# Nanoparticle-Delivered Transforming Growth Factor-$\beta$ siRNA Enhances Vaccination against Advanced Melanoma by Modifying Tumor Microenvironment

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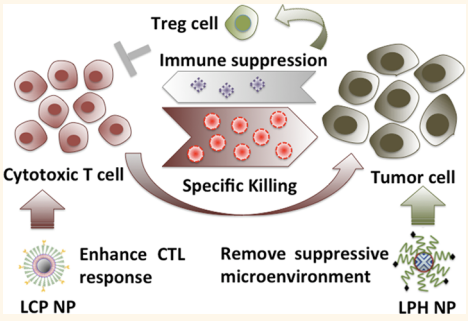
**ABSTRACT** Achievement of potent immunoresponses against self/tumor antigens and effective therapeutic outcome against advanced tumors remain major challenges in cancer immunotherapy. The specificity and efficiency of two nanoparticle-based delivery systems, lipid-calcium-phosphate (LCP) nanoparticle (NP) and liposome-protamine-hyaluronic acid (LPH) NP, provide us an opportunity to address both challenges. A mannose-modified LCP NP delivered both tumor antigen (Trp 2 peptide) and adjuvant (CpG oligonucleotide) to the dendritic cells and elicited a potent, systemic immune response regardless of the existence or the stage of tumors in the host. This vaccine was less effective, however, against later stage B16F10 melanoma in a subcutaneous syngeneic model. Mechanistic follow-up studies suggest that elevated levels of immune-suppressive cytokines within the tumor microenvironment, such as TGF-$\beta$, might be responsible. We strategically augment the efficacy of LCP vaccine on an advanced tumor by silencing TGF-$\beta$ in tumor cells. The delivery of siRNA using LPH NP resulted in about 50% knockdown of TGF-$\beta$ in the late stage tumor microenvironment. TGF-$\beta$ down-regulation boosted the vaccine efficacy and inhibited tumor growth by 52% compared with vaccine treatment alone, as a result of increased levels of tumor infiltrating CD8+ T cells and decreased level of regulatory T cells. Combination of systemic induction of antigen-specific immune response with LCP vaccine and targeted modification of tumor microenvironment with LPH NP offers a flexible and powerful platform for both mechanism study and immunotherapeutic strategy development.




**KEYWORDS:** nanoparticle · peptide vaccine · melanoma · tumor microenvironment · immunotherapy

Immunotherapy has become an attractive strategy and an essential component of a successful antitumor treatment.[1,2] Current immunotherapies are primarily aimed at harnessing both the innate and adaptive immune systems to initiate or boost a strong immune response to tumors and their antigens. On their own, however, most immunotherapeutic approaches fall short of initial expectations when used against aggressive and advanced malignancy.[3] Evidence indicated that during the progression, the surviving tumor cells are able to develop several different mechanisms to avoid immune recognition and elimination,[4,5] making a theoretically efficient immunotherapy become offset. Therefore, development of an effective treatment against advanced tumors remains a major challenge for cancer immunotherapy.

Nanotechnology has had significant impact on a variety of therapeutics for decades.[6−8] Advances in materials and formulation have allowed safer and more efficient delivery of a myriad of drugs. Targeted delivery ensures a specific action and reduced systemic side effects. Nanoparticle-based delivery systems also hold much promise for cancer immunotherapy.[9−12] The flexibility and specificity of nanoparticle-based delivery systems provide an opportunity for both targeting the immune system to initiate a potent immune response and targeting the tumor tissue for a local modification of the tumor microenvironment. We have successfully developed several

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delivery systems that meet the merits. In the present work, we are interested in exploring their potentials to work as an effective immunotherapeutic system against advanced tumors under a rational combination.

