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Original Article

A visible fluorescent nanovaccine based on functional genipin crosslinked ovalbumin protein nanoparticles

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Abstract

Accurate and efficient antigen delivery is crucial for inducing a strong and long-term immune response. A visible protein nanovaccine made from antigen could provide a novel and promising technology for secure and efficient delivery of the antigen with imaging visualization. In this study, a functional nanovaccine based on genipin crosslinked ovalbumin (OVA) fluorescent nanoparticles with chitosan (CS-OVA-NPs) was developed. The nanovaccine can carry abundant antigens by self-crosslinking without additional carriers. The fluorescence imaging technique was applied to monitor and reveal the process of antigen delivery in vivo based on the fluorescence of genipin with a non-invasive and real-time manner. This functional OVA nanovaccine can enhance the uptake of OVA in Dendritic Cells (DCs) and further promote DCs to mature in vitro. In vivo study further indicated CS-OVA-NPs could trigger antigen-specific immune responses, which demonstrated that this fluorescent nanovaccine provided a novel design approach for accurate and efficient vaccine delivery.

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Key words: Antigen delivery; In vivo fluorescence tracking; Protein nanoparticles; Nanovaccine; Ovalbumin

Vaccination, one of the greatest achievements in medicine, has a great advantage of higher efficacy and less side effects on account of antigen-specific immune response, which has provided an extremely available approach to fight with infectious disease depending on the development of an effective adaptive immune response.^{1,2} In order to enhance the efficiency of the immune therapy and reveal the mechanism of the vaccination, the accurate and efficient delivery of the vaccine is a significant challenge for the immune therapy. However, the weak immunogenicity of soluble protein antigens reduces significantly the immune efficacy due to the low delivery efficiency.^{3,4} Thereby, considerable researches have focused on the vaccine delivery systems to protect antigen against enzymatic degradation,⁵ enhance antigen uptake efficiency^{6,7} by professional presentation cells like dendritic cells (DCs) or strengthen cross-presentation in a targeted and sustained

release manner to activate antigen-specific T cells.^{8–11} On the other hand, the accurate vaccine delivery can be located and tracked in real-time by imaging visualization, which can reveal the delivery process objectively.^{12–14} Accordingly, the design and development of an efficient and accurate vaccine delivery system play decisive roles to the immune therapy.

To enhance the efficiency of the immune therapy, nanotechnology has attracted wide attention in vaccine development.^{15–18} The application of nanotechnology has made it possible to design nanovaccine for effective antigen delivery.¹⁹ These nanovaccines are able to induce lasting humoral and cellular immune response by different loading methods including encapsulation, conjugation, absorption or mixing of the antigen for vaccine delivery.^{1,20} However, the low antigens loading of the nanocarriers still limited the effective antigen-specific immune response. Notably, protein-

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based nanoparticles explore a novel strategy for the nanovaccine with abundant loading. Protein-based nanoparticles consisted of the bioactive proteins exhibit the unique ability for the vaccine delivery because the bioactive proteins not only stimulate the immune response as the immune antigens, but also improve the delivery efficiency as the delivery carriers themselves. Protein-based nanoparticles can merge the functions of both the immune antigens and the nanoparticle carriers by their self-assembling or cross-linking beyond of other vaccine delivery systems, which can enhance the antigens loading efficiency without additional nanocarriers. Vaccines made from particles consisting of pure protein antigen have been reported before.^{21,22} Protein particles were produced using a method called dense gas anti-solvent precipitation. Protein particles and others were also generated by heat induced aggregation. In both cases, protein particles were more immunogenic *in vivo* than the soluble form of the same proteins even in the absence of alum and Freund's adjuvant. On the other hand, covalent crosslinking has been applied to protein crystals²³ and tumor antigens,²⁴ resulting in stronger immunogenicity in comparison to soluble or uncrosslinking antigens. Based on the tremendous potential of the protein nanoparticles with antigens for the vaccination, novel protein nanovaccines need to be further explored in order to meet the requirement of the immune therapy. As ovalbumin (OVA) protein has usually been applied a model antigen,^{14,25} a functional OVA nanovaccine deserves for further development as a potential vaccine.

Another important issue for the vaccine delivery system is the accuracy of the vaccine delivery. With the development of medical imaging techniques, imaging guiding visualization can afford an accurate vaccine delivery for the locating, monitoring and tracking with a noninvasive and real time method. They can track the drastic process of the vaccine delivery systems from the injection site to the draining lymph nodes *in vivo*. Understanding the process of vaccine delivery has significant implications for rational vaccine design. By monitoring the vaccine delivery process, the vaccine could be constantly optimized from various aspects including cellular uptake, targeted migration or promoting the maturation. At present, the *in vivo* fate of vaccines after administration has been reported in several different imaging studies including radionuclide imaging, computed tomography (CT), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and fluorescence imaging.^{13,26,27} Fluorescence imaging is unique and advanced compared to other imaging methods owe to its advantageous of the high sensitivity, low radiation, non-invasion and long-term monitoring.^{28–30} In our previous studies, fluorescence imaging has been applied in the non-invasive tracking for the drug delivery system including the drug release and distribution, the location and degradation of the carrier.^{31–33} Several fluorescence drug delivery systems were successfully developed for the tumor therapy without external fluorescence labeling, suggesting the superiority and advance of the accurate delivery with the self-fluorescent delivery system.^{32,33} A considerable number of vaccine tracking studies with fluorescence imaging focus on tracking of DCs, the delivery system or the antigen vaccine, which have approved the important role for the vaccination.^{12,13,34} The combination of fluorescence imaging and vaccine delivery will develop an innovative method for the accurate delivery with imaging visualization.

A functional fluorescent OVA nanovaccine can exert the accurate and efficient delivery of the vaccine with imaging visualization. The functional OVA nanovaccine can strengthen cross-presentation in a targeted and sustained release manner to activate antigen-specific T cells, while the vaccine delivery can be simultaneously tracked and monitored by the *in vivo* fluorescence imaging. In this article, a nanovaccine based on genipin cross-linked functional OVA nanoparticles was designed to meet the requirement of the accurate and efficient delivery of the vaccine with imaging visualization (Figure 1). The nanovaccine can carry abundant antigens by self-crosslinking of antigens and exert accurate delivery with self-fluorescence. The genipin crosslinked OVA nanoparticles combined the functions of antigen, vehicle and fluorescence as a novel nanovaccine. In addition, the self-crosslinked OVA fluorescent nanoparticles were further modified with chitosan to improve the stability and cellular uptake capacity. Meanwhile, using the fluorescent property of genipin, the vaccine delivery and the antigen retention was monitored in real-time from the injected site to draining lymph nodes *in vivo*. They indicated the potential of the genipin crosslinked OVA nanoparticles as a visible and efficient antigen delivery system for induction of antigen-specific immune response.

Methods

Materials

Ovalbumin (OVA) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Chitosan (molecular weight of 5,000–8,000 Da; degree of deacetylation of 90%) was obtained from Golden-Shell Biochemical Co., Ltd. (Yuhuan, Zhejiang, People's Republic of China). Mouse cytokine ELISA kits and fluorochrome-labeled MHC I, CD3e, CD40, CD86, CD11c, CD44, CD62L, CD4, and CD8a, CD8b, CD90.1 antibodies were obtained from eBioscience (San Diego, CA, USA). Recombinant mouse GM-CSF and IL-4 were obtained from Peprotech (Rocky Hill, NJ, USA). PE-labeled anti-mouse intracellular interferon- γ (IFN- γ) antibodies were obtained from BD Biosciences (San Jose, CA, USA). Genipin (98%) was purchased from Xi'an Plant Bio-engineering Corporation (Xi'an, China).

Cells and mice

The bone marrow cells (BMDCs) were obtained from bone marrow cells of C57BL/6 mice according to the previously described method.²⁰ Mouse B16-OVA melanoma tumor cell line was purchased from the Cell Bank of China Academy of Sciences, and cultured according to the manufacture's guidelines. Male C57BL/6 mice (6 weeks old) were housed with free access to food and water. Native OT-I T cells were obtained by separating single-cell suspensions from the spleens of OT-I mice. All animal procedures were conducted following the protocol approved by the Institutional Laboratory Animal Ethics Committee and the Institutional Animal Care and Use Committee (IACUC), Peking Union Medical College, People's Republic of China. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals, Peking Union Medical College, People's Republic of China.

Results

Characterization of nanoparticles

The OVA nanoparticles were prepared by the crosslinked reaction of genipin and the amino groups of OVA itself. To achieve the stable nanoparticles and maintain the fluorescence characteristics, a suitable concentration of genipin was chosen by optimizing the ratio of genipin and OVA. CD spectrum analysis (Figure 2, A) showed no significant structural change between free OVA and OVA-NPs, which indicated the covalent crosslinking did not damage the secondary structure of OVA. From the infrared spectra of OVA-NPs, nonabsorption could be observed in OVA in fingerprint regions from 1000 cm^{-1} to 750 cm^{-1} , where the strong absorption peaks were detected in genipin and the weak absorption peaks in OVA-NPs (Figure 2, B). In addition, the degree of crosslinking was evaluated by the free amino group content of OVA. The free amino group content of OVA is approximate 5.5 times to the OVA NPs sample with equal amount of protein, which demonstrated the degree of crosslinking is approximate 80%. All these results gave the direct evidence about the crosslinking of OVA by genipin. AFM images (Figure 2, C) showed that the morphology of CS-OVA-NPs and OVA-NPs was no detectable morphological difference. The mean diameter of OVA-NPs was $86.34 \pm 3.21\text{ nm}$ and the zeta potential was $-14.2 \pm 0.9\text{ mv}$, while the CS-OVA-NPs had an increased diameter of $157.5 \pm 9.86\text{ nm}$ and the zeta potential dramatically increased to $23.4 \pm 1.69\text{ mv}$ (Figure 2, D) after the modification of CS through ionic gelation method. All these data indicated that positively-charged CS on the surface of OVA-NPs successfully elevated the zeta potential of the nanoparticles, which can improve the stability and the cellular uptake of nanoparticles.

