Polycation-decorated PLA microspheres induce robust immune responses via commonly used parenteral administration routes

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Recombinant viral subunit-based vaccines have gained increasing attention due to their enhanced safety over the classic live-attenuated or inactivated vaccines. The low immunogenicity of the subunit antigen alone, however, requires the addition of an adjuvant to induce immunity. Particulate-based delivery systems have great potential for developing new vaccine adjuvants, compared to traditional aluminum-based saline adjuvants. The physico-chemical properties of particulate vaccines have been extensively investigated; however, few studies have focused on how the administration route of various adjuvant–antigen combinations impacts the efficacy of the immune response. Here, for the first time, the viral Hepatitis B surface antigen (HBsAg) was combined with aluminum-based or cationic-microsphere (MP) based adjuvants to investigate the characteristics of immune responses elicited after immunization via the subcutaneous, intramuscular, or intraperitoneal routes respectively. In vitro, the MP-based vaccine significantly increased dendritic cell (DC) activation with up-regulated CD40 and CD80 expression and IL-12 production compared to alum-based vaccine. After immunization, both MP and alum-based vaccines produced increased IgG titers in mice. The administration route of these vaccines did influence immune responses. The MP-based vaccine delivered via the intramuscular route yielded the highest levels of the IgG2a isotype. The alum-based vaccine, delivered via the same route, produced an IgG1-dominated humoral immune response. Moreover, subcutaneous and intramuscular immunizations with MP-based vaccine augmented Granzyme B, Th1-type cytokines (IL-2, IL-12, and IFN-γ), and Th2 cytokine IL-4 secretions. These results demonstrate that MP-based vaccines have the capacity to induce higher cellular and humoral immune response especially via an intramuscular administration route than an alum-based vaccine.

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1. Introduction

Using vaccines to combat infectious pathogens was a brilliant advancement of the 20th century. Vaccine development has since evolved from using live-attenuated or inactivated viruses to using recombinant viral subunits [1]. While these antigen subunit vaccines have greatly improved vaccine safety, the immunogenicity of the single viral subunit antigen is low and requires the addition of an adjuvant. For example, the Hepatitis B vaccine utilizes the Hepatitis B surface subunit antigen (HBsAg) (derived from Hansenula polymorpha). In this vaccine the antigen uses aluminum hydroxide (alum) as an adjuvant which induces potent immune responses. Alum has been widely used as an adjuvant for many antigens; however, it is not effective with all antigens [2]. An increasing number of studies have demonstrated disadvantages to using alum as an adjuvant; these include the necessity of cold-chain vaccine storage and distribution, and its unsuitability for lyophilization [3]. There are also side effects associated with alum-based vaccines, which include sterile abscesses, eosinophilia, and myositis, generally most of these serious side effects occur only in small percentages of those immunized [4]. Moreover, alum-based vaccines primarily induce antibody dominated humoral immunity, and are unable to generate T cell mediated immune response; which plays a key role in clearing intracellular infectious pathogens, such as the Hepatitis B virus, from the subject [5]. Therefore, a safe and effective alternative vaccine adjuvant that induces an effective T cell mediated immune response is of great interest. Nano- and microspheres fabricated with biodegradable polylactic acid (PLA) are being extensively investigated as potential vaccine-delivery systems. Particles have been shown to facilitate antigen uptake in antigen-presenting cells (APCs), enhance antigen presentation to T cells, and have the ability to elicit strong cellular and humoral immunity responses [6–8].
The physicochemical characteristics of specific particulate delivery systems have been shown to influence the type and quality of immune responses induced in the host. For example, particle size has been linked to efficacy of the immune response induced [9]. Several studies suggested that small sized particles (<500 nm) induce a more robust immune response than large sized particles (>1 μm) [10,11], while other studies concluded that large particles induced better immune responses than small particles [12], yet other research has suggested that similar immune responses occur across particle size ranges [13], or, finally, that an optimal particle-size range was required to induce an optimal immune response [14]. Thus, particle size is one of the important aspects to consider when designing a particle-based adjuvant to generate desirable immune responses. While there are other parameters that are also important to consider, such as antigen and adjuvant formulation, antigen dose, immunization regimen, administration route, and/or application of immune agonists such as the Toll-like receptor (TLR) ligands [15,16].