Inducing a potent systemic immune response against the tumor is the prerequisite of an efficient cancer immunotherapy.[13] We previously reported an enhanced, persistent, *in vivo* antigen loading and activation of dendritic cells in response to a mannose-modified LCP NP-based vaccine containing both tumor-specific antigen and adjuvant.[14] This vaccine evoked a strong *in vivo* cytotoxic T lymphocyte (CTL) response against poorly immunogenic self-antigen tyrosinase-related protein 2 (Trp2) peptide, resulting in potent antitumor effects against melanoma in a subcutaneous xenograft model and a metastasis model. Therefore, the LCP particle offers a promising platform for generating potent systemic immune responses against tumor antigens.

During the progression, the tumor cells change the microenvironment to impede immunotherapy. Thus, approaches to specifically modify or normalize the tumor microenvironment are becoming a vital companion for an effective immunotherapy. LPH NP is another well-established delivery system in our lab that has been optimized for systemic delivery of siRNA to the tumor site with high specificity and efficiency.[15] Targeted modification of tumor microenvironment using LPH NP may allow for tumor cell-specific intervention without the adverse effects and complications from the systemic alteration.

In the present work, a spontaneous and poorly immunogenic B16F10 melanoma was chosen over the artificial xenogeneic tumor models to create a realistic model for *in vivo* evaluation. To test for an immunotherapy against advanced melanoma, the therapeutic efficacy of LCP NP-based vaccine was first evaluated against a later stage melanoma. The systemic and local parameters were investigated to reveal the correlation between a compromised tumor growth inhibition mediated by LCP vaccine and the progression of melanoma. A strategy to augment the efficacy of LCP vaccine was proposed and achieved by reversing the immunosuppressive microenvironment using LPH NP. The efficacy of the combined immunotherapies was compared with solo treatment, and changes in tumor microenvironment were monitored to verify the hypothesis and mechanism.

## RESULTS AND DISCUSSION

Immunotherapy against malignancies has been explored with effort focused on potent induction of systemic responses. Several animal models have been used for cancer vaccine development, generally using a foreign antigen, such as E7 antigen in cervical cancer and OVA peptide in E.G7-OVA tumor.[16−18] The effect of the vaccine typically becomes marginal under a setting of self-antigen (when using transgenic mice), which unfortunately represents the case for most tumor-specific antigens. There are also notable differences in the effects of prophylactic vaccines *versus* therapeutic cancer vaccines, and advanced solid tumor models are rarely studied to evaluate a self-antigen-based vaccine. Here, we report an attempt to address two major challenges, self-antigen and advanced tumor, simultaneously, through use of nanoparticle-based delivery systems.

**Characterization of LCP NP and LPH NP.** To improve the efficiency of the therapeutic vaccine against the self-antigen, Trp2 (SVYDFFVWL), we co-delivered the antigen together with CpG oligonucleotides (ODN) as a potent adjuvant by incorporating them into LCP nanoparticles.[14] LCP NP was developed previously in our lab as a new class of intracellular delivery systems. Formation of the LCP core requires the precipitation of calcium phosphate in the aqueous phase of a microemulsion system. To facilitate encapsulation, phosphorylated serine residues were added to Trp2 peptide to interact with calcium and increase the co-precipitation of Trp2 with calcium phosphate. The increased hydrophilicity of the phosphorylated peptide may also keep it within the aqueous phase and further increase the chance of co-precipitation. The NP surface was functionalized with mannose to achieve an enhanced and prolonged cargo deposit into the lymph nodes. The encapsulation efficiency of p-Trp2 peptide in LCP NP was about 50%, and efficiency of CpG ODN encapsulation was approximate 40%. The final LCP NP were about 30 nm in diameter as determined by TEM after negative staining (Figure 1A), slightly smaller than