UV absorption spectrum (Figure 2, E) showed that the characteristic peak was the main absorption at 590 nm and the modification of CS did not lead to the shift of the absorption peak. Fluorescent spectra analysis (Figure 2, F) indicated that the maximum emission was observed at the wavelength of 615 nm with an excitation wavelength of 596 nm . The fluorescence of nanoparticles was suitable for in vivo fluorescence imaging with the low interference of autofluorescence. All these data indicated that the properties of the CS-OVA-NPs including size, potentials and self-fluorescence were sufficient to be a visible antigen delivery system.

BMDCs viability assay

Cell viability was calculated by comparing free OVA, OVA-NPs or CS-OVA-NPs treated cells with untreated cells. As shown in Figure 2, G, those nanoparticles did not induce cytotoxicity in BMDCs with the OVA concentration ranging from $10\text{ }\mu\text{g/mL}$ to $100\text{ }\mu\text{g/mL}$. Meanwhile, it was observed that there was a slight increase of cell viability for CS-OVA-NPs group at the low concentration range ($10\text{--}50\text{ }\mu\text{g/mL}$). One possible reason is that chitosan promoted the growth of BMDCs to some extent as an additional inflammatory and adjuvanting effect. This could be one of the contributing factors why chitosan nanoparticles had the potential to stimulate innate immune responses.³⁵

Antigen uptake and intracellular localization in BMDCs

In order to investigate the delivery process, confocal fluorescent microscope images were used to track the influence signal of genipin in BMDCs after incubation for 12h. The quantitative flow cytometry detection was further used to measure the cellular uptake of OVA nanoparticles. Confocal images (Figure 3, C) revealed the OVA (red) were delivered into DCs by OVA-NPs or CS-OVA-NPs and the nanoparticles mainly resided in endosomes or lysosomes (green). The lysosome escape played an important role for the following immune responses, especially for the crosspresentation. It was found that most of the CS-OVA-NPs were captured by endosomes/lysosomes, while there were still some CS-OVA-NPs escaped from them. However, the phenomenon was not observed in the OVA-NPs treated cells. The flow cytometry data (Figure 3, A, B) illustrated the low uptake efficiency of OVA-NPs owing to negatively charges, while the relative fluorescence was enhanced more than 4-folds in cells treated with CS-OVA-NPs.

Activation and maturation of BMDCs

Flow cytometry analysis data (Figure 3, D) indicated that CS-OVA-NPs induced the highest level expression of CD40, CD86 and MHC-I in the DCs surfaces, which verified the consequence of high uptake efficiency for DCs maturation. Moreover, MHC-I is a major histocompatibility complex class I molecule, which presents antigenic peptides to cytotoxic T cells. Therefore, the increased expression of MHC-I has an important effect for the cellular immunity. It was worth noted that CD86 and MHC-I expression elicited by OVA-NPs was higher than that elicited by free OVA, which suggested that antigen nanoparticles maybe more available to stimulate immune response due to the specific structure compared with soluble antigen.

In vivo tracking of labeled NPs

As show in Figure 4, A, fluorescence intensity change of OVA nanoparticles appeared clearly in tail-base injection site in a real-time manner. It was observed that the fluorescence of free OVA disappeared quickly. But for OVA-NPs group, especially for CS-OVA-NPs, the fluorescence had a longer retention time in the injection site. By quantitative analysis of the mean fluorescence intensity (Figure 4, B), it was found that approximate 70% or 62% fluorescent signal remained in CS-OVA-NPs or OVA-NPs group 24 hours after injection respectively, while the fluorescence intensity rapidly decayed to 28% in free OVA group. At day 8, no visible fluorescence signal was monitored in free OVA group, while around 10% or 28% of the fluorescence still reserved for OVA-NPs or CS-OVA-NPs, respectively. Moreover, ~18% of fluorescent signal still remained in CS-OVA-NPs group whereas only ~2 % for OVA-NPs group. All these data suggested the self-crosslinked CS-OVA-NPs protected the OVA from quick releasing for the persistence of an effective antigen concentration.

It is necessary that the matured DCs migrate to the lymph node, where the immune responses are initiated. Herein, the fluorescence images of draining lymph nodes demonstrated the

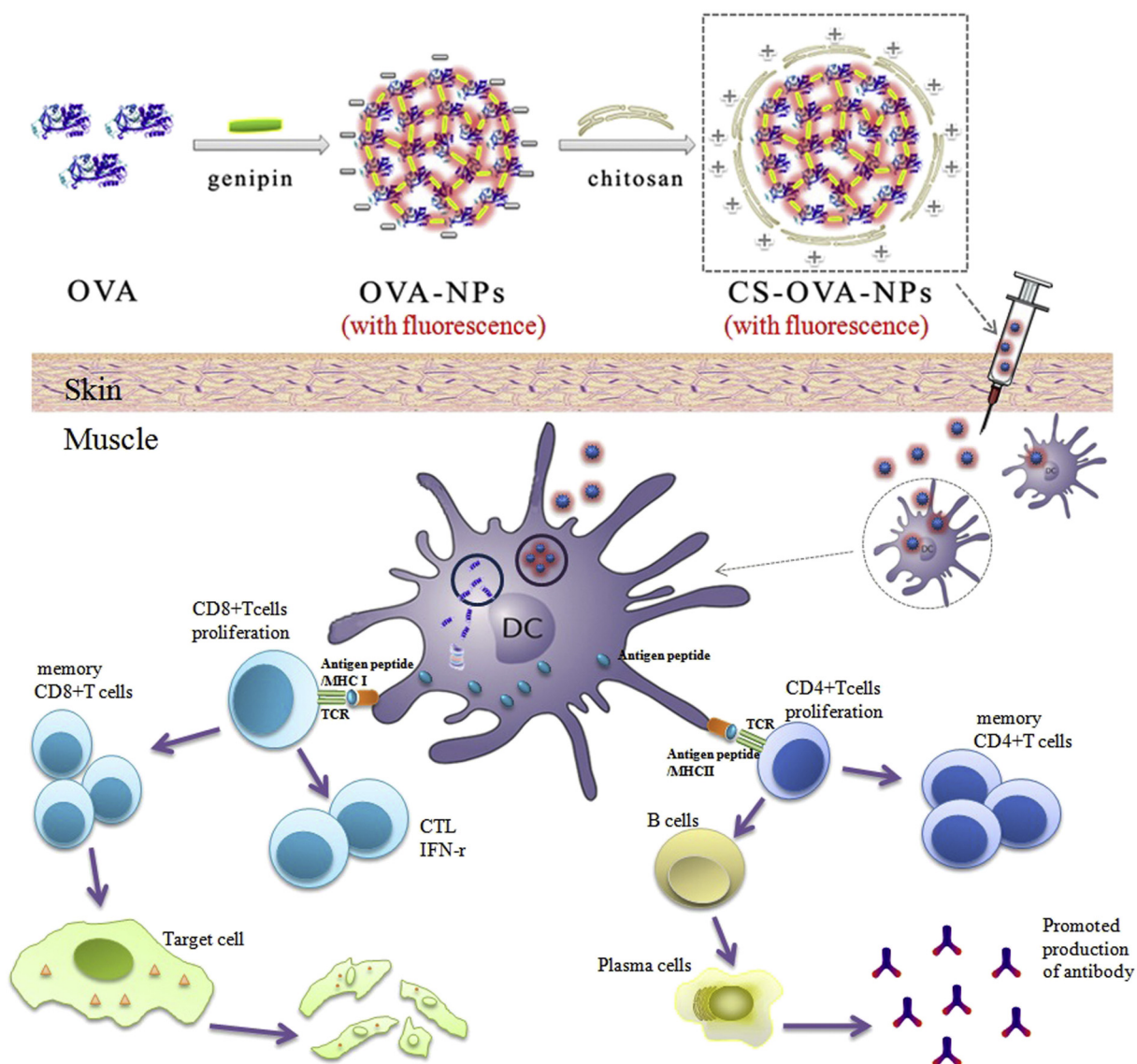


Figure 1. Schematic illustration of the formulation of fluorescent CS-OVA-NPs and the immune response in vivo.

presence of CS-OVA-NPs, OVA-NPs and free OVA from the injection site to draining lymph nodes (Figure 4, C). The fluorescence signal appeared in draining lymph nodes was strong at 24h in CS-OVA-NPs group, but it was very weak in OVA-NPs group and no migration of antigen was observed in free OVA group with the same exposure time. It indicated that CS-OVA-NPs can migrate to the draining lymph nodes more efficiently from the injection sites than OVA-NPs. It was supposed that the more efficient migration of CS-OVA-NPs to lymph nodes was depended on the longer retention time and the higher uptake of CS-OVA-NPs by DCs.