The administration route, in particular, may be a key factor to consider for vaccination as different routes may affect the antigen uptake behavior and the type of induced immune responses. Studies have demonstrated that particles are superior antigen-delivery systems that greatly facilitate APC-mediated antigen uptake, which plays a key role in inducing adaptive immune responses [17]. To our knowledge, only a few studies have investigated how administration routes affect the immune responses induced by different adjuvanted immunogens [18,19]. In previous studies, we designed and prepared a series of PLA MPs with various surface charges, hydrophobicity, and surface groups. We then investigated their ability to influence immune responses in mice [20,21]. Our results demonstrated that positively charged PLA MP based vaccines induced potent antigen specific immune responses in mice [22]. Here, we further investigated how the routes of administration influenced immune responses induced by cationic PLA MP-based hepatitis B vaccines compared to alum based or adjuvant free antigen induced immune responses. We initially tested DC activation after co-incubation with different antigen formulations in vitro. Next, mice were immunized, via the subcutaneous (s.c.), intramuscular (i.m.), and intraperitoneal (i.p.) routes. Our results revealed that the administration route and vaccine formulation strongly affected the Th1-associated IgG2a isotype response. Moreover, the cationic PLA MP-based vaccine induced higher Th1/CTL-associated cytokine expression levels than the alum-based vaccine, especially via the i.m. and s.c. immunization routes. These findings support the future development and clinical application of PLA MPs for subunit-based vaccines.

2. Materials and methods

2.1. Materials

Chitosan chloride (CSC) was obtained from Xiamen Blue Bay Science & Technology Co., Ltd. (Xiamen, China). Polyvinyl alcohol was obtained from Kuraray Co., Ltd. (Tokyo, Japan). Polylactic acid (PLA) was obtained from Shandong Institute of Medical Instruments (Jinan, China). Dichloromethane was obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. Recombinant HBsAg (derived from H. polymorpha) and aluminum hydroxide gel (alum adjuvant) were obtained from Hualan Biological Engineering Incorporation (Henan, China). The micro-bicinchoninic acid protein assay (Micro BCA) Kit was obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Shirasu Porous Glass (SPG) membranes were purchased from SPG Technology Co., Ltd. (Miyazaki, Japan). Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, and IgG2a antibodies were purchased from Abcam Ltd. (Hong Kong). Brefeldin A, PE-anti-mouse CD8 and APC-anti-mouse CD69 monoclonal antibodies were purchased from eBioscience (San Diego, USA).

2.2. MP preparation and characterization

CSC surface-coated PLA (CSC-PLA) MPs were prepared by a two-step-modification method. Briefly, PLA MPs were prepared first by performing SPG premix membrane emulsification and emulsion-solvent evaporation as previously reported [23]. The formed PLA MPs were next washed 3 times using ultrapure water, collected by centrifugation, and lyophilized. For the preparation of CSC-coated PLA MPs, lyophilized PLA MPs were dispersed into a 1% (w/v) CSC solution, and incubated for 4 h, at room temperature, in a shaking incubator. Coated cationic PLA MPs were washed using ultrapure water to remove any unattached polymers, centrifuged, and lyophilized for preservation. The surface morphology of CSC-PLA MPs was observed by scanning electron microscopy (SEM) using a JEM-6700F microscope (JEOL, Tokyo, Japan). The average size and surface charge of these MPs were measured by dynamic light scattering (DLS) using a computerized inspection system (Malvern Zetasizer Nano ZS, Malvern, UK). HBsAg adsorption into MP was performed, with slight modification, as described previously [24]. The antigen adsorption efficiency was calculated as follows:

$$AE(\%) = \left(\frac{m_t - m_f}{m_t}\right) \times 100$$

where AE is the adsorption efficiency, $m_t$ is the total amount of antigen added to the system, and $m_f$ is the amount of antigen in the supernatant.

The endotoxin level in the final formulation was determined by the Limulus amebocyte lysate (LAL) assay method using a commercially available endotoxin assay kit (Pyrosate 0.25 EU/mL) from Associates of Cape Cod (Falmouth, MA, USA) according to the manufacturer’s instructions. All formulations were tested and used only when the endotoxin levels were <0.05 EU/mg MP.

