Nanodiamonds in Oil Emulsions as Effective Vaccine Adjuvants and Antitumor Therapeutic Agents

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Abstract

Background

Vaccination is an effective tool to elicit immunological responses that mediate the protection from infection or disease. Composed of mineral oil and mycobacteria pathogens, complete Freund’s adjuvant (CFA) is one of the most commonly employed adjuvants for antibody production and vaccination due to its high efficiency. However, the dead mycobacteria in CFA can cause many allergic reactions. To avoid these adverse effects, we propose here a new formulation based on the use of nanodiamonds (NDs) as biocompatible non-allergic additives in incomplete Freund’s adjuvant (IFA) instead.

Results

Tested with chicken egg ovalbumin (OVA) in mouse models, the new formulation with 100-nm NDs was found to serve well as a safe and potent vaccine adjuvant that significantly enhanced the immune responses and reduced the consumption of antigens in producing the antibodies of interest. Additionally, the composites showed distinct therapeutic activities, as proven by the OVA/ND/IFA treatment which effectively inhibited the tumor progression of OVA-expressing E.G7 cells inoculated in mice and allowed the animals to survive up to 35 days post tumor-cell challenges.

Conclusions

The dual functionality of ND/IFA makes it useful as adjuvants not only to increase antibody production but also to create single-dose vaccines.

Introduction

Vaccination is an effective public health tool to prevent the spread of infectious disease worldwide [1]. It is playing an ever-increasing role in preventing and controlling epidemics (such as COVID-19) today [2]. The use of vaccines is to elicit immunological responses that mediate the protection from infection or disease. The majority of vaccine antigens currently available and under development are subunits of pathogens or their recombinant molecules with little or no immunostimulatory activities. Therefore, the development of safe and potent immunologic adjuvants that can direct and enhance vaccine-specific immunity is absolutely needed. However, safety has always been a concern for their applications [3, 4]. Despite numerous efforts made in the past, only a handful of adjuvants have been included in licensed human vaccines and few are in clinical trials. Among these vaccine adjuvants, alum (or aluminum salt) is the most widely used, although aluminum is a known neurotoxin and our understanding of its toxicology and pharmacokinetics in the human body is still limited [5, 6]. While new types of adjuvants such as those composed of water-in-oil emulsion, squalene, liposomes, other compounds have been developed, they may have higher local reactogenicity and systemic toxicity than alum alone [7, 8]. How to achieve potent adjuvant effects and yet avoid human reactogenicity or toxicity remains a major challenge in the field of vaccine development to date.
Complete Freund's adjuvant (CFA) is a water-in-oil emulsion containing heat-killed mycobacteria for immunization. It is one of the strongest adjuvants known [9], because the inactivated mycobacteria in CFA effectively attract macrophages and other immune cells to the injection sites whereas the oil acts as an insoluble depot of antigens to achieve long-term immunostimulation. These two characteristics together greatly enhance the immune responses [10, 11]. However, the high reactogenicity and toxicity of CFA have precluded its applicability in human vaccination and, as a result, CFA is most commonly used for antibody production in experimental animals. A way to overcome this limitation is to employ incomplete Freund's adjuvant (IFA), which lacks allergic additives (e.g., inactivated mycobacteria), and mix it with biocompatible, non-allergic, and non-toxic nanoparticles to reduce undesirable side effects. Ideal balance of efficacy and safety is possible to be reached by mixing IFA with synthetic nanoparticles that have been developed over the past few decades for vaccination applications [12, 13]. The approach is appealing because there have been several completed clinical trials using IFA as vaccine adjuvants to treat diseases like human immunodeficiency virus (HIV) infection [14].

