Multifunctional nanoparticles co-delivering Trp2 peptide and CpG adjuvant induce potent cytotoxic T-lymphocyte response against melanoma and its lung metastasis

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Abstract

Immunotherapy has shown the potential to become an essential component of the successful treatment of various malignancies. In many cases, such as in melanoma, however, induction of a potent and specific T-cell response against the endogenous antigen or self-antigen still remains a major challenge. To induce a potent MHC I-restricted cytotoxic T-lymphocyte (CTL) response, cytosol delivery of an exogenous antigen into dendritic cells is preferred, if not required. Lipid–calcium–phosphate (LCP) nanoparticles represent a new class of intracellular delivery systems for impermeable drugs. We are interested in exploring the potential of LCP NPs for use as a peptide vaccine delivery system for cancer therapy. To increase the encapsulation of Trp2 peptide into the calcium phosphate precipitate core of LCP, two phosphoserine residues were added to the N-terminal of the peptide (p-Trp2). CpG ODN was also co-encapsulated with p-Trp2 as an adjuvant. The NPs were further modified with mannose to enhance and prolong the cargo deposit into the lymph nodes (LNs), which ensured persistent antigen loading and stimulation. Compared with free Trp2 peptide/CpG, vaccination with LCP encapsulating p-Trp2 and CpG resulted in superior inhibition of tumor growth in both B16F10 subcutaneous and lung metastasis models. An IFN-γ production assay and in vivo CTL response study revealed that the improved efficacy was a result of a Trp2-specific immune response. Thus, encapsulation of phosho-peptide antigens into LCP may be a promising strategy for enhancing the immunogenicity of poorly immunogenic self-antigens for cancer therapy.

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1. Introduction

Active immunotherapy has shown the potential to become an essential component of a successful antitumor treatment, especially for aggressive and metastatic cancers [1]. Not only can the immune system be harnessed to specifically target existing tumor cells, but it may also provide long-term memory against the recurrence [2]; both represent a path to a durable and long-lasting response. However, in the context of various immune evasion mechanisms developed by cancer cells [3,4], the induction of a potent and specific T cell response is still a major challenge to immunotherapy.

Antigen presenting cells, especially dendritic cells (DCs), play a central role in cancer immunotherapy [5]. DCs process and present tumor antigens on their surface via MHC-I molecules. Upon activation and maturation, antigen presenting DCs are able to migrate to the lymph nodes and activate an antigen-specific cytotoxic T-lymphocyte (CTL) response, which is important for tumor eradication. DC-based cancer vaccines, that is, ex-vivo matured DCs pulsed with tumor lysates or specific antigens, have been vigorously investigated. Unfortunately, the results of clinical trials have come short of the initial expectations [6,7]. Mechanism studies suggested that optimal antigen loading and activation of DCs is of great importance to the initiation of an efficient CTL response against malignancy. However, many DC-based vaccines may not fulfill these requirements [8,9], and failure in any step of the process will compromise the efficacy, or even in contrast induce an antigen-specific tolerance [10]. Nanoparticle-based vaccine systems provide several advantages over DC-based cancer vaccines by: 1) requiring less labor-intensive and sophisticated procedures, 2) loading and activating DCs directly under the physiological condition without disturbing the loop of immune response initiation, 3) enhancing target delivery and cellular uptake by DCs to trigger a strong immunostimulatory cascade while avoiding tolerance or toxicity induced by systemic delivery of high dose antigen and adjuvant, and 4) offering encapsulation of multiple components and simultaneous delivery of antigens, immune-adjuvants...
and target ligands. Furthermore, nonviral vaccine carriers have also been regarded as a preferable strategy from the safety standpoint compared to viral carriers.

