown to be very attractive as an adjuvant in protein and DNA vaccine applications.[22–26] Therefore, phagocytic uptake of the PMMA/CRT-E7 DNA complexes by macrophages with subsequent production of cytokines such as TNF-α shown in this study might play an adjuvant role in enhancing the immune responses toward the target antigen.

It is known that both direct transfection of epidermal APCs and cross-priming mechanisms that occur between APCs and macrophages are involved in the generation of antitumor immunity in gold particle-mediated epidermal delivery of DNA vaccines.[5] In addition to the direct transfection of DNA to APCs by using the gene gun, it may be possible to increase delivery of DNA to APCs by using a particulate formulation of DNA because of the nonspecifically phagocytic capacity of such cells. In support of this hypothesis, Singh and coworkers showed that intramuscular injection of poly(lactide-*co*-glycolide) microspheres of about 1 μm diameter with surface-adsorbed DNA substantially induced higher levels of immune responses compared with that of naked DNA.[31] Theoretically, the PMMA 4/EGFP DNA complexes can be easily phagocytosed by macrophages.[7–9] However, Figure 6 shows that PMMA 4 particle-mediated transfection on macrophages was not satisfactory. Therefore, the observed tumor protection in this study might not due to phagocytic uptake of DNA with subsequent transfection of target antigen by the macrophages. The mechanism for PMMA particle-mediated DNA vaccine in suppressing cervical cancer is not clear at this time. It is postulated that the increased cytokine production after phagocytic uptake of the PMMA/CRT-E7 DNA complexes by macrophages [Fig. 5(b)] might improve the "cross-priming" effects that occur through the crosstalk between macrophages and professional APCs. Direct delivery of PMMA/CRT-E7 DNA complexes into professional APCs through the gene gun is another possible explanation for the observed tumor protection effects in this study.

CONCLUSION

A series of PMMA particles (PMMA 1–5) were synthesized in this study for a DNA vaccination study. It was found that PMMA particles with different sizes and surface charges could result in different tumor protection effects. Among them, PMMA 4 particles (particle size: 460 ± 160 nm; surface charge: +11.5 ± 1.8 mV) stimulated the highest level of TNF-α production by macrophages *in vitro* and yielded the best results of tumor protection *in vivo*. Although the underlying mechanism remains elusive, our results possess the potential for great translation and implementation of polymer particles in DNA vaccination because the safety of many polymer biomaterials has been approved by the FDA.

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