The nanoparticles that have been applied for vaccination can be roughly classified into two types [12, 13]: (i) organic nanoparticles including liposomes and polymers; (ii) inorganic nanoparticles include aluminum hydroxides, mesoporous silica, magnetic nanoparticles, gold nanoparticles, and nanodiamonds (NDs). Of particular interest are aluminum hydroxides and mesoporous silica, which have been experimentally demonstrated to be useful as antigen carriers as well as self-adjuvants for vaccine delivery [15–18]. However, the toxic levels of these nanoparticles in the human body remain unclear. NDs, on the other hand, are chemically inert and have excellent biocompatibility and exceptionally low cytotoxicity. They have found practical applications in biology and nanomedicine due to their high surface-area-to-volume ratios, tunable surface chemistry, and the capability of emitting near-infrared fluorescence from color centers [19, 20]. Additionally, NDs have been demonstrated to be able to improve the efficacy of many chemotherapeutic agents by increasing their dispersibility in water, enhancing sustained release, shielding the drug from inactivation, and bypassing the mechanisms of chemoresistance [21–23]. These significant improvements have inspired further research on the sustained release of other therapeutic molecules such as growth factors, peptides, and genes both in vitro and in vivo. A recent study has shown that NDs can serve as an efficient delivery system for immunostimulatory cytosine-phosphate-guanine oligonucleotides with great potential for cancer immunotherapy applications [24]. Another study demonstrated that NDs can be readily taken up by immune cells (including natural killer cells and monocytes), resulting in no compromise in cell viability and immune cell activation, thereby useful for targeted anti-tumor immunotherapy [25]. Furthermore, fluorescent nanodiamond (FND) particles surface-conjugated with immunomodulatory molecules are promising candidate agents with trackable and traceable capabilities to stimulate and manipulate the immune systems [26, 27].

Chicken egg ovalbumin (OVA) was chosen as the model antigen for this study because the protein is a well-characterized target antigen for CD8+ T cells (e.g., cytotoxic T lymphocytes), which specifically recognize the OVA 257–264 peptides and thus offer an excellent opportunity to study antigen-specific T cell immunity [28]. The biocompatible, non-allergic, and non-toxic nanoparticles used in this work were
monocrystalline NDs of 100 nm in diameter. Their surfaces were first oxidized in air and subsequently
carboxylated by acid treatment to facilitate their conjugation with OVA through electrostatic attraction,
hydrogen bonding, van der Waals force, and hydrophobic interactions. The conjugation is expected to
help increase the uptake of antigens by antigen-presenting cells (APCs) through endocytosis of NDs and
thus enhances the antibody production. To achieve this goal, we first noncovalently conjugated OVA with
NDs in phosphate-buffered saline (PBS) to form stable complexes to enable sustained release of the
surface-bound immunogens either \textit{in vitro} or \textit{in vivo}. We then mixed the antigen-containing buffers with
IFA to yield emulsions, followed by subcutaneous injection of the emulsions into healthy mice and
monitoring the animals’ immune responses. Finally, to demonstrate the immunotherapeutic effects of the
vaccine, we inoculated tumor-free mice with E.G7-OVA, which was derived from the mouse lymphoma cell
line EL4 containing a single copy of an inserted gene for constitutive synthesis and secretion of chicken
egg OVA in the cells. How the vaccination inhibited the tumor growth was then examined closely over a
time period of more than 1 month. A significant enhancement in antibody production and an effective
suppression of tumor growth were discovered by use of this new ND-IFA-based adjuvant system.

\section*{Results And Discussion}

\textbf{Characterization of OVA-ND complexes.} NDs before and after mixing with OVA were analyzed for their
size distributions and zeta potentials. Transmission electron microscopy (TEM) of bare NDs first revealed
that the particles were irregular in shape and varied considerably in size (inset in Fig. 1). Dynamic light
scattering measurements showed that bare NDs have a mean hydrodynamic diameter of \textasciitilde 100 nm and a
zeta potential of \textasciitilde 45 mV (Fig. 1). The average diameter of the particles increased by about 20 nm after
mixing with OVA in water, indicating that these NDs have been successfully coated with OVA by physical
adsorption. As the isoelectric point of OVA is 4.5 \textsuperscript{[29]}, meaning that the protein molecules are negatively
charged in PBS buffer (pH 7.4), the change of the zeta potential from \textasciitilde 45 mV of ND to \textasciitilde 23 mV of OVA-
ND implied that forces (such as hydrophobic forces) other than electrostatic interaction play important
roles in the protein adsorption process.