MHC I-restricted cytotoxic T-lymphocyte (CTL) is important for eradicating the growth of tumor cells and preventing the recurrence of cancer [11,12]. To induce an MHC I-restricted CTL response, delivery of an exogenous antigen to the cytosol of DCs is preferred, if not required [5]. Lipid/Calcium/Phosphate nanoparticles (LCP NPs) were developed previously in our lab as a new class of intracellular delivery systems for imperative drugs. The sub-cellular distribution indicated that LCP NPs could efficiently release their cargo into the cytoplasm [13]. In the present work, we are interested in exploring the potential of LCP NPs as a peptide vaccine delivery system for cancer therapy.

The spontaneous and poorly immunogenic B16F10 melanoma was chosen over the artificiap xenogeneic tumor models to create a realistic model for evaluation, as sensitizing CTLs against endogenous antigen or self-antigen poses major challenges to therapeutic vaccines. Tyrosinase-related protein 2 (Trp2) has been identified as one of melanoma tumor-associated antigens that is also expressed by the normal melanocytes. Trp2 peptide (amino acids 180–188 of the Trp2 protein, SVYDFFWVL), one of the epitopes of this protein, is restricted by both murine major histocompatibility complex (MHC) class I H-2Kb and human HLA-A2, making it an attractive model for preclinical studies. The preparation of LCP NPs consisted of mixing 1 mL of CaP cores and 200 μL of 20 mM DSPE-PEG-2000 or 20 μL of 20 mM DSPE-PEG-2000/DSPE-PEG-Mannose (1:1). After evaporating the chloroform, the residual lipid was dispersed in 1 mL chloroform and stored in a glass vial for further modification.

The preparation of LCP NPs consisted of mixing 1 mL of CaP cores with 200 μL of 20 mM DOTAP/Cholesterol (1:1), with 20 μL of 20 mM DSPE-PEG-2000 or 20 μL of 20 mM DSPE-PEG-2000/DSPE-PEG-Mannose (1:1). After evaporating the chloroform, the residual lipid was dispersed in 100 μL of 5% glucose.

Both CaP cores and final LCP NPs were observed using transmission electron microscopy (JEOL 100CX II TEM, JEOL, Japan). Particle size and zeta potential were measured with a Malvern Zetasizer Nano ZS in water (Malvern, Worcestershire United Kingdom). 125I-labeled Trp2 peptide and 125I-labeled p-Trp2 peptide was prepared with IODOGEN (Thermo Scientific, Rockford, IL) as previously described [18]. The encapsulation efficiency of Trp2 peptide or p-Trp2 peptide was measured by adding trace amounts of 125I-labeled Trp2 peptide or 125I-labeled p-Trp2 peptide, respectively, and counted using a gamma counter. The encapsulation efficiency of CpG ODN was determined using trace amounts of Texas Red-labeled ODN.

2.3. Lymph node accumulation

LCP NPs encapsulating 125I-labeled p-Trp2 peptide and Texas Red-labeled ODN were prepared as described above. Female C57BL/6 mice of age 6–8 weeks were purchased from the National Cancer Institute.
2.4. Tumor growth inhibition

On day 0, female C57BL/6 mice of age 6–8 weeks old were inoculated subcutaneously with 2x10^5 B16F10-luc cells on their lower back. Formulations in 5% glucose solution were subcutaneously injected into the contralateral side of the lower back on day 4 and day 11, respectively. Tumor volume was measured using digital calipers (Thermo Fisher Scientific, Pittsburgh, PA) and animal weight was monitored every 2–3 days. Tumor volume was calculated as (0.5 × length × width × height). Humane sacrifice of mice was performed after tumors reached 20 mm in one dimension. At the endpoint, tumors were harvested and subjected to fluorescent imaging and gamma counting.

2.5. Metastasis inhibition

Six to eight week-old female C57BL/6 mice were inoculated intravenously with 2x10^7 B16F10-luc cells on day 0. Formulations in 5% glucose solution were subcutaneously injected into the lower back on day 4 and day 11, respectively. At the endpoint, mice were sacrificed and their lungs were harvested. Tumor nodules exceeding 2 mm in diameter were counted manually. Luciferase activity of the whole lung was measured with Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions.