T-cell activation and antigen-specific immune response assay

The splenocytes were harvested in mice after the third immunization and restimulated by OVA for 3 days, the

proportion of CD4⁺ or CD8⁺ T cells was measured by flow cytometry. As shown in Figure 5, the proportion of CD4⁺ and CD8⁺ T were both markedly enhanced in mice immunized with CS-OVA-NPs compared with OVA-NPs and free OVA.

Cellular immunity is greatly necessary for the treatment of infectious diseases and tumor immunotherapy. Interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) are typical markers of cellular immunity. Herein, the activity of antigen-specific CD8⁺ T cells was evaluated based on the fraction of IFN- γ -secreting CD8⁺ T cells in splenocytes restimulated ex vivo with SIINFEKL. Intracellular cytokine staining indicated that there was a statistically elevated level of IFN- γ ⁺CD8⁺ T cells in CS-OVA-NPs treated group with average percentage of 2.78%. Immunization with CS-OVA-NPs resulted in a 2-fold increase in IFN- γ production of CD8⁺ T cells compared with OVA-NPs (1.32%) (Figure 6, A). Meanwhile, the increased

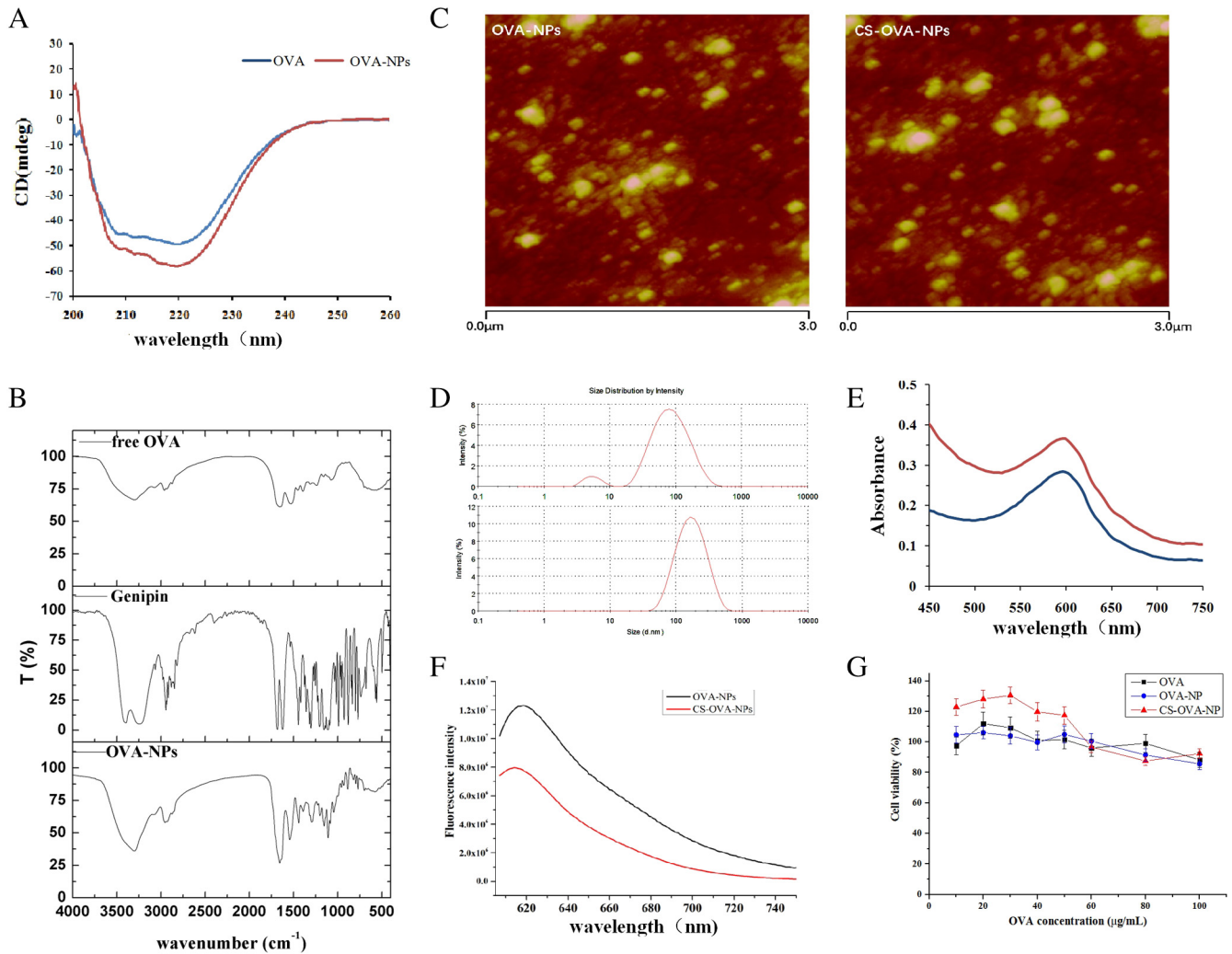


Figure 2. Characterization of different OVA nanoparticles. (A) far-UV CD spectra of free OVA and OVA-NPs. (B) Infrared spectra of free OVA, genipin and OVA-NPs. (C) Atomic force microscopy (AFM) images. (D) size distribution, (E) UV-vis spectra and (F) fluorescence emission spectra of OVA-NPs and CS-OVA-NPs. (G) Cytotoxicity of OVA nanoparticles after 24h of incubation with BMDCs. Data are presented as the mean $\pm$ SD (n=6).

secretion of IFN- γ in splenocytes supernatant further confirmed the T cell activation by ELISA assay (Figure 6, B). The combined application of intracellular staining and ELISA assay verified that CS-OVA-NPs could induce a higher percentage of CD8⁺ T cells with the ability to synthesize cytokines IFN- γ , as well as the cytokines could be further released to the extracellular environment to work for the cytotoxic T lymphocyte responses. Similar to the improved IFN- γ secretion, significant secretion of TNF- α was detected in serum in the mice immunized with CS-OVA-NPs (Figure 6, G), whereas no obvious difference was observed between OVA-NPs and free OVA group.

CS-OVA-NPs enhanced antibody response in vivo

The effectiveness of CS-OVA-NPs and OVA-NPs on antibody response was measured by ELISA 7 days after immunization. Antibody production of IgG, IgG1 and IgG2a in immunized mice was detected from blood serum. The results demonstrated that CS-OVA-NPs induced higher antigen-

specific IgG, IgG1 and IgG2a production than OVA-NPs and free OVA (Figure 6, C), but no significant difference was found in the three antibody production between OVA-NPs and free OVA. The results indicated only protein nanoparticles were not enough to induce strong humoral immune response without the addition of adjuvant. IgG2a antibody revealed a Th1-polarized immune response, and the ratio of IgG2a/IgG1 represented a Th1-biased immune response.³⁶ As shown in Figure 6, D, CS-OVA-NPs induced stronger IgG2a responses compared with OVA-NPs or free OVA. Taken together, the effectiveness of CS-OVA-NPs on antibody response maybe attribute to the sustained antigen release from CS-OVA-NPs. All these responses may increase the function of B cells to produce antibody.

In vivo antigen cross-presentation

To evaluate whether CS-OVA-NPs could induce antigen cross-presentation in vivo, CS-OVA-NPs, OVA-NPs or free