2.3. Bone marrow dendritic cell (BMDC) culture and stimulation

Mouse BMDCs were prepared as described previously, with slight modifications [25]. In brief, bone marrow (BM) cells were isolated from the femur and tibia of BALB/c mice. After red blood cell (RBC) lysis, BM cells were cultured in RPMI-1640 medium, supplemented with GM-CSF (10 ng/mL) and IL-4 (50 ng/mL), at 37 °C, for 6 days to acquire immature DCs. Medium was changed every other day. On day 7, the percentage of CD11c+ cells was >85%, as verified by flow cytometry. Immature DCs (5 × 10^6 cells/mL) were stimulated with free HBsAg, antigen adsorbed MP or antigen adsorbed alum formulations for 24 h. The concentrations of HBsAg, alum and MP in the stimulated medium were 1 μg/mL, 60 μg/mL and 50 μg/mL, respectively. At the end of the stimulation, DCs were harvested and incubated with PE-anti-mouse CD40 and APC-anti-mouse CD80 monoclonal antibodies, on ice, for 30 min. CD40 and CD80 expression levels in stimulated medium were 1 μg/mL, 60 μg/mL and 50 μg/mL, respectively. After performing SPG premix membrane emulsification and emulsion-solvent evaporation as previously reported [23]. The formed PLA MPs were next washed 3 times using ultrapure water, collected by centrifugation, and lyophilized. For the preparation of CSC-coated PLA MPs, lyophilized PLA MPs were dispersed into a 1% (w/v) CSC solution, and incubated for 4 h, at room temperature, in a shaking incubator. Coated cationic PLA MPs were washed using ultrapure water to remove any unattached polymers, centrifuged, and lyophilized for preservation. The surface morphology of CSC-PLA MPs was observed by scanning electron microscopy (SEM) using a JEM-6700F microscope (JEOL, Tokyo, Japan). The average size and surface charge of these MPs were measured by dynamic light scattering (DLS) using a computerized inspection system (Malvern Zetasizer Nano ZS, Malvern, UK). HBsAg adsorption into MP was performed, with slight modification, as described previously [24]. The antigen adsorption efficiency was calculated as follows:

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2.4. Animals and immunization

2.4.1. Animals

Female BALB/c mice (4–6 weeks old) were purchased from Vital River Laboratories (Beijing, China). Mice were specific pathogen-free and were treated according to the regulations of Chinese law and the local ethical committee (Animal Care and Use Program Guidelines of Peking University). Briefly, mice were housed throughout the study in climate-controlled (temperature: 23 ± 2 °C; relative humidity: 60%)
and photoperiod controlled (12 h light–dark cycles) housing. Mice were fed standard pelleted rodent chow supplemented with grain and had free access to drinking water. The study included 10 experimental groups (n = 6 mice/group): free HBsAg treatment, HBsAg alum-based, or HBsAg MP-based vaccine formulations administered via s.c., i.m., or i.p. route; untreated mice were used as a control. A two-step immunization regimen (“primary and boost”) was employed; each animal was treated with 4 μg of antigen on days 0 and 28. The total immunization program was sustained for 35 days. The alum concentrations in alum-based vaccine were 2.5 mg/ml and MP concentrations in MP-based formulation were 5 mg/mL. Each animal received 0.1 mL vaccine solutions composed of HBsAg, HBsAg plus alum or HBsAg plus microparticles, respectively.

2.4.2. Collection of sera from vaccinated mice and supernatant from splenocyte cultures

Blood samples were collected from mice retro-orbital plexus on days 15 and 35 after primary immunization. Sera were collected by centrifugation (10,000 rpm for 10 min), and stored at −80 °C until analysis. On day 35, mice were sacrificed, spleens were aseptically removed, and cell suspensions were prepared by gently grinding the spleen on a fine-wire screen. RBCs were removed using ACK Lysis Buffer (Beijing Cell Chip Biotechnology Co., Ltd. Beijing, China), followed by washing (using the medium) and centrifugation (1000 rpm for 5 min). Cells were diluted to 6 × 10⁶ cells/ml in RPMI-1640 medium, containing 10% FBS and 1% penicillin–streptomycin. Cells were incubated with 5 μg/mL HBsAg for 60 h, after which cell culture supernatants were collected.

2.4.3. Determination of HBsAg-specific IgG, IgG1, and IgG2a levels

HBsAg-specific antibody levels were determined by indirect ELISA as described previously [26]. Briefly, 96-well microtiter plates (Corning, Shanghai, China) were incubated at 4 °C, in moist chambers, overnight. Next, plates were washed 3 times with PBS-T (0.01% w/v Tween-20 in PBS) to remove the unbound antigen. The unbound free sites of each well were blocked with 2% w/v bovine serum albumin (BSA) (200 μL) in PBS-T (blocking buffer) for 2 h, at 37 °C, and wells were then washed 3 times with PBS-T. Serial dilutions of each serum sample (100 μL/well) from individual mice were added to the wells in duplicate, and incubated for 1 h, at 37 °C. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, or IgG2a was added to the wells, and the plates were incubated for 1 h, at 37 °C. After washing, peroxidase activity was visualized by adding 100 μL of substrate solution (TMB, H₂O₂) to each well. The reaction was stopped by adding 2 M H₂SO₄ (50 μL) to all wells. The color produced by the reaction was measured at 450 nm, using an Infinite M200 microplate spectrophotometer, and a reference absorbance at 620 nm. ELISA titers were expressed as the maximum dilution ratio whose absorbance was 2.1 × more than that of the control serum (1:100 dilution).