2.6. Interferon gamma (IFN-γ) production

Female C57BL/6 mice were immunized with different formulations and sacrificed seven days later. Spleen and vaccine draining lymph nodes were steriley harvested from each mouse and processed into single cell suspensions. IFN-γ production was measured with BD™ ELISPOT assay system (BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Briefly, cells were seeded at different cell densities into 96-well plates that were pre-coated with capture antibody and incubated in RPMI 1640 media supplemented with NEAA and antibiotics/antimycotics. At 2 and 4 days, the plates were removed and IFN-γ was detected by adding detection antibody followed by enzyme conjugate. Signals were developed using BM™ ELISPOT substrate set and enumerated manually after imaging.

2.7. In vivo cytotoxic T Lymphocyte (CTL) Assay

The in vivo CTL assay was performed according to the previous protocol with slight modifications [19,20]. C57BL/6 mice were injected intravenously with 2x10^7 B16F10-luc cells on their lower back. Formulations in 5% glucose solution were subcutaneously injected into the contralateral side of the lower back on day 4 and day 11, respectively. At the endpoint, tumors were harvested and subjected to fluorescent imaging. The number of CFSE<sup>high</sup> and CFSE<sup>low</sup> were determined, and the in vivo Trp2 specific lysis percentage was enumerated according to a published equation [19].

\[
\text{% specific lysis} = \frac{(\text{Ova} \times x - \text{Trp2})}{(\text{Ova} \times x)} \times 100\%
\]

where \(x = \frac{\text{Trp2}}{\text{Ova}}\) from naive mice.

2.8. Statistical Analysis

Data were analyzed statistically using a one-way ANOVA and a two-tailed Student’s t-test. All two-group statistical analyses were done by comparing with the control group unless specified with markings. Differences in data were considered statistically significant if the p value was less than 0.05.

3. Results

3.1. Characterization of LCP NPs containing p-Trp2 peptide and CpG ODN

In order to break the immune-tolerance and improve the efficiency of the Trp2 peptide vaccine, one of the strategies is to co-deliver the antigen and a potent adjuvant to the antigen presenting cells by incorporating them together into a nanoparticle. Our previous studies have already shown that LCP NPs can efficiently encapsulate nucleic acids [13,21], which could be applied to CpG ODN, a potent adjuvant. However, the encapsulation efficiency of Trp2 peptide into LCP NPs was relatively low, only about 10%. To facilitate the encapsulation of Trp2 peptide antigen, phosphate groups were introduced into the peptide by adding phosphorylated serine residues to the N-terminal (Fig. 1A). In vivo, the nanoparticles encapsulating both p-Trp2 peptide and CpG ODN showed a dense core structure –20 nm in diameter when observed by TEM (Fig. 1B). Changes in the composition of CaP cores due to the addition of various amounts of p-Trp2 peptide and CpG ODN did not affect the size and morphology (data not shown). Encapsulation efficiency and loading capacity were determined using trace amounts of 125I-labeled p-Trp2 peptide and Texas Red-labeled ODN. Encapsulation efficiency of p-Trp2 peptide was consistent over a range of input doses (~65% efficiency), while the loading capacity increased constantly to approximate 500 μg/mL preparation. Addition of CpG ODN decreased the encapsulation efficiency of p-Trp2 from 65% to 50%, while the trapping efficiency of CpG ODN was about 40%.

The final LCP NPs containing both p-Trp2 peptide and CpG ODN were about 30 nm in diameter, determined by TEM after negative staining (Fig. 1C), which was slightly smaller than the measurement of the hydrodynamic diameter (40–45 nm) obtained by dynamic light scattering. The difference could be a result of the fact that TEM images are obtained under dehydrated conditions. Incorporation of 10% DSPE-PEG or 10% DSPE-PEG/Mannose-PEG/DSPE decreased the zeta potential to about 15 mV. More importantly, it stabilized the final particle to maintain well dispersed in 5% glucose over a week at 4°C.