As a member of nanocarbon family, the surface of NDs can be conveniently modified with a variety of
oxygen-containing groups such as \textasciitilde COOH, \textasciitilde COH, \textasciitilde COOC\textasciitilde, etc. by extensive washes in strong oxidative
acids. Uniquely, the acid-washed NDs exhibit an exceptionally high affinity for a wide range of protein
molecules including bovine serum albumin (BSA), myoglobin, cytochrome c, lysozyme, and luciferase
\textsuperscript{[30–32]}. Moreover, the structural integrity of these proteins is retained, as demonstrated by the catalytic
activities of lysozyme and luciferase after adsorption on NDs \textsuperscript{[31, 32]}. Chicken OVA is a phosphorylated
glycoprotein consisting of 385 amino acid residues with a molecular weight of 42.7 kDa or a total
molecular weight of 45 kDa including the carbohydrate and phosphate portions \textsuperscript{[29]}. To evaluate the
amount of OVA that could be loaded on the acid-washed NDs, we measured the changes in optical
absorbance of unbound OVA at 280 nm before and after mixing with the nanoparticles (Supplementary
\textbf{Figure S1}). For OVA adsorbed on 100-nm NDs, we determined a protein loading capacity of OVA:ND = 1:8
(weight ratio) at saturation. Assuming a spherical shape for the adsorbent, this high loading capacity
suggests that each 100-nm ND (weight of ~1.8 fg/particle) can accommodate more than 3000 OVA molecules on surface.

**Immune responses.** The *in vivo* experiments were started by mixing 5 µg OVA with 60 µg NDs and dispersing the OVA-conjugated NDs in PBS, CFA, or IFA prior to subcutaneous injection of the mixtures into BALB/C mice. The corresponding control experiments consisted of 5 µg OVA in PBS, CFA, or IFA, respectively (Figs. 2 and 3). Figure 2A shows the timeline of immunization and blood collection in this experiment. The water-in-oil emulsions formed small nodules and appeared as soft capsules at the injection sites upon immunization. We evaluated OVA-specific IgG antibody responses in the sera of the immunized mice with enzyme-linked immunosorbent assays (ELISA) after the second and third immunizations with OVA and OVA/ND in CFA. As shown in Fig. 2B and 2C, the OVA/ND/CFA treatments induced a significantly higher amount of OVA-specific IgG antibodies in the mouse sera than OVA/CFA alone by 3.5- and 1.6-fold, respectively, after the second and third immunizations. It is demonstrated that the addition of NDs in CFA is able to elicit highly efficient and protective immune responses against OVA in the mouse body, in line with a previous report that NDs can enhance immune responses against recombinant HA/H7N9 in mice [33].

Next, we investigated the dose dependence of the immune response by employing OVA/CFA and OVA/ND/CFA containing 25 µg OVA. The amount of NDs used accordingly increased to 300 µg. Indeed, a 2-fold increase of the OVA-specific IgG antibody production was found in the OVA/CFA treatment (Fig. 2A). However, the response did not exceed that of the 5-µg treatment with OVA/ND/CFA. Notably, further increase of the OVA dose failed to boost the immune response in the OVA/ND/CFA treatment. The result suggested a saturation effect, where no higher levels of anti-OVA could be reached irrespective of the doses of OVA applied. An important implication of this finding is that the use of NDs as additives in CFA can help reduce the consumption of antigens in producing the antibodies of interest, which is a valuable feature for industrial production of antibodies.

We explored further if the same level of immune response by OVA/ND/CFA could be maintained without the need of allergic components such as inactivated mycobacteria in CFA. The dosage groups of 5 µg and 25 µg OVA were tested in parallel in this experiment. As shown in Fig. 3, we did not found significant differences in the results between OVA/ND/IFA and OVA/ND/CFA treatments in these two groups, indicating that the substitution of dead mycobacteria by NDs as additives in the mineral oil not only can improve the safety but also can maintain the efficacy of the vaccine adjuvant. This new combination of substances is expected to work well also as immune drug delivery vehicles to promote directed antitumor activities with minimal systemic toxicity [27].