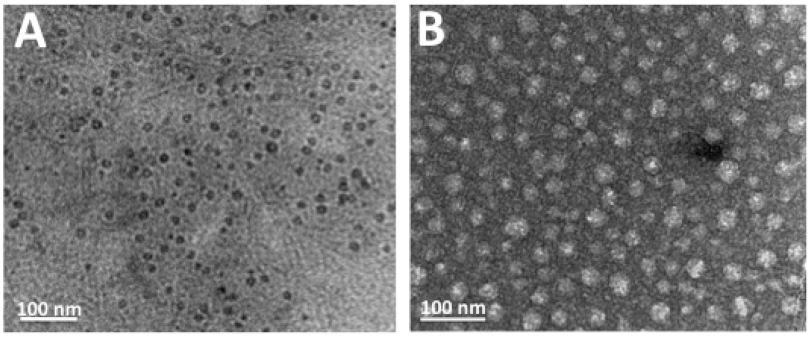


**Figure 1. TEM images of LCP NP (A) and LPH NP (B) after negative staining.**

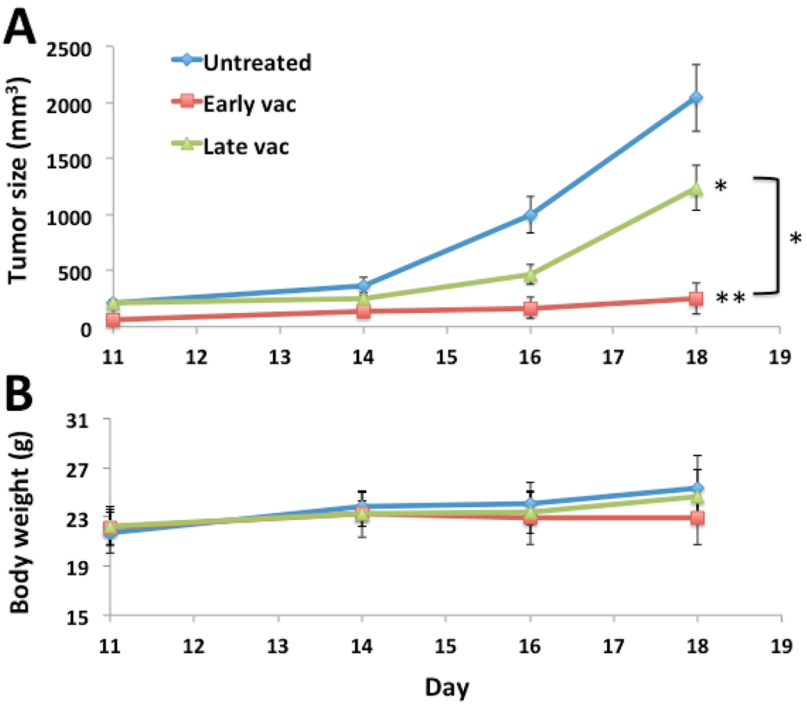


**Figure 2. Antitumor activity of LCP vaccine against different stages of B16F10 melanoma. C57BL/6 mice were inoculated with $2 \times 10^5$ B16F10 cells on day 0. LCP vaccine was injected on day 4 (Early vac) and day 13 (Late vac). Tumor growth (A) and body weight (B) were measured every 2 to 3 days for 18 days. $n = 5$, $*P < 0.05$, $**P < 0.001$. Statistical analyses were done by comparing with the untreated group unless specified with markings.**

the hydrodynamic diameter (40−45 nm) obtained by dynamic light scattering. The zeta potential was about 15 mV. Each component in the LCP NP is designated to improve the stability of NP, enhance the antigen delivery efficiency, or boost the immune response. Absence of any component would result in a compromised antitumor efficacy.[14]

Targeted LPH NP were previously optimized for systemic delivery of siRNA to tumor site.[15] LPH NP are prepared through a stepwise self-assembly process. A mixture of siRNA and HA was condensed with protamine. The complex with slightly negative surface charge was then coated with cationic liposome *via* electrostatic interaction. Cationic liposome coated NP was subsequently PEGylated to reduce protein binding to evade uptake by the reticuloendothelial system, allowing for extended circulation time in the blood. A targeting ligand, anisamide, was added to enhance the payload delivery to sigma receptor overexpressing cancer cells. The final LPH-NP was about 50 nm in diameter with a surface charge of 25 mV, as determined by using Zetasizer. The decreased surface charge of LPH-NP compared with cationic liposomes (~50 mV) was indicative of efficient PEGylation. TEM of LPH-NP after uranyl-acetate staining showed a particle size around 40 nm (Figure 1B).