2.4.4. Determination of ex vivo splenocyte activation and cytokine production

After co-incubation with HBsAg as described above, splenocytes were collected and stained with AlexaFluor488-anti-mouse CD4, PE-anti-mouse CD8, and APC-anti-mouse CD69 monoclonal antibodies. After washing, surface CD69 expression on CD4 and CD8 T cells was measured using a CyAn™ ADP flow cytometer. For the intracellular IFN-γ analysis, co-incubated splenocytes (additionally adding brefeldin A into the medium in the last 8 h according to the manufacturer’s protocols) were collected, stained with AlexaFluor488-anti-mouse CD4, PE-anti-mouse CD8, and APC-anti-mouse IFN-γ monoclonal antibodies, and detected by flow cytometry. IL-2, IL-4, IL-10, IL-12, interferon (IFN)-γ, Granzyme B and tumor necrosis factor (TNF)-α cytokine levels in splenocyte culture supernatants (incubation did not include brefeldin A) were measured using Platinum ELISA Kits (eBioscience) according to the manufacturer’s protocols.

2.5. Statistical analysis

All experiments were performed in triplicate, and the data were presented as the mean ± standard deviation (SD). Statistical differences between groups were determined by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test. Values of p < 0.05 were considered statistically significant.

3. Results

3.1. MP preparation and characterization

To investigate the influence of administration routes on the efficacy of alun and MP induced immune responses, we prepared the cationic CSC-coated PLA MPs with a mean size of 826 nm (Fig. 1). Results from our previous study demonstrated that electrostatic interactions played a key role in HBsAg adsorption into MPs and potential for positively charged MP usage in HBsAg vaccine delivery [22]. Here, the cationic CSC attached to the MP surface, creating a positive surface charge of approximately +16 mV (Fig. 1), thus significantly enhancing HBsAg adsorption (81.6%) as compared with uncoated MPs (<5%, data not shown). The alun adjuvant generated a similar potential for HBsAg adsorption (~67%) compared to the CSC-PLA MPs.

3.2. Antigen-adsorbed MP activate DCs in vitro

MPs are known to enhance APC-mediated immune responses. Both CD40 and CD80 act as co-stimulatory molecules in DC maturation and activation. IL-12 secretion is involved in T cell stimulation and function, facilitating naive T cell differentiation into Th1 cells [27]. Therefore tested the ability of the three antigen formulations to activate DCs by analyzing CD40 and CD80 expression levels and IL-12 secretion levels in vitro. The DCs incubated with HBsAg MP-based vaccine (MP-Ag) formulation induced significantly higher CD40 and CD80 expression levels than either the free antigen or the HBsAg alun-based vaccine (alum-Ag) (Fig. 2A–D). MP-Ag also augmented IL-12 secretion levels compared to DCs treated with alum-Ag, which generated the lowest IL-12 levels of the 3 formulations, further illustrating the ability of MP-Ag to enhance DC activation (Fig. 2E). Together, these results indicate that the HBsAg MP-based vaccine had the greatest efficacy in DC activation.

3.3. HBsAg MP vaccine elicited relatively high antibody responses via tested administration routes in mice

Given the enhanced CD40, CD80 and IL-12 levels in DCs, we postulated that the MPs may induce enhanced antigen-specific humoral and cellular
immune responses as a vaccine adjuvant. To begin testing this possibility, we evaluated the influence of administration route on humoral immune responses in vivo, after primary and boost vaccination by measuring HBsAg-specific total IgG. On day 15 after primary immunization, detectable IgG titers were observed in all treatment groups across all three administration routes. The i.m. route induced relatively high IgG titers when compared with the s.c. (lowest IgG titer) and i.p. (moderate IgG titer) routes (Fig. 3A). MP and alum-based vaccines generated significantly higher IgG levels, via all 3 injection routes, than the HBsAg alone group, evidencing that recombinant HBsAg requires the addition of an adjuvant to induce a vigorous immune response.

On day 7 after boost immunization (day 35 after primary immunization), all formulations induced significantly elevated IgG titers compared with data from day 15 after primary immunization (Fig. 3B). Consistent with the above results, i.m. and i.p. immunization routes induced higher IgG titers compared to the s.c. route; MP-Ag and alum-Ag induced IgG titers >20,000 while free antigen groups only generated titers of <9000. MP-Ag and alum-Ag vaccines induced similar IgG titers via the i.m. and i.p. routes of which the i.m. injection generated the much higher IgG responses. This result further demonstrated that the recombinant HBsAg subunit needs the addition of an adjuvant to induce potent antigen-specific IgG responses while both MP and alum are
helpful. In contrast, only low level of IgG titers was induced via the s.c. administration. Together, these results suggest that the HBsAg MP-based vaccine induces relatively high IgG levels via the three tested administration routes, especially via the i.m. and i.p. routes.