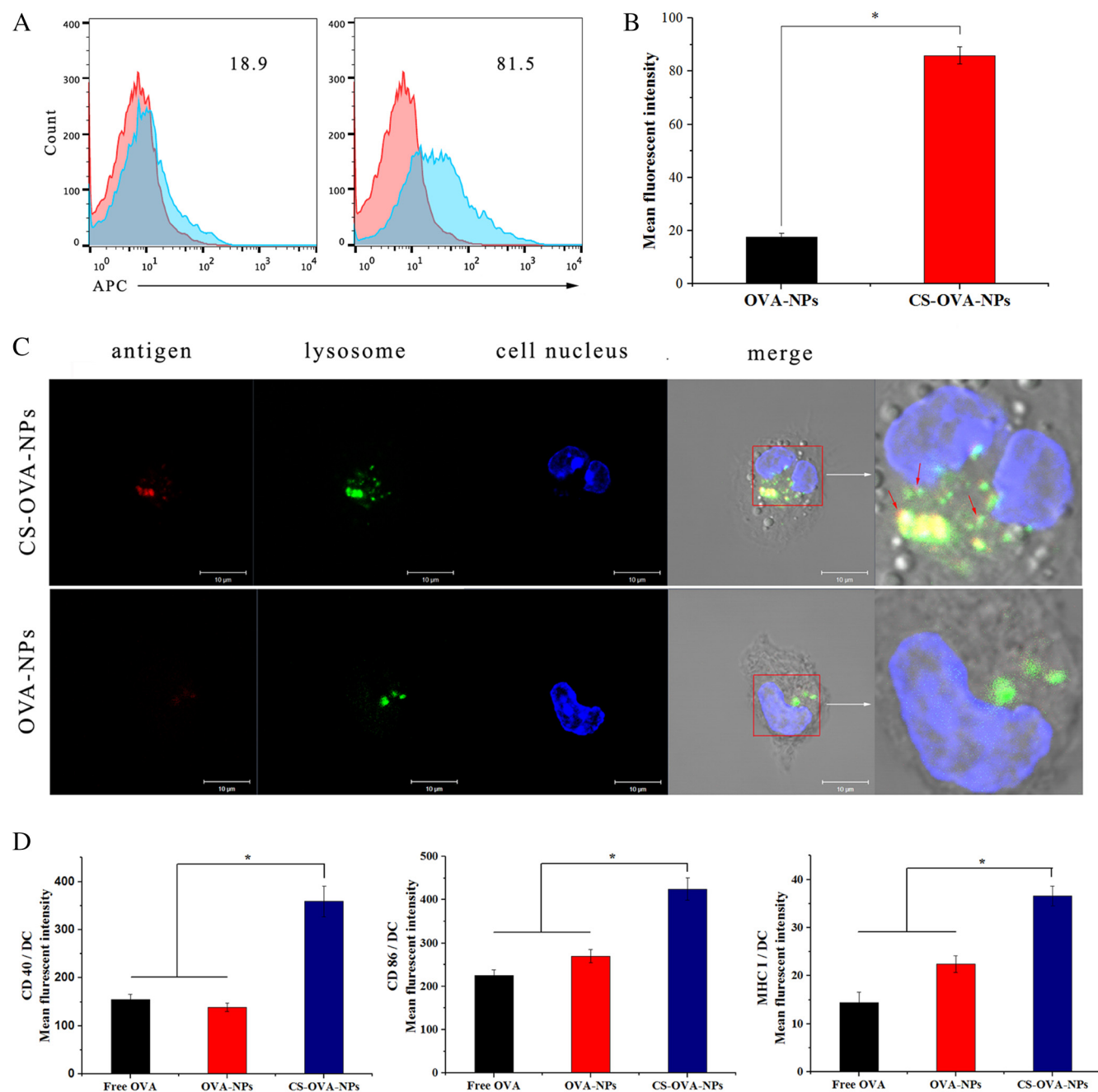


Figure 3. (A) Cellular uptake of OVA-NPs and CS-OVA-NPs in BMDCs was analysed by flow cytometer. (B) The quantitative analysis of mean fluorescence intensity (MFI). (C) CLSM images of BMDCs after 12h incubation with OVA- NPs or CS-OVA-NPs. Red: genipin, green: lysotracker green, blue:DAPI. (D) Enhanced DC maturation after CS-OVA-NPs treatment. Quantification of CD40⁺, CD86⁺ and MHC I⁺ expression in DCs (CD11c⁺) after incubation with free OVA, OVA-NPs and CS-OVA-NPs for 24h by flow cytometry (* $p < 0.05$).

OVA were administered to mice. One day later, OVA-specific CD8⁺ T cells (OT-I cells) were injected intravenously, the precursor frequency of OVA-specific T cells was artificially increased to a high level. The proliferation of these transferred OT-I T cells in the spleen was used to reflect the effect of in vivo cross-presentation of OVA antigens. As shown in Figure 6, E, a significant expansion of OT-I cells was observed in the spleen of the mice immunized with CS-OVA-NPs compared with OVA-NPs treated group. Almost no expansion was observed in the

mice immunized with free OVA. These results indicated the enhanced antigen uptake by the application of CS-OVA-NPs could effectively induce the activation of peripheral lymphocytes including the injected OT-1 cells. Further, the proliferation of CD8⁺ T cells in the spleen (Figure 6, F) verified the ability of OVA self-crosslinked nanoparticles to induce antigen cross-presentation to OVA specific CD8⁺ T cells, which was very important to the tumor immunotherapy based on cellular immunity.

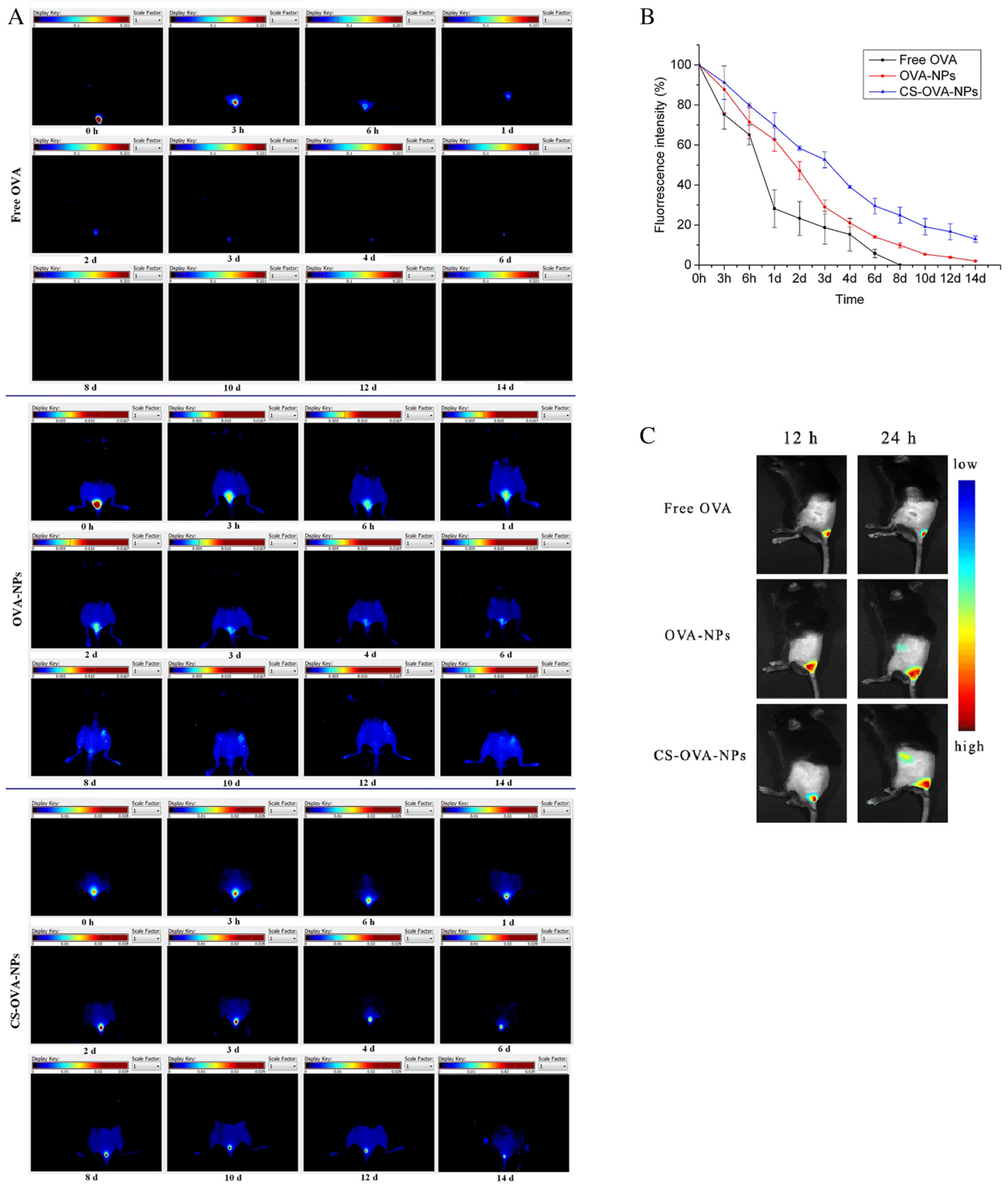


Figure 4. In vivo visualization of free OVA, OVA-NPs and CS-OVA-NPs in the injection site and draining lymph nodes based on the influence signal of genipin. (A) Fluorescence imaging for in vivo tracking of OVA antigen and (B) quantitative analysis of fluorescence intensity. Fluorescent signal at the injection site expressed as a percentage of initial signal. Statistical analysis was performed, $n=3$ mice per group. (C) Antigen transport into draining lymph nodes.