3.2. Enhanced and prolonged lymph node accumulation mediated by Mannose-LCP NPs

Since the lymph nodes (LNs) are where activated APCs can interact with T-cells and B-cells and then initiate the immune response, accumulation of p-Trp2 peptide and ODN in the LNs was investigated. LNs from mice injected with free p-Trp2 peptide and non-labeled ODN (Control), particles without mannose (Non-target LCP) or particles with mannose (Mannose-LCP) were harvested for distribution study. Texas Red-labeled ODN was used for fluorescent imaging. As shown in Fig. 2, while there was no detectable signal in the control group, accumulation of cargo in the LNs was observed after particle injection. Furthermore, the fluorescent signal was only seen in the LNs near the
3.3. Inhibition of tumor growth by therapeutic vaccination with Mannose-LCP NPs

The therapeutic efficacy of Mannose-LCP NPs was first evaluated in a B16F10 melanoma model. C57BL/6 mice were inoculated subcutaneously on day 0. All tested formulations were injected subcutaneously on the contralateral side on day 4 and 11, respectively. Equal amounts of antigen (50 μg) and/or CpG ODN (20 μg) were applied if indicated. Tumor size and body weight were monitored every 2–3 days for 20 days when the tumors in the control group reached 20 mm in one dimension.

As shown in Fig. 3, both empty Mannose-LCP NPs and Mannose-LCP NPs encapsulating CpG had no therapeutic effect on this melanoma model, while Mannose-LCP NPs encapsulating p-Trp2 peptide showed a modest tumor inhibition, suggesting that an antigen is necessary to induce immune response against the tumor. Free p-Trp2 peptide/CpG exhibits only minor effects on tumor inhibition. When compared to the control, statistically significant inhibition of tumor growth was observed in the group treated with Mannose-LCP-NPs encapsulating both p-Trp2 peptide and CpG, with \( p < 0.001 \). There was no decrease in body weight observed in any of the groups, indicating that there is no toxicity associated with treatment with Mannose-LCP NPs. Slight weight gain in some groups may result from the tumor growth.

To compare the immunogenicity of p-Trp2 peptide with Trp2 peptide, the therapeutic efficacy of free p-Trp2/CpG was also evaluated in this melanoma model. Combined with CpG ODN, free p-Trp2 peptide was also able to elicit a minor tumor inhibition effect (Fig. S3). No significant difference was observed at the end point between two peptide-treated groups in terms of the tumor growth (\( p > 0.05 \)), suggesting that this particular phosphorylation did not alter the immunogenicity of the Trp2 peptide.

3.4. Inhibition of tumor metastasis by therapeutic vaccination with Mannose-LCP NPs

Metastatic melanoma is one of the most aggressive skin cancers with extremely short survival times in patients [22]. To evaluate the therapeutic efficacy of Mannose-LCP NPs on melanoma metastasis, a well-established B16F10 metastatic model, in which lung metastasis develops spontaneously after intravenous inoculation, was utilized. C57BL/6 mice inoculated on day 0 were vaccinated on day 4 and 11, respectively, with tested formulations with or without antigen (50 μg) and/or CpG ODN (20 μg). Lungs were harvested on day 20 and observable nodules (exceeding 2 mm in diameter) were counted manually (Fig. 4A and B). Since the B16F10-luc cells were stably transfected with the firefly luciferase gene, the tumor volume was also quantified by measuring luciferase activity in the homogenates of the whole lungs (Fig. 4C).