3.3.2. HBsAg-specific IgG1 (Th2-associated) and IgG2a (Th1-associated) antibody responses

Th1-associated immunity is known to enhance cell-mediated immune responses. The activation of Th1-like T cells facilitates IgG2a generation, and Th2 polarization regulates IgG1 induction. To determine the effect of administration routes on Th1/Th2 polarization, IgG1 and IgG2a isotype levels were measured by indirect ELISA in serum harvested on day 35 after primary immunization (Fig. 4). MP-Ag induced the highest IgG2a compared to both the alum-Ag and free HBsAg vaccines. The i.m. MP-Ag group generated IgG2a titers of >2500 which was the highest among these groups. Although the s.c. MP-Ag group induced IgG2a titers about 600, this was still 2-fold above the alum-Ag vaccine. MP-Ag formulation induced comparable IgG1 titers with alum-based vaccine among all three immunization routes which were significantly higher than the Ag group. In the comparison of the three administration routes, the i.m. immunization was more conducive to the induction of HBsAg-specific IgG1 and IgG2a. Overall, these results indicate that the HBsAg MP-based vaccine formulation augmented the Th1-associated IgG2a response in all three administration routes, while the alum-based vaccine mainly induced an IgG1-dominated antibody response.

3.4. HBsAg MP vaccine activation of CD4 and CD8 T cells and enhancement of cytokine production upon ex vivo splenocyte re-stimulation

3.4.1. CD4 and CD8 T cells

To continue evaluating the MP and alum adjuvants’ efficacy of generating adaptive immune responses we investigated CD4 and CD8 T cell activation after vaccination. Given that in vivo T cell activation and in vitro T cell activation augment CD69 expression, we evaluated CD69 expression in CD4 and CD8 T cells, after ex vivo re-stimulation of harvested splenocytes with HBsAg (Fig. 5). The CD69+CD8+ T cell frequency in ex vivo re-stimulated splenocytes from the alum-Ag group was higher (3%) than the other vaccine formulations given via the s.c. immunization route (Fig. 5A). In both i.m. and i.p. groups, MP-Ag and alum-Ag induced similar CD4 T cell activation. These results were consistent with the IgG antibody responses shown above, likely because CD4 T cell activation provides help for B cell- and CD8 T cell-mediated immune responses. In measuring CD8 T cell responses, MP-Ag significantly enhanced CD8 T cell activation over the other formulations. This effect was most pronounced in the s.c. and i.m. groups. Overall the i.m. and s.c. immunization MP-Ag groups generated >3% of CD69+CD8+ T cells in ex vivo re-stimulated splenocytes which was significantly higher than the alum and Ag groups (Fig. 5A). This was also higher than splenocytes from MP-Ag i.p.-immunized mice. This result may be indicative of the i.m. and s.c. administration routes promoting CD8 T cell-mediated immune responses. To further confirm these results, intracellular IFN-γ levels in CD4 and CD8 T splenic cells were analyzed (Fig. 5B–C). Both IFN-γ+CD4 and IFN-γ+CD8 T cell
populations were elevated in mice immunized with MP-Ag and alum-Ag vaccines. After s.c. immunization, the MP-Ag group induced comparable IFN-γ+CD4 and IFN-γ+CD8 T cells with alum-based vaccine which was significantly higher than the Ag group. MP-Ag formulation also induced significantly higher IFN-γ+CD4 and IFN-γ+CD8 T cells than the other two immunization groups via the i.m. route. The alum-based vaccine generated relatively high levels of IFN-γ+CD4 T cells via s.c. administration. Low levels of IFN-γ positive CD4 and CD8 T cells were detected via the i.p. route. These results support the HBsAg MP-based vaccine as being superior to the traditional alum-based vaccine in its ability to induce CD8 and CD4 T cell activation via the parenteral administration routes.

### 3.4.2. Cytokine secretion levels from splenocytes after ex vivo re-stimulation

The impact of administration routes on functional cellular immune responses was further investigated by profiling cytokine production resulting from ex vivo re-stimulation of splenocytes. Th1-polarizing cytokines (IL-2 and IL-12), Th1/CTL effector cytokines (IFN-γ, TNF-α, and Granzyme B), and Th2 cytokines (IL-4 and IL-10) were all evaluated.