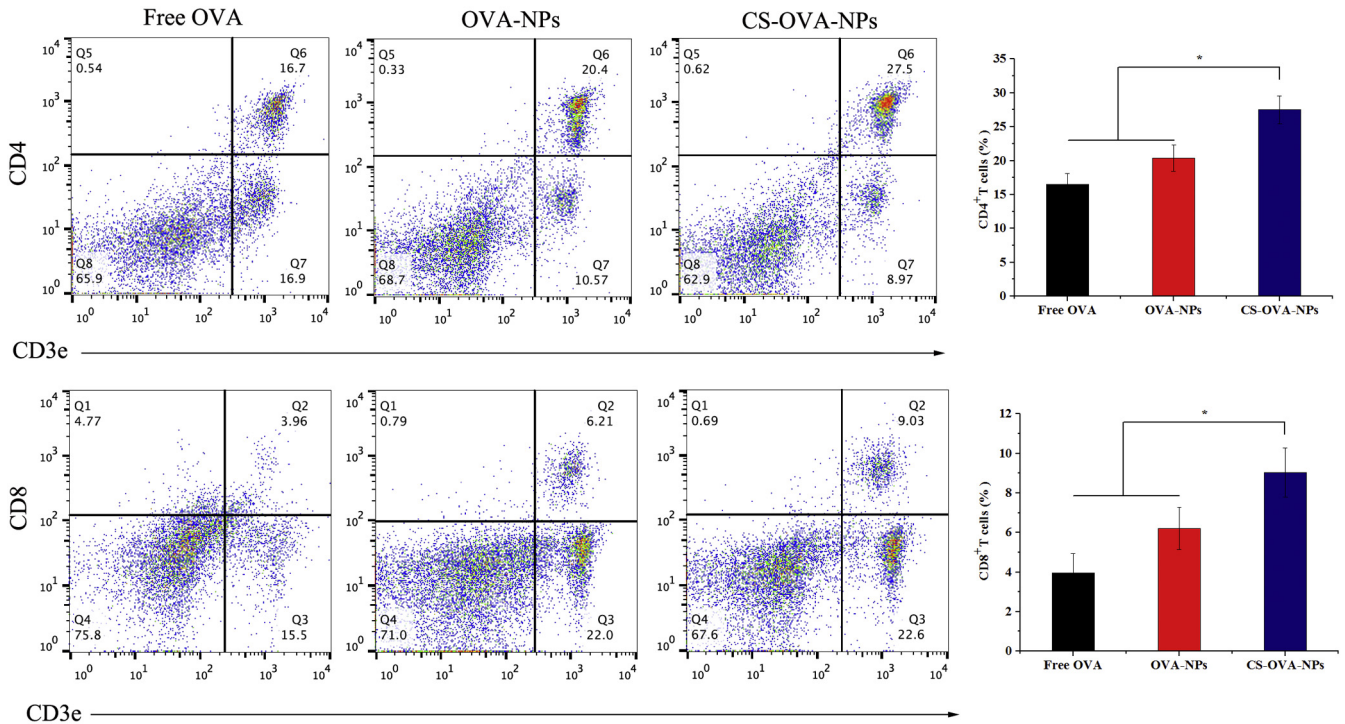


Figure 5. The activation of CD8⁺ and CD4⁺ T cells in spleen ex vivo after restimulation with OVA (50 µg/mL) 14 days after the third immunization. Data are expressed as the mean ± SD (n = 6) (*p < 0.05).

Cytotoxic T lymphocyte responses

After 7 days immunized with CS-OVA-NPs, OVA-NPs or free OVA, the splenocytes were isolated to restimulate with OVA and incubate with B16-OVA melanoma cells, then the cytotoxicity was measured of CTLs to tumor cells. As shown in Figure 6, H, an enhanced cytotoxic response induced by CTLs could be observed in splenocytes immunized with CS-OVA-NPs at a E/T ratio of 20:1 compared with OVA-NPs and free OVA. All these data illustrated that the CS-OVA-NPs could induce antigen-specific immune responses and destroy cancer cells, which suggested its great importance in cancer immunotherapy.

CS-OVA-NPs enhanced memory T cells responses in vivo

Immunological memory consists of memory T and B cells, of which memory T cells are the foundation of immunological memory. The ability of adaptive immune response is closely associated with the production of antigen-specific effector T cells (T_{em}, CD44^{hi} CD62^{lo}) and central memory T cells (T_{cm}, CD44^{hi} CD62^{hi}), with T_{cm} exhibiting more powerful responses than T_{em} when encountering the pathogen. To evaluate the responses of memory T-cell, the percentage of memory T-cell was measured in mouse splenocytes restimulated with antigen following immunization with CS-OVA-NPs, OVA-NPs or free OVA. As shown in Figure 7, CS-OVA-NPs elicited significant higher number of T_{cm} cells compared with OVA-NPs or free OVA. These results indicated that CS-OVA-NPs could induce stronger memory CD4⁺ and CD8⁺ T cells response and provide better protection from reinfection.

Discussion

The low antigens loading of the nanocarriers still limited the effective antigen-specific immune response. The development of protein-based nanoparticles can solve the problem to some extent. These nanoparticles were made entirely of crosslinked antigens, which could not only significantly increase antigens loading efficiency but also enhance antigen immunogenicity and immune protection.³⁷ Furthermore, fluorescence imaging plays a crucial role in studying the immunological mechanism of vaccine with a noninvasive and real time pattern. Herein, in order to build a novel nanovaccine to meet the requirement of accurate and efficient vaccine delivery with imaging visualization, a genipin crosslinked functional OVA nanoparticle was designed, which combined the advantages of abundant antigen, delivery vehicle and fluorescence monitoring.

First of all, the stable self-crosslinked OVA nanoparticles (CS-OVA-NPs) made of OVA proteins was obtained through the covalent crosslinking and modification of CS. OVA is negatively charged at neutral pH due to its pI of 4.9. It is unfavorable for the stability and cellular uptake because of the negative charged condition of cell membrane. In order to improve the stability and cellular uptake efficiency of OVA nanoparticles, the functionalization of OVA nanoparticles is a suitable strategy. Chitosan (CS) is a cationic polysaccharide for a variety of applications including immunization.^{35,38,39} Earlier studies indicated that CS nanocarriers had been used as feasible vehicles for in vivo delivery of proteins or peptides because they were convenient to control the characteristics, such as particle size, size distribution,

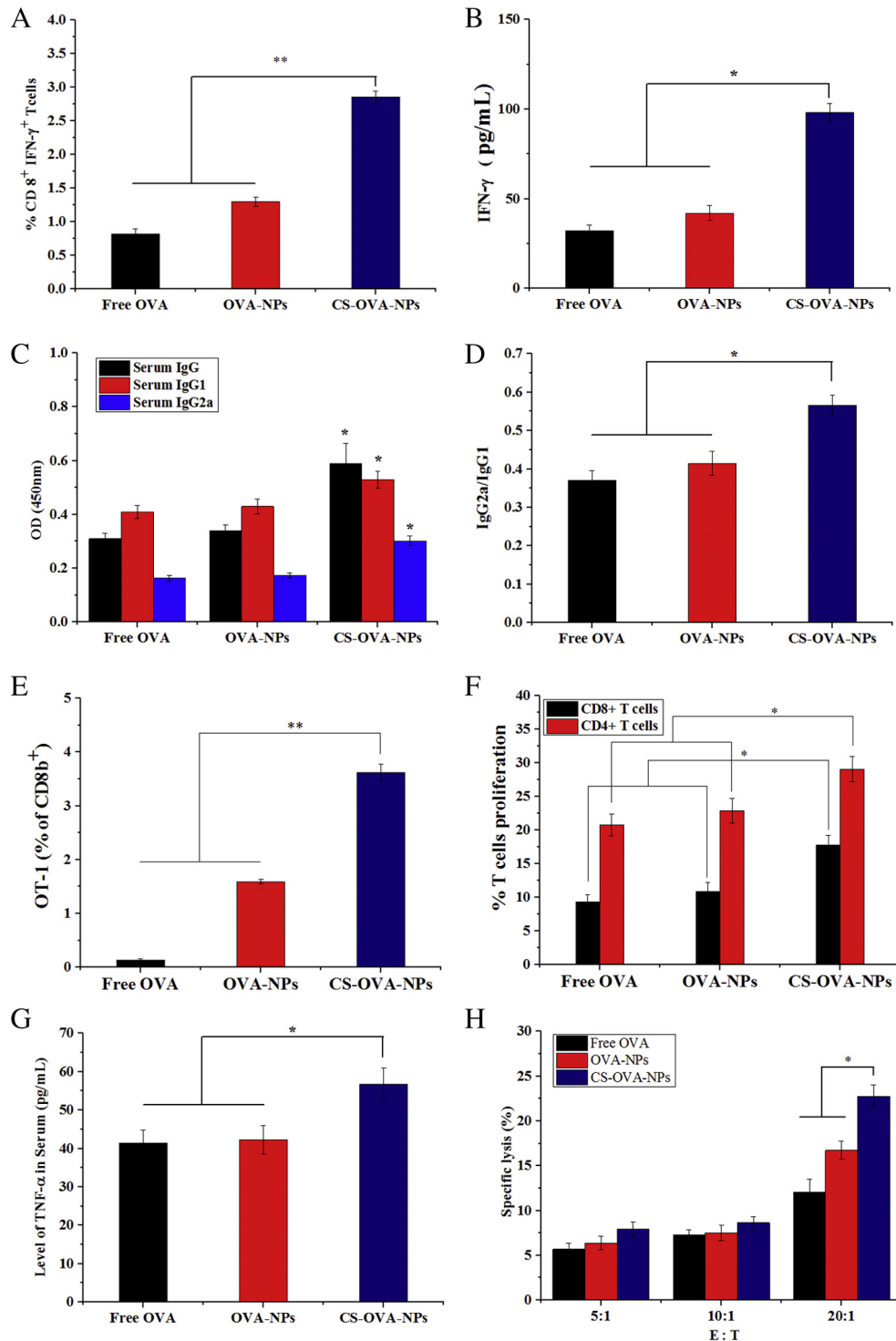


Figure 6. The proportion of IFN-γ⁺ CD8⁺ T cells was measured after 3h of resitimulation by intracellular staining and flow cytometry (A). IFN-γ and TNF-α expression were measured by ELISA (B and G). Antigen-specific total IgG, IgG1 and IgG2a levels were measured in the serum of mice immunized with free OVA, OVA-NPs and CS-OVA-NPs (C). The IgG1/ IgG2a ratio was presented (D). Expansion of OT-1 (E) and proliferation of OVA-specific CD4⁺ and CD8⁺ T cells (F) in spleen was determined by ex vivo flow cytometry for the in vivo crosspresentation. CTL response was measured by incubating resitimated splenocytes with B16-OVA tumor cells, the activity was represented by the mean percentage specific lysis of tumor cells. E:T means effector:target. Data are expressed as the mean ± SD (n = 6) (*p < 0.05).