As shown in Fig. 4, compared with the other groups, significantly fewer lung metastasis nodules developed after vaccination with...
were harvested for imaging (A). Tumor nodules exceeding 2 mm in diameter were counted with 5% glucose, free Trp2/CpG, empty LCP, LCP-p-Trp2, LCP-CpG or LCP-p-Trp2/CpG was given on day 4 and day 11, respectively. Mice were sacrificed on day 20 and lungs were harvested for imaging (A). Tumor nodules exceeding 2 mm in diameter were counted manually (B) and luciferase activity of the whole lung was also measured (C). n = 5, *, **: P < 0.05.

Mannose-LCP NPs containing p-Trp2 and CpG ODN. Empty Mannose-LCP NPs and Mannose-LCP NPs encapsulating CpG ODN without an antigen, failed to exhibit any inhibition effect. Limited therapeutic effect was observed in the groups treated with free Trp2 peptide/CpG ODN and Mannose-LCP NPs containing only p-Trp2 peptide.

3.5. IFN-γ production by lymphocytes from vaccinated mice

The antigen-specific immune response induced by Mannose-LCP NPs containing both an antigen and an adjuvant was proposed to be the cause for the tumor growth inhibition. Among all the lymphocytes, IFN-γ + T cells are considered to be the most important cell population in the mediation the tumor inhibition upon immunotherapy [23,24]. IFN-γ production induced by the tumor antigen was analyzed using a BD™ ELISPOT assay system. Seven days after vaccination with various formulations, mice were sacrificed, and the spleen and vaccine draining LNs (inguinal in this case) were extracted and processed into single cell suspensions. Cells were stimulated with 5 μM Trp2 or Ova peptide, a control peptide. Production of IFN-γ was detected using BD™ ELISPOT substrate set and manually enumerated after imaging (Fig. 5). Consistent with the results from therapeutic studies, there was no detectable IFN-γ production to Trp2 pulse in the spleen or LNs of naïve mice, mice treated with empty particles or Mannose-LCP NPs encapsulating CpG ODN, which suggested that these treatments failed to induce Trp2-specific immune response. Highest production was observed in the group treated with Mannose-LCP NPs containing p-Trp2 peptide and CpG ODN, and there was no significant difference between levels in the spleen and draining LNs. No signal was detected from Ova-pulsed cells for any group in spleen or LN, indicating the IFN-γ production was specific to Trp2 peptide.

3.6. In vivo cytotoxic T lymphocyte assay

Primary CTL is important in stopping the growth of tumor cells, while memory CTL is essential in the prevention of cancer recurrence [11,12]. An assay for antigen-specific CTL response may provide more evidence that elucidates how and how well the vaccine formulation works. In vivo CTL response assay was performed. Splenocytes from naïve mice were pulsed with Trp2 peptide and Ova peptide and labeled with either high or low levels of CFSE, respectively (Trp2-pulsed CFSEhigh, Ova-pulsed CFSeqlow). Seven days after vaccination, mice were intravenously injected with a mixture containing equal amounts of Trp2-pulsed CFSEhigh and Ova-pulsed CFSeqlow. The specific lysis of Trp2-pulsed CFSEhigh was analyzed using flow cytometry 16 h after the adoptive transfer (Figs. 6A and S4). The mice immunized with free peptide (Trp2 or p-Trp2 peptide) plus CpG ODN (Fig. S4) or Mannose-LCP NPs encapsulating p-Trp2 peptide were able to generate a modest Trp2-specific killing (about 30%). Mice treated with empty particles or particles containing only CpG ODN did not exhibit any noticeable Trp2-specific CTL response. In contrast, mice receiving Mannose-LCP NPs containing p-Trp2 and CpG ODN efficiently eliminated about 60% of Trp2-pulsed target cells, suggesting this vaccine formulation elicited a potent Trp2-specific in vivo CTL response compared with others. The same results were obtained when target cells were pulsed with p-Trp2 peptide (Fig. 6B).