#### 3.4.2.1. Th1-polarizing cytokines

IL-2 is necessary for T cell growth, proliferation, and differentiation into effector T cells, while IL-12 is involved in the differentiation of naive T cells into Th1 cells as well as activating natural killer (NK) cells and T lymphocytes [28]. Mice immunized with MP-Ag via the s.c. and i.m. routes exhibited significantly higher

![Fig. 5. Characterization of T cell activation.](image-url)
IL-2 levels in ex vivo re-stimulated splenocytes than mice immunized with the other vaccines (Fig. 6). The i.p. group displayed lower levels of IL-2, as all three immunization groups generated similar IL-2 levels. The MP-Ag group via s.c. immunization generated the highest IL-2 level among all the immunization groups. Increased IL-12 levels were also detected in splenocyte cultures from MP-Ag-immunized mice compared to the other formulations, across all 3 administration routes (Fig. 6). As IL-12 secretion can enhance Th1-polarization leading to cell-mediated immune responses. Our results suggest that the HBsAg MP-based vaccine produces an enhanced cellular immune response compared to the traditional alum-based vaccine. This advantage was especially robust in the s.c. and i.m. immunization groups.

3.4.2.2. Th1/CTL effector cytokines. Biodegradable PLA-based MPs have been widely used as vaccine adjuvants to enhance Th1 immune responses. Therefore, we analyzed secretion of the Th1/CTL effector cytokines IFN-γ, TNF-α, and Granzyme B (Fig. 7). The MP-Ag vaccine induced significantly higher Granzyme B levels, via the s.c. and i.m. routes, than the alum-Ag vaccine. There was a pronounced difference in the s.c. route group, where MP-Ag immunization generated >800 pg/mL.
Granzyme B upon splenocyte re-stimulation, compared to the alum-Ag immunization group (~400 pg/mL). All formulations induced low-level Granzyme B secretion in the i.p. group. Here the alum-Ag induced slightly higher secretion, but it was not significantly different from the other two formulations.

Both the MP-Ag and alum-Ag vaccines, administered by the s.c. route induced an ~30-fold higher production of IFN-γ (~3000 pg/mL), after ex vivo re-stimulation than the free HBsAg group. The MP-Ag vaccine induced a ~10-fold higher production of IFN-γ (>2000 pg/mL), in the i.m. route group, than the other formulations. The i.p. immunization groups generated the lowest IFN-γ secretion of the three immunization routes. The MP-Ag vaccine induced the highest levels of IFN-γ (~150 pg/mL) in this immunization group.
3 different administration routes, while the differences among the type of vaccine antigen seemed to have little effect on TNF-α secretion. The s.c. and i.m. routes were more favorable for the induction of TNF-α than the i.p. route. Overall, these results indicate that HBsAg MP-based vaccine could induce significantly higher Th1/CTL effector cytokine secretion than alum-based vaccines across the 3 administration routes. Thus MP-based vaccines could elicit a superior cellular immune response after vaccination.

3.4.2.3. Th2 cytokines. The Th2 cytokines IL-4 and IL-10 were also evaluated. These cytokines induce differentiation of naïve helper T cells (Th0 cells) into Th2 cells, stimulate proliferation of activated B and T cells, and differentiate B cells into plasma cells; all of which ultimately contribute to humoral-mediated immune responses [29]. As shown in Fig. 8, MP-Ag immunization augmented IL-4 levels in ex vivo re-stimulated splenocytes compared to alum-Ag or free antigen groups in the s.c. and i.p. groups. I.p. injection of the alum-Ag and MP-Ag vaccines generated relative low levels of IL-4 compared with the s.c. and i.m. routes. In contrast to the IL-4 results, alum-Ag induced significantly higher IL-10 levels than MP-Ag did; this difference was markedly increased in the s.c. and i.p. groups. All vaccine formulations generated similar IL-10 levels (~500 pg/mL) in the i.m. group. These data indicate that the HBsAg MP-based vaccine induced high IL-4 levels via all 3 vaccination routes, while the alum-based vaccine only induced elevated IL-10 levels.