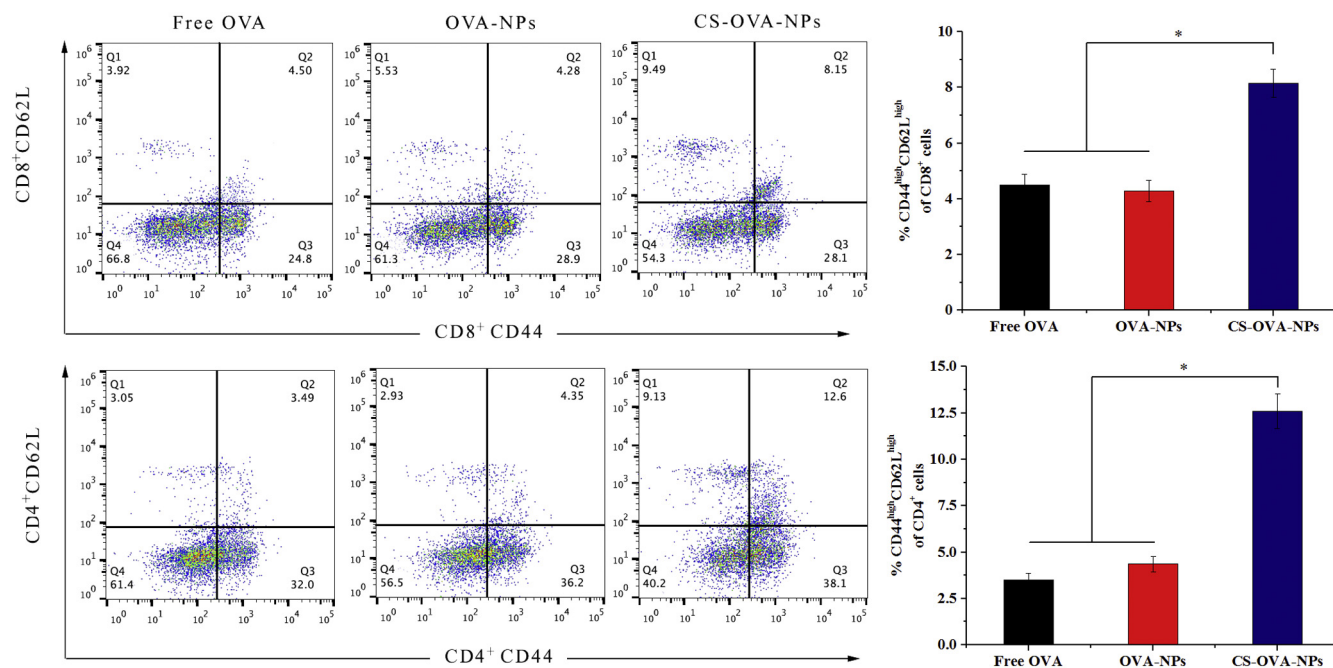


Figure 7. Proportion of central memory (TCM, CD44^{Hi} CD62L^{Hi}) in CD4⁺ T cells and CD8⁺ T cells restimulated with OVA (50 µg/mL) 14 days after the third immunization. The proportions of the CD44^{Hi} CD62L^{Hi}/CD4⁺ T cells, CD44^{Hi} CD62L^{Hi}/CD8⁺ T cells were determined by flow cytometry. Data are expressed as the mean $\pm$ SD (n = 6) (*p < 0.05).

and surface charge, which can significantly influence the release and immune effects.⁴⁰ Moreover, chitosan nanoparticles could exhibit adjuvant effects by stimulating innate immune.⁴¹ Thereby, CS was selected to modify on the surface of OVA-NPs for the better stability and cellular uptake efficiency. CS-OVA-NPs themselves were expected to be antigen delivery carriers, which not only were more convenient to control the antigen dosage, but also avoided the possible toxicity by the addition of other carrier material.

Generally, imaging fluorescent labels were conjugated, encapsulated or covalent crosslinked to the delivery system to provide fluorescence signal in vitro and in vivo including ICG or quantum dots,^{12,42} and a variety of fluorescent labeling was employed for in vivo antigen or vehicle tracking.^{13,43} However, the spontaneous release or structural damage of these external fluorescent labels may lead to an adverse influence in vivo. Covalent cross-linking even might damage the structure and function of the delivery system or the antigen vaccine. All these factors would significantly affect the sensitivity and accuracy of fluorescence imaging. Therefore, developing an innovative method for the accurate delivery with visualization is particularly important for vaccine delivery. Genipin, an effective naturally occurring crosslinking agent, has been used in crosslinking of amine-group-containing biomaterials, such as chitosan, gelatin, silk protein, collagen, etc., which is much less toxic than glutaraldehyde and many other commonly used synthetic crosslinking reagents.⁴⁴ What's more, the formation of blue fluorescent pigments after reaction with amino acids affords the suitable fluorescent signals for tracking proteins or nanocarriers in vitro or in vivo.^{45,46} Unlike the external fluorescent labels, the intrinsic pigment would provide more

stable and accurate information for antigens or carriers in vitro and in vivo. The fluorescence of genipin made it possible for the accurate and efficient delivery of the vaccine with imaging visualization.

The cellular uptake results indicated that OVA could be real-time monitored by the fluorescent signal of genipin. The colocalization of OVA and endo/lysosomes could be clearly observed inside the DCs, which confirmed that the optical property of the genipin-crosslinked OVA nanoparticles could completely meet the requirement of cell imaging. What's more, the intrinsic pigment would generate more stable fluorescent signal for visualized tracking on the cellular level as the ingredient of the nanoparticles than the external fluorescent label. It was also worth noted that a little part of CS-OVA-NPs escaped from lysosome, while OVA-NPs almost entirely resided in endosomes or early lysosomes. This phenomenon suggested that the endosomal escape mainly be caused by the surface modification of chitosan. Previous research also reported that chitosans have a similar capacity like PEI to mediate endosomal escape through the proton sponge effect.⁴⁷ Although no obvious endosomal escape was observed here, the small amount of CS-OVA-NPs escaped from lysosome still might be dealt with via the MHC-I antigen presentation pathway, then induce potent cellular immunity response. Furthermore, the sustained antigen release and gradual migration from the injection site to draining lymph nodes were successfully visualized in vivo. Therefore, the genipin crosslinked functional OVA nanoparticle successfully merged the function of antigen, vehicle and fluorescence.

To assess the effects of different OVA nanoparticles on DCs maturation, the expression of adhesion-related (CD40) and costimulatory (CD86) molecules and MHC class I molecules

were analyzed in DCs by flow cytometry. All these data confirmed that covalent cross-linking OVA nanoparticles did not influence antigen activity and the ability of DCs to mature, which was an important prerequisite for the further immune response study in vivo. CD4⁺ T cells were crucial helper cells for CD8⁺ CTL and also could be effector cells or partners with NK cells to clear the infected cells, as well as CD8⁺ T cells are critical effectors for cancer immunotherapy. Herein, CS-OVA-NPs and OVA-NPs could significantly improve the T cells activation compared to the soluble antigen. Tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ) are typical marker of cellular immunity, which play important roles in inhibiting the growth of tumor. In the study, the production level of TNF- α and IFN- γ indicated that CS-OVA-NPs were more effective in inducing secretion of cytokines and all these results led to the enhanced cytotoxic T lymphocyte responses. Memory T cells are important components of memory immune response and they can produce a faster and stronger immune response at a second encounter with the invader than the first time. The quantity and quality of the memory T cells have an important impact on the long-term immunological protection. It is necessary that vaccines can induce as large a memory T-cell population as possible. Herein, CS-OVA-NPs elicited significant higher number of memory CD4⁺ and CD8⁺ cells compared with OVA-NPs or free OVA. It suggested that CS-OVA-NPs could generate a long-time immunological memory to ensure persistent protection of reinfection in vivo.

Simplicity, high efficiency and visualization are three crucial factors for vaccine delivery, which were combined in our CS-OVA-NPs nanovaccine. The nanovaccine can carry abundant antigens by self-crosslinking of antigens and exert accurate delivery with self-fluorescence. CS-OVA-NPs exhibited great application potential as attractive antigen delivery vehicles with higher OVA loading efficiency. The fluorescent characteristics of CS-OVA-NPs also met the requirement for visualization in one step cross-linking without any external fluorescence labeling. However, the results in this study indicated that only altering the presentation of protein antigens from a soluble to a nanoparticulate (OVA-NPs) form was not completely sufficient for increasing immune effect. Thus functional nanoparticles would be further optimized like making them escape more efficiently from endosomes/lysosomes or prolonging retention time for stronger cellular immunity. In further study, the immune effects of nanoparticles need to be improved by the optimization of the nanoparticles.

In summary, a visible antigen delivery platform based on genipin crosslinked OVA protein nanoparticles was developed for delivery, tracking and vaccination with the advantages of self-fluorescent, biocompatible and low-toxic properties. On the one hand, functional OVA self-crosslinked nanoparticles enhanced the uptake of OVA in DCs and further promote DC activation and maturation in vitro. In vivo, genipin crosslinked functional OVA nanoparticle triggered antigen-specific immune response including CD4⁺ and CD8⁺ T-cell responses, IgG antibody production, enhanced memory T-cell generation and CTL effect. On the other hand, the self-fluorescence of OVA nanoparticles was used for the real-time tracking of cellular uptake in vitro and monitoring of the migration of DC into the

lymph nodes and sustained release of antigen in vivo. Genipin cross-link functional OVA nanoparticles are attractive nanovaccine for accurate and efficient protein antigen delivery with real-time tracking visualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nano.2018.02.007>.