**Inhibition of Tumor Growth by Vaccination at Different Stages of Tumor Progression.** Previous studies indicated that LCP vaccination at early stage (4 days after tumor inoculation) resulted in a potent growth inhibition of B16F10 subcutaneous tumors and lung metastases.[14] In the present work, we are particularly interested in the potential of LCP as a therapeutic vaccine for advanced melanoma. We evaluated the therapeutic effect of the LCP NP-based vaccine at different tumor stages in a B16F10 melanoma model. C57BL/6 mice were subcutaneously inoculated with B16F10 melanoma cells on day 0 and LCP vaccine was given on day 4 or day 13, respectively, when the tumors were at different stages of progression. Tumor size and body weight were monitored every 2 to 3 days for 18 days.

As shown in Figure 2, B16F10 melanoma progressed aggressively without any treatment. Tumors reached 20 mm in one dimension on day 18, with an average size over 2000 $mm^3$. Compared with the untreated control, both vaccinated groups showed a tumor growth inhibition, but to varying extents. Consistent with previous study, early vaccination (Early vac) on day 4, when the tumors were still on early development stage, exhibited a significant inhibition of tumor growth ($p < 0.001$). Whereas in the case of late vaccination (Late vac), as the same vaccine was given when the tumor size had already reached 200 $mm^3$, its efficacy was much less potent. No decrease in body weight was observed in any of the groups, indicating that there is no toxicity associated with the treatments. Slight weight gain in some groups may result from the tumor growth.