4. Discussion

Using recombinant viral subunit antigens rather than live attenuated or inactivated viruses to generate vaccines greatly improves vaccine safety. Using recombinant antigens alone, however, induces weak immune responses due to low immunogenicity, thus requiring the addition of an adjuvant to generate effective anti-viral immune responses. Particulate adjuvant delivery systems, such as PLA- and PLGA-based nano- and microparticles, exhibit superior biocompatibility and biodegradability, and hold great promise as adjuvants in the development of new and more effective vaccines [30]. Previous studies have reported how physicochemical properties of these particles, size, surface morphology, surface charge, and hydrophobicity influence resulting immune responses. To date, no studies had defined the role of immunization routes play in vaccine efficacy. Previously, we reported that positively charged PLA MP HBsAg vaccine augmented cellular and humoral immune responses in mice [22]. The route of administration may be an especially pertinent factor to consider for particulate delivery systems, as nano- or micro-sized particles can easily be up taken by various APCs or be engineered to target specific tissues and/or cells [31]. Also different vaccine formulations may significantly affect antigen movement to secondary lymphatic organs, such as the lymph nodes or spleen, which regulate adaptive immune responses [32]. In the present study, we addressed this factor for the first time by comparing the humoral and cellular adaptive immune responses induced by the cationic HBsAg PLA MPs, HBsAg alum, and free HBsAg vaccines in mice, via 3 commonly used (s.c., i.m., and i.p.) parental administration routes.

Previous studies demonstrated that HBV does not activate DCs and impairs CpG-induced DC maturation and function [33]. Moreover, peripheral-blood plasmacytoid dendritic cells (pDCs) in HBV carriers express lower levels of CD40 and CD80 than healthy subjects, leading to the inability of DCs to induce an effective anti-viral immune response against the virus [34]. Our results showed that DCs treated with MP-Ag formulation significantly augmented surface CD40 and CD80 expression levels, as well as T cell-stimulating cytokine IL-12 secretion compared to the alum-Ag vaccine. Antigen adsorbed cationic MP-based formulation could be a potential vaccine candidate that enhances DC activation, as well as promoting antigen processing and presentation to induce potent anti-viral immune responses.

First, different adjuvant formulations, containing HBsAg, were assessed with respect to their potential to elicit the antibody response following primary and boost immunization in mice via the s.c., i.m., or i.p. administration route. Primary immunizations induced relatively low HBsAg-specific IgG titers in all treatment groups. After booster immunizations on day 28, all three vaccine formulations induced significantly elevated IgG titers, especially in the MP and alum-adjuvanted groups. These results reflect the need for repeated immunization, and the requirement of an adjuvant in HBsAg vaccines to generate robust antibody responses. Among the different routes, the i.m. route induced higher IgG titers than the s.c. or i.p. route. Previous studies have demonstrated that i.m. routes have significantly higher anti-HBs mean titers than those found using a subcutaneous route [35,36]. The aforementioned results suggest that i.m. immunization facilitates antigen uptake in antigen-presenting cells culminating in related lymphoid organs mounting a strong antibody response against the antigen.

Similar to the IgG response, the proportion of IgG1 and IgG2a isotype depended not only on administration route but also vaccine formulations. The i.m. immunization route induced potent IgG2a and IgG1 titers in all vaccine formulations when compared with the other two immunization routes. Conversely the s.c. immunization route generated relative low titers. MP-based vaccine tended to induce significantly higher IgG2a antibody titers, especially via the i.m. route, while alum-based vaccine predominantly induced an IgG1 antibody response. Our results were in agreement with other studies showing that MP-based antigen delivery systems are favorable for generating cellular immune responses that induced elevated IgG2a responses [37]. There are several possible mechanisms that explain these results. One is that MP-based antigen formulation is favorable for antigen uptake and DC activation. Activated DCs generate augmented IL-12 levels, one of the key factors promoting Th1 cell polarization [38] and enhances the IgG2a response. A second one, from other study, suggests that an IgG2a response depended on the route of administration, and is likely explained by the antigen-dose-sensitivity of the two IgG isotypes, as the dose of foreign antigen can influence whether a cell-mediated or humoral immune response is elicited: The stimulation of the Th1 phenotype and IgG2a antibody requires relative higher antigen doses, while the Th2 phenotype and IgG1 antibody are responsive to very low doses [39]. Higher amounts of antigen increased the possibility and duration of antigens interacting with both T and B cells, thus facilitating isotype switching [40]. MP adsorbed antigen formulations may provide high amounts of available antigen for DCs, thus generating enhanced adaptive immune responses. This mechanism may also explain the potent Th1 cytokines, IFN-γ, TNF-α, and Granzyme B expression levels induced by the MP-based antigen formulation. Previous studies showed that HBV DNA vaccination via an i.m. immunization route predominantly generated an IgG2a antibody response, and induced the high IgG titers [41]. Our results suggest that the MP-based vaccine formulation favors the generation of Th1 immune responses, especially via i.m. administration.