4. Discussion

Although vaccines represent some of medicine’s greatest successes against infectious agents, several major challenges still exist to their use in treating pre-existing tumors. These challenges could be a reflection of poor platforms that fail to elicit optimal antigen processing or presentation by dendritic cells or suboptimal adjuvants. In the present work, we constructed a novel nanoparticle-based vaccine co-encapsulating both a tumor-specific antigen and a potent adjuvant. Enhanced and prolonged cargo deposit to the draining lymph nodes was achieved through modification of the surface of the nanoparticles with mannose. In vivo studies in poorly immunogenic B16F10 melanoma models demonstrated that the nanoparticle-based vaccine was able to elicit a strong antigen-specific CTL response against both pre-existing and metastatic tumor cells in vivo, which may represent a promising vaccine formulation for cancer therapy.

Recently, some protein/peptide vaccines have been studied in clinical trials for the treatment of cervical cancer, breast tumors, nasopharyngeal tumors and melanoma [25–27]. However, peptide/protein vaccines usually show low immunogenicity [28]. Free proteins/peptides are likely to have poor pharmacokinetic properties and may be rapidly
cleared before they are able to reach the dendritic cells. Risk of inducing specific tolerance may also exist after the systemic delivery of high doses of proteins/peptides, especially in the absence of appropriate DC activation [10]. Therefore, efficient lymph targeting is needed to overcome these drawbacks. In the case of low immunogenic peptides, such as Trp2 peptide, an effective strategy to develop immunotherapeutic efficacy is even more crucial. Efforts have been made through employing modifications to Trp2 peptide by altering the epitope [29], replacing the P1 and/or P2 residues [30], or conjugating to another peptide [31] or ligand [32], or with limited improvement in the vaccine activity.

Several nanoparticle-based Trp2 delivery systems have been developed. Unlike most of the epitope peptides, Trp2 peptide is hydrophobic and thus difficult to formulate into an aqueous solution or a delivery vehicle. PLGA nanoparticles with a low encapsulation of Trp2 peptide showed prophylactic activity, but only marginal therapeutic benefit against the melanoma [33,34]; AVE3 liposomes have also been tested, and compromised therapeutic efficiency might result from a low dose and separate administration of the adjuvant [35]. A cationic lipid, DOTAP-adjuvanted Trp2 formulation was developed previously in our lab. Although the formulation inhibited tumor growth in a model of advanced melanoma, the solubility and the encapsulation efficiency of the Trp2 peptide still present a problem to the success of the formulation [36].

LCP particles that consist of a calcium phosphate core and an asymmetric lipid bilayer were first developed in our lab for the purpose of siRNA delivery. The particles also showed high encapsulation efficiency for ODN (about 50%), which can serve as a potent adjuvant. Modification was made to Trp2 peptide to facilitate the encapsulation, while leaving the original MHC I binding sequence unchanged (Fig. 1). As we described, the formation of the LCP core relies on the precipitation of calcium phosphate in the aqueous phase of the micro-emulsion system. The phosphate groups on the peptide can interact with calcium and increase the chance of p-Trp2 peptide to co-precipitate with calcium phosphate. Increased hydrophility may also help the peptide stay within the aqueous phase and further increase the chance to form co-precipitates with calcium phosphate. Characterization results indicated that LCP can successfully and efficiently load large amounts of both antigen peptide (500 μg/mL) and CpG adjuvant (200 μg/mL). The particles were stable at 4 °C for at least one week. Theoretically, any hydrophobic or hydrophilic peptide could be encapsulated, along with the CpG adjuvant, into LCP NPs after modification with phosphoserine, making it a powerful delivery platform for peptide vaccines with high flexibility.