References

- Karch CP, Burkhard P. Vaccine technologies: From whole organisms to rationally designed protein assemblies. *Biochem Pharmacol* 2016;**120**:1–14.
- Rappuoli R. Vaccines: science, health, longevity, and wealth. *S A* 2014;**111**:12282.
- Sokolova V, Knuschke T, Kovtun A, Buer J, Epple M, Westendorf AM. The use of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin to induce dendritic cell maturation and T cell activation. *Biomaterials* 2010;**31**:5627–33.
- Kang TH, Monie A, Wu LS, Pang X, Hung CF, Wu TC. Enhancement of protein vaccine potency by in vivo electroporation mediated intramuscular injection. *Vaccine* 2011;**29**:1082–9.
- Akagi T, Shima F, Akashi M. Intracellular degradation and distribution of protein-encapsulated amphiphilic poly(amino acid) nanoparticles. *Biomaterials* 2011;**32**:4959–67.
- Stano A, Scott EA, Dane KY, Swartz MA, Hubbell JA. Tunable T cell immunity towards a protein antigen using polymersomes vs. solid-core nanoparticles. *Biomaterials* 2013;**34**:4339–46.
- Mogroo J, da Costa CA, Gaspar R, Florindo HF. Modulation of dendritic cells by nanotechnology-based immunotherapeutic strategies. *J Biomed Nanotechnol* 2016;**12**:405–34.
- Kaminskas LM, Porter CJ. Targeting the lymphatics using dendritic polymers (dendrimers). *Adv Drug Deliv Rev* 2011;**63**:890–900.
- Wilson JT, Keller S, Manganiello MJ, Cheng C, Lee CC, Opara C, et al. pH-Responsive nanoparticle vaccines for dual-delivery of antigens and immunostimulatory oligonucleotides. *ACS Nano* 2013;**7**:3912–25.
- Eby JK, Dane KY, O'Neil CP, Hirose S, Swartz MA, Hubbell JA. Polymer micelles with pyridyl disulfide-coupled antigen travel through lymphatics and show enhanced cellular responses following immunization. *Acta Biomater* 2012;**8**:3210–7.
- Lee YT, Ko EJ, Kim KH, Hwang HS, Lee Y, Kwon YM, et al. Cellular immune correlates preventing disease against respiratory syncytial virus by vaccination with virus-like nanoparticles carrying fusion proteins. *J Biomed Nanotechnol* 2017;**13**:84–98.
- Heo MB, Lim YT. Programmed nanoparticles for combined immunomodulation, antigen presentation and tracking of immunotherapeutic cells. *Biomaterials* 2014;**35**:590–600.
- Rahimian S, Kleinovink JW, Fransen MF, Mezzanotte L, Gold H, Wisse P, et al. Near-infrared labeled, ovalbumin loaded polymeric nanoparticles based on a hydrophilic polyester as model vaccine: In vivo tracking and evaluation of antigen-specific CD8(+) T cell immune response. *Biomaterials* 2015;**37**:469–77.
- Xiang J, Xu L, Gong H, Zhu W, Wang C, Xu J, et al. Antigen-loaded upconversion nanoparticles for dendritic cell stimulation, tracking, and vaccination in dendritic cell-based immunotherapy. *ACS Nano* 2015;**9**:6401–11.
- Thomann-Harwood LJ, Kaeuper P, Rossi N, Milona P, Herrmann B, McCullough KC. Nanogel vaccines targeting dendritic cells: contributions of the surface decoration and vaccine cargo on cell targeting and activation. *J Control Release* 2013;**166**:95–105.
- De Geest BG, Willart MA, Hammad H, Lambrecht BN, Pollard C, Bogaert P, et al. Polymeric multilayer capsule-mediated vaccination induces protective immunity against cancer and viral infection. *ACS Nano* 2012;**6**:2136–49.

17. Liang R, Xie J, Li J, Wang K, Liu L, Gao Y, et al. Liposomes-coated gold nanocages with antigens and adjuvants targeted delivery to dendritic cells for enhancing antitumor immune response. *Biomaterials* 2017;**149**:41–50.
18. Zhang C, Shi G, Zhang J, Song H, Niu J, Shi S, et al. Targeted antigen delivery to dendritic cell via functionalized alginate nanoparticles for cancer immunotherapy. *J Control Release* 2017;**256**:170–81.
19. Baleeiro RB, Schweinlin M, Rietscher R, Diedrich A, Czaplewska JA, Metzger M, et al. Nanoparticle-based mucosal vaccines targeting tumor-associated antigens to human dendritic cells. *J Biomed Nanotechnol* 2016;**12**:1527–43.
20. Liu L, Ma P, Wang H, Zhang C, Sun H, Wang C, et al. Immune responses to vaccines delivered by encapsulation into and/or adsorption onto cationic lipid-PLGA hybrid nanoparticles. *J Control Release* 2016;**225**:230–9.
21. White PJ, Anastasopoulos F, Church JE, Kuo CY, Boyd BJ, Hickey PL, et al. Generic construction of single component particles that elicit humoral and cellular immune responses without the need for adjuvants. *Vaccine* 2008;**26**:6824–31.
22. Speidel K, Osen W, Faath S, Hilgert I, Obst R, Braspenning J, et al. Priming of cytotoxic T lymphocytes by five heat-aggregated antigens in vivo: conditions, efficiency, and relation to antibody responses. *Immunol* 1997;**27**:2391–9.
23. St Clair N, Shenoy B, Jacob LD, Margolin AL. Cross-linked protein crystals for vaccine delivery. *SA* 1999;**96**:9469–74.
24. Wang Y, Wang XY, Subjeck JR, Kim HL. Covalent crosslinking of tumor antigens stimulates an antitumor immune response. *Vaccine* 2010;**28**:6613–20.
25. Hou L, Yang X, Ren J, Wang Y, Zhang H, Feng Q, et al. A novel redox-sensitive system based on single-walled carbon nanotubes for chemophotothermal therapy and magnetic resonance imaging. *Nanomedicine* 2016;**11**:607–24.
26. Tan Y, Xie L, Wang Z, Zhang N, Zou C, Chen ZY, et al. Epsilon-caprolactone modified polyethylenimine for highly efficient antigen delivery and chemical exchange saturation transfer functional MR imaging. *Biomaterials* 2015;**56**:219–28.
27. Ta HT, Li Z, Hagemeyer CE, Cowin G, Zhang S, Palasubramaniam J, et al. Molecular imaging of activated platelets via antibody-targeted ultra-small iron oxide nanoparticles displaying unique dual MRI contrast. *Biomaterials* 2017;**134**:31–42.
28. Appel AA, Anastasio MA, Larson JC, Brey EM. Imaging challenges in biomaterials and tissue engineering. *Biomaterials* 2013;**34**:6615–30.
29. Niu X, Chen H, Wang Y, Wang W, Sun X, Chen L. Upconversion fluorescence-SERS dual-mode tags for cellular and in vivo imaging. *ACS Appl Mater Interfaces* 2014;**6**:5152–60.
30. Wang Y, Chen L. Quantum dots, lighting up the research and development of nanomedicine. *Nanomedicine* 2011;**7**:385–402.
31. Dong X, Wei C, Liu T, Lv F, Qian Z. Real-time fluorescence tracking of protoporphyrin incorporated thermosensitive hydrogel and its drug release in vivo. *ACS Appl Mater Interfaces* 2016;**8**:5104–13.
32. Dong XWC, Chen H, Qin J, Liang J, Kong D, Liu T, et al. Real-time imaging tracking of a dual fluorescent drug delivery system based on zinc phthalocyanine-incorporated hydrogel. *ACS Biomater Sci Eng* 2016;**2**:2001–10.
33. Dong X, Chen H, Qin J, Wei C, Liang J, Liu T, et al. Thermosensitive porphyrin-incorporated hydrogel with four-arm PEG-PCL copolymer (II): doxorubicin loaded hydrogel as a dual fluorescent drug delivery system for simultaneous imaging tracking in vivo. *Drug Deliv* 2017;**24**:641–50.
34. Noh YW, Jang YS, Ahn KJ, Lim YT, Chung BH. Simultaneous in vivo tracking of dendritic cells and priming of an antigen-specific immune response. *Biomaterials* 2011;**32**:6254–63.
35. Carroll EC, Jin L, Mori A, Munoz-Wolf N, Oleszycka E, Moran HB, et al. The vaccine adjuvant chitosan promotes cellular immunity via DNA sensor cGAS-STING-dependent induction of type I interferons. *Immunity* 2016;**44**:597–608.
36. Jusforgues-Saklani H, Uhl M, Blachere N, Lemaître F, Lantz O, Bousso P, et al. Antigen persistence is required for dendritic cell licensing and CD8⁺ T cell cross-priming. *J Immunol* 2008;**181**:3067–76.
37. Wang L, Hess A, Chang TZ, Wang YC, Champion JA, Compans RW, et al. Nanoclusters self-assembled from conformation-stabilized influenza M2e as broadly cross-protective influenza vaccines. *Nanomedicine* 2014;**10**:473–82.
38. Zhang X, Zhang H, Yin L, Hu R, Qiu T, Yin Y, et al. A pH-sensitive nanosystem based on carboxymethyl chitosan for tumor-targeted delivery of daunorubicin. *J Biomed Nanotechnol* 2016;**12**:1688–98.
39. Yang Y, Wang S, Wang Y, Wang X, Wang Q, Chen M. Advances in self-assembled chitosan nanomaterials for drug delivery. *Biotechnol Adv* 2014;**32**:1301–16.
40. Shi GN, Zhang CN, Xu R, Niu JF, Song HJ, Zhang XY, et al. Enhanced antitumor immunity by targeting dendritic cells with tumor cell lysate-loaded chitosan nanoparticles vaccine. *Biomaterials* 2017;**113**:191–202.
41. Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O’Neil CP, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. *Nat Biotechnol* 2007;**25**:1159–64.
42. Sen D, Deerinck TJ, Ellisman MH, Parker I, Cahalan MD. Quantum dots for tracking dendritic cells and priming an immune response in vitro and in vivo. *PLoS One* 2008;**3**:e3290.
43. Cruz LJ, Tacke PJ, Zeelenberg IS, Srinivas M, Bonetto F, Weigelin B, et al. Tracking targeted bimodal nanovaccines: immune responses and routing in cells, tissue, and whole organism. *Mol Pharm* 2014;**11**:4299–313.
44. Lin YH, Tsai SC, Lai CH, Lee CH, He ZS, Tseng GC. Genipin-cross-linked fucose-chitosan/heparin nanoparticles for the eradication of *Helicobacter pylori*. *Biomaterials* 2013;**34**:4466–79.
45. Hwang YJ, Larsen J, Krasieva TB, Lyubovitsky JG. Effect of genipin crosslinking on the optical spectral properties and structures of collagen hydrogels. *ACS Appl Mater Interfaces* 2011;**3**:2579–84.
46. Qiu J, Li J, Wang G, Zheng L, Ren N, Liu H, et al. In vitro investigation on the biodegradability and biocompatibility of genipin cross-linked porcine acellular dermal matrix with intrinsic fluorescence. *ACS Appl Mater Interfaces* 2013;**5**:344–50.
47. Richard I, Thibault M, De Crescenzo G, Buschmann MD, Lavertu M. Ionization behavior of chitosan and chitosan-DNA polyplexes indicate that chitosan has a similar capability to induce a proton-sponge effect as PEI. *Biomacromolecules* 2013;**14**:1732–40.