**Antitumor therapeutics.** The new formulation of NDs in oil emulsions is applicable as antitumor therapeutic agents as well. To demonstrate the application, we employed the mouse lymphoma cell lines, EL4 and E.G7-OVA. The E.G7-OVA cells are able to express OVA and have been widely used in cancer immunotherapy studies. Depicted in Fig. 4A is the timeline for the injection of OVA/ND/IFA, followed by the inoculation of EL4 and E.G7-OVA cells in C57BL/6 mice. By referring to the unvaccinated groups, we
found that the treatment with OVA/ND/IFA in the EL4 model was unable to delay the tumor growth (Fig. 4B). In contrast, the OVA/ND/IFA treatment could effectively inhibit the tumor progression in the E.G7 model over 3 weeks post inoculation of the cells (Fig. 4C). Notably, half of the mice (4 out of 7 mice) in the E.G7 model maintained tumor-free for more than 15 days after cell inoculation (Fig. 4D) and survived up to 35 days post tumor cell challenges (Fig. 4E). In Fig. 4F, we show photographs of the tumors isolated on day 24 from vaccinated and non-vaccinated mice. The difference in tumor size between these two groups (in triplicate) of mice is substantially, about 10 times in total volume. Taken together, these results indicate that the presently developed nanovaccines with ND/IFA as adjuvants are promising agents for cancer immunotherapy.

To further assess the therapeutic potential of OVA/ND/IFA, we investigated the in vivo immunostimulatory activity of the agent with just one dose in each mouse. Single-dose therapy has several advantages over multiple-dose therapy, including greater patient compliance, less risk of side effects, and lower costs [34]. In particular, knowing the effectiveness of the single-dose vaccines composed of either whole viruses, protein subunits, viral vectors, or nucleic acids (RNA and DNA) is critically important in the prevention and control of COVID-19 infections today [35]. Additionally, in protecting livestock (such as cattle, sheep, pigs, and goats) from infectious diseases, single-dose veterinary vaccine makes it easier for suppliers to streamline the production process and distribution of the agents to rural areas [36].

In this single-shot experiment, mice were first administrated with OVA/ND/IFA via subcutaneous injection and then examined by measuring the production of anti-OVA IgG in the mouse sera on a weekly basis. We found that the OVA/ND/IFA treatment could dramatically induce the production of OVA-specific IgG antibodies on day 28 and day 35 after the administration (Fig. 5). Compared with the OVA/ND and OVA/IFA groups using the same amount of antigens, the OVA/ND/IFA treatment boosted the levels of anti-OVA IgG by 432 and 6 times on day 28, respectively. The enhancement factor further increased to 1717 and 19 times on day 35. It is demonstrated that the addition of NDs can greatly improve the effectiveness of IFA as a single-dose vaccine adjuvant, which is capable of sustaining its immunostimulatory activities over an extended period of time.

Finally, we explored whether or not the addition of NDs in IFA altered the mechanism of the immune response elicited by IFA alone, which is known to proceed predominantly through the Th2 pathway (i.e. humoral immune response) [17, 37]. We addressed the question by performing ELISA assays for cytokines in the sera of C57BL/6 mice after injection with OVA/ND/IFA. As shown in Fig. 6, only a small difference in the interleukin 2 (IL-2) level was found between the control and treatment groups, whereas a marked elevation of the interleukin 4 (IL-4) concentration in the vaccinated group was detected. Furthermore, by replacing NDs with FNDs in the adjuvants, we were able to clearly identify the presence of FNDs in mouse spleens through background-free detection of far-red fluorescence at ~ 700 nm in the tissue digests (Supplementary Figure S2 and ref. [38] for details). All the results led us to a possible predominant mechanism for the initiation of the immune response by the ND/IFA-based vaccine as follows: (i) formation of nodules with loose structure in mouse tissues after subcutaneous injection of
the antigen-loaded ND/IFA emulsion, in which the adjuvants act as a depot; (ii) sustained release of the antigens from NDs in the water phase of the emulsions; (iii) active and continuous recruitment of immature immune cells to the depot; (iv) uptake of the antigen-loaded NDs by the immune cells through endocytosis; and (v) promotion of Th2 response, where helper T cells bind with the antigen presenting cells and activate the development of B cells into antibody-producing plasma cells in spleens. The proposed mechanism is depicted in Fig. 7.