***In Vivo* Cytotoxic T Lymphocyte Response after Vaccination.** Primary CTL responses are important in stopping

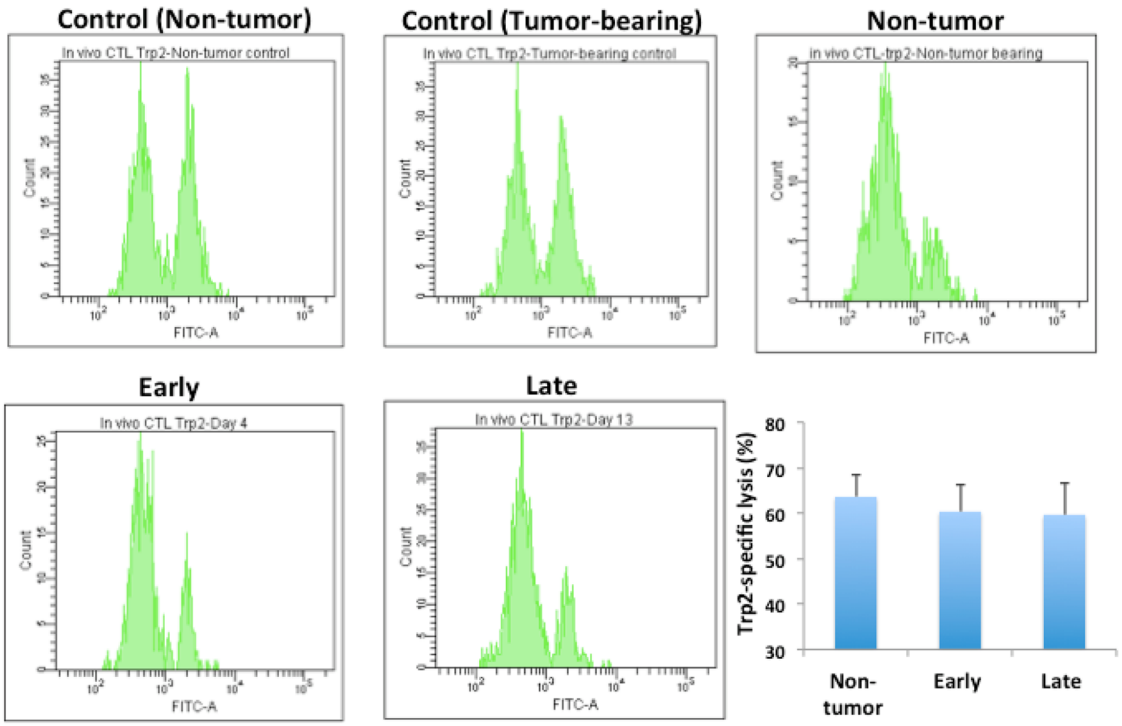


**Figure 3.** ***In vivo*** **CTL response after vaccination under various conditions. C57BL/6 mice were inoculated with 2 × $10^5$ B16F10 cells SC on day 0. LCP vaccine was given on day 4 (Early) and day 13 (Late). Target cells pulsed with Ova or Trp2 were stained with low (Ova) or high (Trp2) concentrations of CFSE, mixed, and injected into vaccinated mice. After 18 h, splenocytes were analyzed by flow cytometry and enumerated according to a published equation.** ***n*** **= 3, and a representative graph from each group is shown.**

tumor growth,[13] and previous studies have confirmed that the LCP vaccine is able to elicit an antigen-specific CTL response against Trp2 peptide. Although advanced tumors are known to be difficult to treat, reduced efficacy of late vaccination still raises the question of why and how could the same vaccine work differently on the same type of tumor at different stages? To provide a possible answer, an assay for antigen-specific CTL responses under various conditions was performed. C57BL/6 mice were inoculated subcutaneously on day 0, and LCP vaccines were given on day 4 or day 13, respectively. Splenocytes from naïve mice were pulsed with Ova or Trp2 peptide and labeled with different levels of carboxyfluorescein succinimidyl ester (CFSE) (Ova-pulsed $CFSE^{low}$, Trp2-pulsed $CFSE^{high}$). Seven days after vaccination, mice were intravenously injected with a mixture containing equal amounts of Ova-pulsed $CFSE^{low}$ and Trp2-pulsed $CFSE^{high}$. Specific lysis of Trp2-pulsed splenocytes was analyzed using flow cytometry. As shown in Figure 3, compared with non-tumor bearing mice (Control (Non-tumor)), there was no detectable Trp2-specific CTL response within the tumor-bearing mice (Control (Tumor-bearing)), indicating that Trp2 peptide is indeed a self-antigen and there is an immune tolerance to Trp2 in the tumor-bearing mice. All the mice receiving LCP vaccine showed an efficient elimination of the Trp2-pulsed target cells, suggesting that LCP vaccine is able to break the pre-existing immune tolerance to Trp2 peptide. A similar percentage of specific lysis was obtained in groups of tumor bearing mice (Early) and non-tumor bearing mice (Non-tumor). No significant difference was observed between the early vaccination (Early) group and the late vaccination (Late) group.

For a tumor/self-antigen as Trp 2 peptide, it is mostly likely that not only the pre-existing tolerance but also the immune suppression gained along with the tumor progression will interfere with the immunogenesis. However, our *in vivo* CTL response study indicated that LCP NP-based vaccine elicited a potent systemic immune response regardless of the existence and stage of tumors, ruling out the possibility that compromised efficacy resulted from unsuccessful induction of antigen-specific CTL response at a late stage. This result also suggested that the nanoparticle-based vaccine formulation was able to break both the pre-established and acquired immune tolerance, inducing a strong antigen-specific immune response against advanced tumor.