In a previous study, we have demonstrated that MP-Ag facilitated antigen uptake by APCs [22]. Cationic microparticles favored antigen adsorption and internalization into macrophages, and facilitated the phagocytic antigen localization independent of the lysosome and degradation in proteasome promoting antigen cross-presentation via MHC I which potentially improves cell-mediated immunity. In the present study we report an increase in DC maturation and activation. Given these results the possibility exists that activated DCs enhanced antigen processing and presentation to CD4 and CD8 T cells, which is known to promote T cell activation with the help of co-stimulatory molecules [42,43]. Here, administration routes had little effect on CD69 expression in CD4 T cells, while vaccine formulation significantly affected IFN-γ expression in CD4 and CD8 T cells. MP-Ag formulation induced high levels of IFN-γ and IFN-γ′ CD8 T cells, after ex vivo re-stimulation, especially via the s.c. and i.m. routes. These results agree with previous reports of MP-based antigen delivery systems facilitating antigen presentation via MHC-I activation of CD8 T cell mediated immune responses [44,45]. It is likely that cationic MP with surface antigen adsorption activates the CD8 T cell mediated immune response [46].
Cheng et al. previously demonstrated that i.m. immunization may occur through both direct presentation and cross-presentation of antigens [47], which favors CD4 and CD8 T cell activation [48].

Other studies suggested that the adjuvant potential of our prepared cationic MP may be related to the surface coated chitosan chloride, as chitosan is a kind of polysaccharide derived from the deacetylation of chitin [49]. Three innate immune receptors, Toll-like receptor (TLR) 2, Dectin-1, and the mannose receptor, have been implicated in mediating a variety of immune responses to chitin. Carla et al. have demonstrated that chitin is a size-dependent stimulator of macrophage IL-17A production and IL-17AR expression and these responses are TLR-2 and MyD88-dependent [50]. TLR2 has been found to contribute to sensing of chitin by keratinocytes [51]. Chitosan was also shown to activate the NLRP3 inflammasome, leading to robust IL-1β responses by a phagocytosis-dependent mechanism [52]. Together these mechanisms may also contribute to the potent adjuvant property of chitosan chloride coated microparticles.

Studies have demonstrated that vaccination via the s.c. route could promote Th1-type cytokine expression by facilitating antigen uptake by DCs and cross-presentation after migration into the lymph nodes, and ultimately enhance cellular immune responses [53]. Our results showed that MP-Ag immunization induced significantly higher IL-12 and IL-2 expression levels than those induced by the other formulations across all three routes, suggesting the promotion of T cell proliferation and Th1 polarization [54]. Enhanced Granzyme B and Th1-type cytokine (IFN-γ and TNF-α) expression in this group might be dependent on Th1 polarization. TNF-α and IFN-γ are considered crucial cytokines for the induction of Th1 and CD8 T cell responses, and TNF-α plays an essential role in HBsAg-specific CTL proliferation, which leads to TNF-α and IFN-γ secretion [55]. Granzyme B is a key molecule that mediates CTL destruction of virus-infected cells [56]. These results again confirmed that the MP-based vaccine is a potential vaccine delivery system that can elicit effective Th1 and CD8 T cell mediated immune responses when delivered via the s.c. or i.m. immunization route. The MP-based vaccine also induced relatively low Th2 type IL-4 cytokine expression, in all three routes compared to Th1-type cytokine induction. This may be due to the antagonism that occurs between Th1 and Th2 cytokines, where high Th1 cytokine levels can suppress IL-4 secretion [57]. The alum-based vaccine induced high IL-10 expression levels compared to the MP-based vaccine, when administered via the s.c. and i.p. routes. These results might explain the inability of the alum-based vaccine to generate high levels of Th1-type cytokines, where high IL-10 secretion could antagonize IFN-γ expression [58]. The i.p. immunization group only generated limited antibody response and cytokine expression levels independent on vaccine formulations, indicating that it is not an ideal vaccine administration route for hepatitis B vaccine.

5. Conclusion

Our current research demonstrates that both vaccine formulations and administration routes play important roles in immunization, and the type and strength of the immune response elicited. MP adjuvanted antigen formulations generated significantly higher IgG antibody responses than free antigen, especially via the i.m. immunization route. The administration route also played an important role in generating a Th1-associated IgG2a isotype response. Here, we observed that the MP-based vaccine induced significantly higher IgG2a levels, when administered via the i.m. route, than the alum-based vaccine, which mainly induced an IgG1-dominated antibody response. The MP-based vaccine, administered via the s.c. and i.m. routes, also promoted CD8 T cell activation and Th1-type cytokine expression. These responses are critical for effective clinical anti-viral immunity. The i.m. immunization is the more suitable administration route for MP-based antigen formulation to generate potent antibody and cytokine mediated immune responses. By contrast the i.p. immunization route induced relatively weak HBsAg-specific immune responses. Together, these results may help to guide the future development and clinical applications of MP-based vaccine formulations for subunit antigens, ultimately producing more effective and robust subunit-based vaccines.

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