Conclusions

We have demonstrated that the new formulation consisting of ND (diameter of ~ 100 nm) mixed in IFA is capable of generating effective and durable immune responses in mice. Without the need of allergic additives (i.e. dead mycobacteria), the addition of highly biocompatible NDs in the oil emulsion can not only retain the adjuvanticity of IFA but also significantly reduce the level of side effects. Compared with existing products, the ND-in-oil adjuvant has three major advantages: high safety, low side effects, and low demand in antigen. By applying OVA as the model antigen in small animals like mice, our studies clearly show that ND/IFA can serve well as an active vaccine platform to induce sustained and potent immune responses. Additionally, the adjuvants are useful as antitumor therapeutic agents, as proven by the OVA/ND/IFA treatment which effectively inhibits the tumor progression of OVA-expressing E.G7 cells inoculated into mice. Further research, development, and optimization of the ND-based new formulation into single-dose vaccines may find real-world applications of this technology in diverse areas, particularly in the care and protection of domesticated animals.

Materials And Methods

Chemicals and reagents. OVA, CFA, IFA, PBS, and all other chemicals were from MilliporeSigma and used without further purification. Mouse OVA-specific IgG antibodies were obtained from Abcam.

NDs. Monocrystalline synthetic diamond powders with a nominal size of 100 nm were obtained from Element Six. To remove metallic impurities and graphitic carbon atoms on the surface, the diamond powders were first oxidized in air at 490°C for 2 h, followed by microwave cleaning in concentrated \( \text{H}_2\text{SO}_4 - \text{HNO}_3 \) (3:1, v/v) solution at 100°C for 3 h to functionalize the ND surface with \(-\text{COOH}\) groups [31].

OVA-conjugated NDs. OVA-conjugated NDs were synthesized via a simple mixing of 5 µL of antigen solution (1 or 5 mg/mL) with 30 µL of ND suspension (2 or 10 mg/mL) in a shaker for 1 h at room temperature. Excess amounts of OVA were removed by centrifugal separation and water wash.

Particle characterization. Hydrodynamic sizes and zeta potentials of air-oxidized, acid-washed NDs and OVA-conjugated NDs were measured with a particle size and zeta potential analyzer (DelsaNano C, Beckman Coulter). The morphologies of NDs on copper grids were imaged with a transmission electron microscope (H-7650, Hitachi) operating at an acceleration voltage of 75 kV.
**Cell cultures.** EL4 and E.G7-OVA cells were obtained from Bioresource Collection and Research Center, Taiwan, and used together with the mouse models. The E.G7-OVA cells with the OVA expression were derived from the C57BL/6 mouse lymphoma cell line, EL4, transfected with pAc-neo-OVA plasmids [39]. The EL4 cells were grown in DMEM culture medium (Thermo Fisher Scientific) complemented with 10% horse serum and Antibiotic-Antimycotic. The E.G7-OVA cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 10 mM HEPES, 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol and 0.4 mg/mL G418 at 37°C in a humidified atmosphere of 5% CO₂.

**Immunization.** BALB/C mice and C57BL/6 mice (female, 6–8 weeks) obtained from BioLASCO, Taiwan, were immunized by subcutaneous injection of 100 µL solutions or emulsions containing either (i) OVA in PBS, (ii) OVA in CFA, (iii) OVA in IFA, (iv) OVA/ND in PBS, (v) OVA/ND in CFA, or (vi) OVA/ND in IFA on day 1, 14, and 28. The water-in-oil emulsions were prepared by mixing 35 µL of OVA/ND suspension in PBS with 65 µL of CFA or IFA. The immunogens and the adjuvants were thoroughly emulsified by pipetting up and down before injection. All mice were maintained under pathogen-free conditions and treated benevolently to eliminate or reduce suffering.