**Changes of Cytokine Levels within the Tumor Microenvironment.** Increasing evidence has demonstrated that cancer cells constantly alter the local microenvironment during the progression.[19] We then set to examine the local parameters. To evade the immunosurveillance and elimination, cancer cells usually go about generating a malignancy supportive while immune suppressive microenvironment during the progression. They are able to release some cytokines to directly suppress

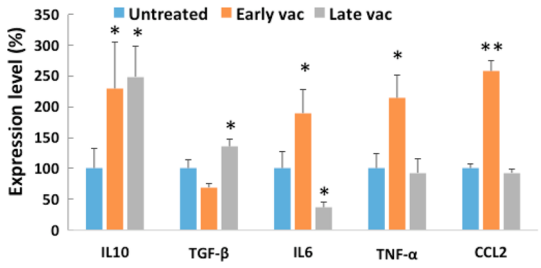


**Figure 4. Tumor local cytokine level after vaccination. C57BL/6 mice were inoculated with 2 × $10^5$ B16F10 cells on day 0. LCP vaccine was given on day 4 (Early vac) or day 13 (Late vac). Mice were sacrificed on day 18, and tumors were collected for cytokine detection using RT-PCR. $n = 5$, *$P < 0.05$, **$P < 0.001$. Statistical analyses were done by comparing with the untreated group.**

the immune effective cells locally to counteract the systemic immune response.[20] To test whether the difference of the efficacy was from the altered cytokine levels within the tumor microenvironment, we harvested melanoma tumors from vaccinated mice, and measured cytokine levels. The tumors from control group were collected at the end point of the therapeutic study as well. All the tumors were inoculated and harvested on the same day to allow a same period of time for progression. Tumor local cytokine levels were determined by RT-PCR (Figure 4). Compared with control, elevated local cytokine levels were seen for all the tested cytokines except TGF-$\beta$ after early vaccination, indicating a local response occurred after treatment. While tumor local TNF-α and CCL2 levels were significant increased after early vaccination, minimal changes were seen after late vaccination. In contrast, the late vaccination group showed a notable increase in TGF-$\beta$ and IL-10. Since both IL-10 and TGF-$\beta$ are anti-inflammatory cytokines that have an inhibitory effect on effector T cells, these results suggested that the up-regulated levels of IL-10 and TGF-$\beta$ might contribute to an immune suppressive local microenvironment, potentially the cause of the compromised effectiveness of the vaccine.

TNF-α, IL-6, and CCL2 are pro-inflammatory cytokines produced by both tumor and stromal cells (including immune cells). The effect of these cytokines on tumor elimination or progression is still controversial. For example, high local levels of TNF-α prove to be beneficial[21] and, combined with other cytokines, such as IL-12, can have synergistic antitumor effects.[22] In contrast, evidence also indicates that TNF-α produced chronically at low levels may promote tumor development.[23] Since a given signaling molecule may act through different pathways in different cell types, opposite conclusions may be explained by different experimental systems. In our experiments, local TNF-α, IL-6 and CCL2 levels were elevated significantly after early vaccination and correlated with enhanced antitumor activity, suggesting that a positive local immune response echoed after the systemic induction (Figure 3). On the other hand, as an anti-inflammatory cytokine produced by both tumor and stromal cells, TGF-$\beta$ exhibits dual functions as well on tumor progression.[24] It is recognized, however, that TGF-$\beta$ can promote tumor progression by suppressing immune effector cells, such as cytotoxic T cells and natural killer cells, and promoting negative regulatory cells, such as Treg cells. Our results demonstrate that the compromised effects of late vaccination correlate with an elevated TGF-$\beta$ at mRNA level, confirming the negative role of TGF-$\beta$ in cancer immunotherapy.

**Augmentation of the Therapeutic Efficacy of LCP Vaccine by Down-Regulating TGF-$\beta$ in the Tumor Microenvironment.** On the basis of the above observations, we proposed that the efficacy of the LCP vaccine against advanced melanoma could be enhanced by down-regulating the suppressive cytokines, such as TGF-$\beta$, within the tumor microenvironment. To test this hypothesis, tumor-specific silencing of TGF-$\beta$ was achieved by systemic delivery of anti-TGF-$\beta$ siRNA with LPH NP, and the therapeutic efficacy of the combined treatments was evaluated.