Although the therapeutic studies showed that formulating p-Trp2 peptide and CpG adjuvant into LCP nanoparticles enhanced immune response against melanoma (Figs. 3 and 4), one may still raise the question as to whether the phosphorylation would affect the immunogenicity of Trp2 peptide. It has been reported that phosphorylated peptides can be transported by TAP, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL [38]. Furthermore, there is a strong cross-reactivity between phospho-peptide and non-phospho-peptide. In our study, to compare the immunogenicity of p-Trp2 peptide with Trp2 peptide, the therapeutic efficacy of free p-Trp2/CpG was first evaluated. No significant difference was observed at the end point in terms of the tumor growth (p > 0.05), suggesting that the phosphorylation did not alter the immunogenicity of the Trp2 peptide (Fig. S3). In the IFN-γ production study, Trp2 peptide was used instead of p-Trp2 peptide as the stimulator to detect the antigen-specific response (Fig. S5). Successful induction of Trp2-specific IFN-γ production after vaccination with formulated p-Trp2 peptide suggested again that the phosphorylation did not affect the immunogenicity of the Trp2 peptide. Also notably, the mice immunized with either of the free peptides (Trp2 or p-Trp2 peptide) plus CpG ODN (Fig. S4) induced comparable Trp2-specific killing (about 30%). When both Trp2 peptide and p-Trp2 peptide pulsed splenocytes were generated for the in vivo CTL response study (Fig. 6), similar results were obtained after the same treatments, respectively. Thus, phosphorylation of the Trp2 peptide did not affect its presentation in the context of the MHC-I molecule.

Two major strategies have been applied in cancer immunotherapy: non-specific immune activation and tumor-specific immune activation. The first one includes cytokine, interferon and TLR agonist treatment. In the present therapeutic studies, vaccination with LCP NPs containing a CpG adjuvant did not show any efficacy in reducing melanoma growth, which suggested that non-specific immune activation was not sufficient to sensitize the CTLs against a self-antigen. The DCs are the key players in tumor-specific immune activation. Upon antigen encounter, the DCs must also receive a suitable activation (‘maturation’) signal, allowing them to differentiate extensively to promote immunity as opposed to tolerance. Presentation of antigens by DCs at the steady state (without activation signal) may promote tolerance through regulatory T cell production, which would oppose an antitumor response. In the present work, LCP NPs containing p-Trp2 peptide alone also exhibited modest CTL response (Fig. 6) and tumor inhibition activity (Figs. 3 and 4), indicating that the particle may serve as a mild DC-activation signal. Indeed, our previous study has revealed that cationic lipid DOTAP not only plays the role of a carrier in delivering the peptide antigen, but also serves as a potent vaccine adjuvant to stimulate immune response and initiate DC-T cell interactions [37]. Calcium phosphate may also act as a minor adjuvant [39]. Detailed mechanism studies will be performed in the future. CpG ODN was still chosen as the major adjuvant here because it has the capacity to augment immune response against tumor [36]. It also has been reported that persistent TLR signals are required for reversal of regulatory T cell-mediated CD8 tolerance [40]. Distribution studies showed that Mannose-LCP particles were able to enhance and prolong the cargo deposit to the LNs (Fig. 2), providing a persistent antigen loading and TLR signal, which could be another explanation as to why the current NP formulation broke the tolerance and elicited a strong CTL response against a self-antigen.

There is still a notable difference in success between prophylactic vaccines and therapeutic cancer vaccines, as upon interaction with immune system, the surviving tumor cells are able to develop several mechanisms to avoid recognition and elimination, such as down-regulation of MHC molecules. The efficacy of a therapeutic vaccine may also be offset by changes in the tumor microenvironment, for example, an increased level of Treg cells and up-regulation of TGF-β [41]. The LCP particle has offered a promising platform for enhanced CTL response against tumor specific antigen. Combination of this efficient vaccine with the treatments aiming to change the tumor microenvironment could be the future direction of cancer immunotherapy.
5. Conclusion

Mannose-LCP, NP-based vaccines allow for the persistent, in vivo loading and activation of dendritic cells, generated a strong in vivo CTL response against poorly immunogenic self-antigen Trp2 peptide, and resulted in a potent anti-tumor immune response against the melanoma. Mannose-LCP NPs are a promising vaccine formulation for cancer therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.08.021.

References