**Antitumor therapeutics.** C57BL/6 mice were subcutaneously immunized with the vaccine formulation as solutions or emulsions for 3 times at 2-week intervals and then challenged by E.G7-OVA lymphoma cells (5 × 10⁵ cells) or OVA-negative EL4 tumor cells (5 × 10⁵ cells) on day 7 post the final immunization. The tumor growth was monitored starting from day 10 post tumor cell inoculation. For the single-dose therapeutic treatment, anti-OVA IgG was examined on a weekly basis after the immunization.

**IgG antibody assays.** Mouse blood was collected from the submandibular veins of vaccinated or non-vaccinated mice on various days after immunization. OVA-specific IgG antibody responses of the immunized mice were evaluated by using ELISA with the collected mouse sera measured in a microplate reader (GloMax, Promega).

**Declarations**

**Ethics approval and consent to participate**

All the procedures related to animal experiments were approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine (NTUCM).

**Consent for publication**

Not applicable.

**Availability of data and materials**

All the data are available on request from the corresponding author (H.C.C.).
Competing interests

The authors declare no competing interests

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Authors’ contributions

HHL, MDP, HHH, and HCC designed the research. HHL, CYW, FJH, FZL, and YKS prepared and characterized NDs and OVA-NDs. HHL, CYW, FJH, and HHH performed animal experiments and associated assays. HHL, CYW, MDP, HHH, and HCC wrote and revised the manuscript. All authors approved the nal version of the manuscript.

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References


Figures
Figure 1

Hydrodynamic sizes of bare NDs and OVA-conjugated NDs in deionized distilled water. Values given in the parentheses of annotations are zeta potentials. Inset: TEM image of bare NDs. Scale bar: 100 nm.
Figure 2

(A) Timeline of the experimental procedures using OVA/ND/CFA for anti-OVA production in mice. (B) Concentrations of OVA-specific IgG antibodies in mouse sera after subcutaneous injection of 5 μg and 25 μg OVA along with their adjuvants in mice after the second immunization. (C) Concentrations of OVA-specific IgG antibodies in mouse sera after subcutaneous injection of 5 μg and 25 μg OVA along with their adjuvants in mice after the third immunization. Values in (B) and (C) are means ± standard deviations for 3 mice/group.
Abilities of producing OVA-specific IgG antibodies by the treatments with OVA/ND in IFA or CFA subcutaneously administered into mice. Values indicated are the amounts of OVA used in the immunization. Values are means ± standard deviations for 3 mice/group.
Figure 4

(A) Timeline of the experimental procedures for using OVA/ND/IFA as antitumor therapeutic agents in mice. (B, C) Changes of the tumor volumes of EL4- (B) or E.G7-OVA- (C) inoculated mice with the tumor sizes measured every 2 days. (D) Comparison of the percentages of the tumor-free mice between non-vaccination groups and vaccination groups in E.G7-OVA-inoculated mice. (E) Comparison of the survival rates between non-vaccination groups and vaccination groups in E.G7-OVA-inoculated mice. (F)
Comparison of the tumors isolated on day 24 between non-vaccination groups (top) and vaccination groups (bottom) of the E.G7-OVA-inoculated mice. Scale bar: 1.5 cm. Values in (B) – (E) are means ± standard deviations for 7 mice/group.

Figure 5

Increases of the concentrations of OVA-specific IgG antibodies in mice subcutaneously administered with four different therapeutic agents. Only one dose of the therapeutic agent is applied in the individual assay. Values are means ± standard deviations for 3 mice/group.
Changes of the IL-2 and IL-4 levels in mice subcutaneously administered with OVA/ND/IFA. The control and treatment groups consist of non-vaccinated and vaccinated mice, respectively. Values are means ± standard deviations for 3 mice/group.
Figure 7

Proposed predominant mechanism for the induction of immune response by the ND/IFA adjuvants.

Supplementary Files

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- OVA.JNBSI.pdf