As shown in Figure 5A, treatment with anti-TGF-$\beta$ siRNA alone exhibited a modest inhibitory effect on tumor growth, while control siRNA had no effect at all, ruling out any effect from the delivery vehicle itself. Though additional TGF-$\beta$ silencing did not have any effect with early vaccination (Figure 5C, Early vac compared with LPH (TGF-$\beta$) + Early vac, $P > 0.05$), it enhanced the efficacy of late vaccination significantly (Figure 5D, Late vac compared with LPH (TGF-$\beta$) + Late vac, $P < 0.05$). Notably, when combined with TGF-$\beta$ silencing, late vaccination showed a comparable efficacy with early vaccination (Figure 5E, LPH (TGF-$\beta$) + Late vac compared with LPH (TGF-$\beta$) + Early vac, $P > 0.05$). No decrease in body weight was observed in any of the groups (Figure 5B), indicating that there is no toxicity associated with any treatments. Down-regulation of TGF-$\beta$ within the tumor after gene silencing treatment was further confirmed by RT-PCR (Figure 6).

It is reported that TGF-$\beta$ can act as a tumor suppressor at early stage by inhibiting tumor cell proliferation and inducing apoptosis, while its role as a tumor promoter comes at later stage.[25] We would like to spare the tumor suppressor part of TGF-$\beta$ and focus only on the tumor promoter part. Meanwhile, from the drug delivery perspective, since EPR (enhanced permeability and retention) effect is the primary driving force for the high accumulation of the drug in the tumor tissue, NPs based therapy are usually administered to the animal when palpable tumors are formed and the leaky neo-vasculature is present in the tumor. For these reasons, TGF-$\beta$ siRNA treatment was started on day 13 when tumors had progressed to a later stage. TGF-$\beta$ siRNA alone did not show a significant effect, suggesting that removal of the immune suppressor alone was not sufficient for tumor elimination. The antitumor