A visible fluorescent nanovaccine based on functional genipin crosslinked ovalbumin protein nanoparticles

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Supplementary Methods

Preparation and characterization of CS-OVA NPs

The OVA-NPs was prepared by cross-linked reaction of genipin and OVA. Genipin solution (1% w/v, 1mL) was added to OVA solution (5mg/mL, 2.5 mL) to stir at 200rpm/min at 25 °C over night. The color of the system changed from slight yellow to blue, and the solution was dialyzed (MWCO 3500Da) against de-ionized water for 24h to remove excess genipin. The CS-OVA-NPs were further prepared by ionic gelation method of CS on the surface of OVA-NPs with a ratio of 8:1 (CS/OVA). The degree of crosslinking was evaluated by the free amino group content of OVA. The free amino group concentration was calculated using a standard curve method according to the OVA samples.

OVA and OVA-NPs were characterized by far-UV CD spectroscopy (J-815 CD spectrometer, JASCO, JAPAN), multimode microplate spectrum photometer (Varioskan™ Flash, ThermoFisher Scientific, USA), infrared spectroscopy (IR) (Nicolet 2000, Thermo Fisher, USA), atomic force microscopy (AFM) (Multi Mode8, Veeco Instruments, USA) and NY 90-Plus particle size and zeta potential analyzer (Nano ZS, Malvern Instruments, UK).

Viability assay of BMDCs

Cell viability was measured by CCK-8 assay in the presence of various concentrations of free OVA, OVA-NPs and CS-OVA-NPs. DCs were seeded into 96-well culture plates (1×10^5 cells/well) in growth medium for 2h, then the cells were incubated with free OVA or nanoparticles for 24 h. After that, CCK-8 solution was added to each well and incubated for 4 h at 37 °C, OD450 was measured using a Multifunction Microplate Reader, (The Varioskan™ Flash; Thermo Fisher Scientific, USA), with the results presented as the percentage of that of the untreated cells.

Cellular uptake and intracellular trafficking of OVA-NPs and CS-OVA-NPs

For cellular uptake assay, DCs were cultured in six-well culture plates (1×10^5 cells/well) for 2 h. The cells were treated with OVA-NPs and CS-OVA-NPs (OVA concentration: 20 µg/mL) for 12 h. Then, the cells were harvested and washed for further FACS analysis using a Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). To investigate the location of OVA nanoparticles in DCs, after incubating with OVA-NPs or CS-OVA-NPs, the cells were stained. Confocal luminescence imaging of DCs was obtained with CLSM (LSM710; Carl Zeiss Meditec AG, Jena, Germany).

Activation and Maturation of BMDCs.

DCs were harvested and stained with anti-CD11c, anti-CD40, anti-CD86 or anti-MHC-I, antibodies for 30 min at 4°C after incubation for 24h. Then, these DCs were washed with PBS and the expression of CD40, CD86 and MHC-I on CD11c⁺ DCs was determined by flow cytometry (BD Biosciences, San Jose, CA). All samples were done in triplicate.

In vivo tracking of labeled NPs

For in vivo imaging, C57BL/6 mice were injected with free OVA, OVA-NPs or CS-OVA-NPs (50 mg of OVA) to the back with hair removed. OVA-NPs and CS-OVA-NPs were tracked with the fluorescent signals of genipin, while free OVA was monitored by the extra labeling of rhodamine B due to the lack of crosslink with genipin. At interval, the fluorescent intensity at the injection site was measured by in vivo imaging system (Maestro 2, CRI, USA) with an exposure time of 200 ms at an excitation wavelength of 595 nm. Meanwhile, the migration of matured DCs to the lymph node was observed by in vivo imaging. In order to quantitatively analyze the variation of fluorescence intensity over time, the fluorescent signal at different time was presented as the percentage of the initial recorded fluorescent intensity value.

In vivo immunization

Six-week-old male C57BL/6 mice were injected with free OVA, OVA-NPs or CS-OVA-NPs (each containing 50 µg of OVA). The mice were immunized at days 0, 7, and 14. Then the mice were sacrificed 14 days after the third immunization, the blood and spleens were collected for the following immunological tests.

ELISA detection and flow cytometry evaluation of T-cell response

Blood samples were collected from the angular vein 14 days after the third immunization. Serum was separated and stored at -80°C for the ELISA assay. OVA-specific serum antibodies (total IgG and IgG1, IgG2a subtypes) and cytokine TNF-α were measured by ELISA. Splenocytes (2×10^6 cells) from each experimental group were harvested and restimulated with OVA (50 µg/mL) for 72 h at 37 °C. After washing, cells were stained with fluorescein-labeled antimouse antibodies against CD3e, CD4, CD8, CD44, and CD62L and IFN-γ for 30 min at 4 °C. T cells activation was evaluated by the proportion of CD4⁺ and CD8⁺ cells. The percentages of central memory T cells (CD44^{Hi} CD62L^{Hi}) and CD8⁺ IFN-γ cells were measured by flow cytometer (BD Biosciences, San Jose, CA). Cytokines secretion of IFN-γ in the supernatant was determined by ELISA (eBioscience). All samples were

measured in triplicate.

In vivo antigen cross-presentation

To evaluate the antigen presentation in vivo, C57BL/6 mice were vaccinated with a 50 μ L suspension of free OVA, OVA-NPs or CS-OVA-NPs (each containing 50 μ g of OVA). One day after vaccination, OVA-specific OT-I CD8⁺ T cells (1×10^5 cells) were injected intravenously via the tail vein in 200 μ L PBS. Mice were sacrificed 4 days after injection to analyze the expansion of the transferred OVA-specific T cells by flow cytometry. Splenocytes were harvested and stained with fluorescently labeled antibodies for CD8b and the OT-I congenic marker CD90.1. T (cell Thy1.1, eBioscience, lot#17-0900-82) expansion was expressed as the number of transferred (OT-I) T cells as a percentage of total CD8⁺ T cells.

CTL assays

Splenocytes (2×10^6 cells) from different groups were harvested and restimulated with OVA (50 μ g/mL) in media containing 10 U/ml recombinant mouse IL-2 for 72 h at 37 °C. After washing three times with PBS, B16-OVA target cells (5×10^3 cells/well) treated with mitomycin were added to splenocytes and cultured together in 96-well culture plates at a proper E:T ratio. After incubation for 4 h, the percentage of specific lysis of target cells by effective cells was calculated according to % specific lysis = ((experimental LDH release - spontaneous LDH release)/(maximum LDH release - spontaneous LDH release)) $\times$ 100%.

Statistical Analysis.

Data were expressed as the mean $\pm$ standard deviation (SD). Difference analysis between groups was made by Student's t test or ANOVA followed by Tukey's multiple comparison. P values of 0.05 or less were considered to be statistically significant.