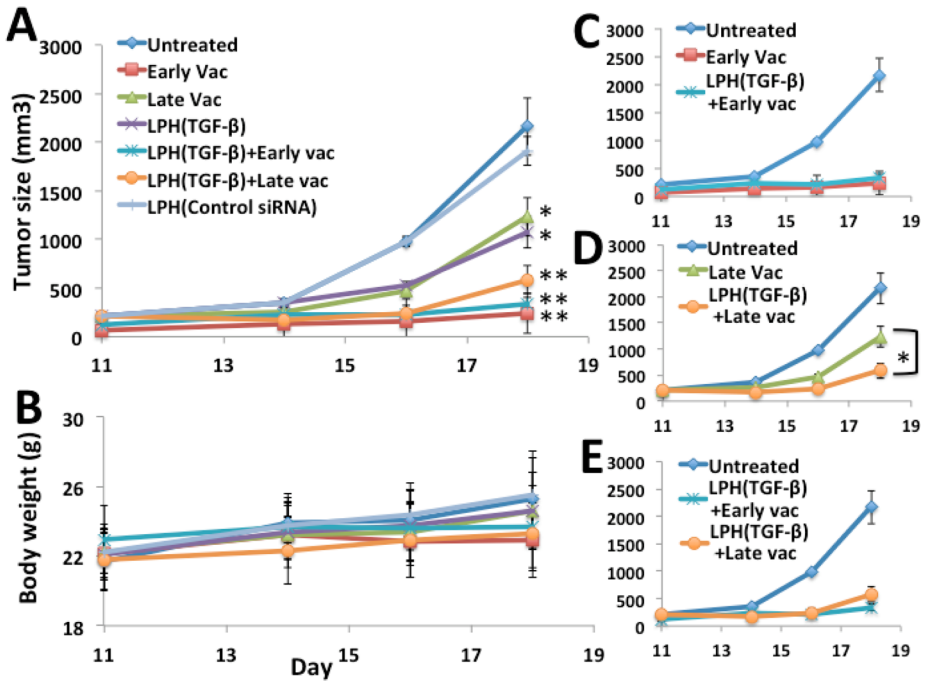


**Figure 5. Antitumor activity against different stages of B16F10 melanoma. C57BL/6 mice were inoculated with 2 × $10^5$ B16F10 cells SC on day 0. LCP vaccine was given on day 4 (Early vac) or day 13 (Late vac). LPH NP containing siRNA (0.6 mg/kg) against TGF-$\beta$ was injected intravenously on days 13, 15, and 17. Tumor growth (A, C, D, E) and body weight (B) were measured every 2 to 3 days for day 18 days. $n = 5$, $^*P < 0.05$, $^{**}P < 0.001$. Statistical analyses were done by comparing with the untreated group unless specified with markings.**

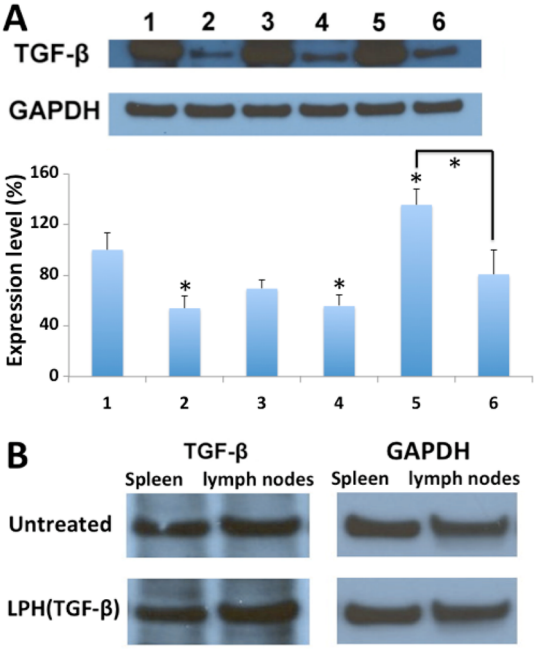


**Figure 6. TGF-$\beta$ levels after treatments. C57BL/6 mice were inoculated with 2 × $10^5$ B16F10 cells SC on day 0, and received the following treatments: 1, Untreated; 2., LPH(TGF-$\beta$); 3, Early vac; 4, LPH(TGF-$\beta$) + Early vac; 5, Late vac; 6, LPH(TGF-$\beta$) + Late vac. Mice were sacrificed on day 18; tumor tissues (A) and lymph organs (B) were harvested for Western blotting and RT-PCR. For the Western blotting, only one representative sample was presented. For the RT-PCR, $n = 5$. Statistical analyses were done by comparing with the untreated group unless specified with markings.**

activity of TGF-$\beta$ inhibition is dependent on the host immune response, while tumor associated antigens were well tolerated without vaccination.

Strategies to disrupt the immune suppressive microenvironment have already been proposed and tested for enhancement of anticancer effects, generally using antibodies or small molecule inhibitors.[5,26] One of the concerns would be that undesired and unexpected systemic immune disruption might occur after systemic administration of these agents. The systemic effect may complicate interpretation of data obtained for the tumor. LPH nanoparticle-based delivery system provides a powerful tool to allow local alteration of a specific molecule/signal without interrupting its systemic functions.[15] It also provides the possibility of studying the effect of tumor microenvironment change without the influence of systemic perturbation. RT-PCR result confirmed an efficient down-regulation of TGF-$\beta$ in the tumor tissues. No significant change of TGF-$\beta$ was observed in the spleen or lymph nodes (Figure 6B), indicating the absence of a systemic effect. There was no decrease in body weight in any treatment groups, suggesting that there is no toxicity associated with the treatments of nanoparticles.

**Reversed Suppressive Tumor Microenvironment by Silencing TGF-$\beta$.** TGF-$\beta$ regulates immune responses and maintains immune homeostasis through its impact on multiple immune cell lineages.[27−29] In general, TGF-$\beta$

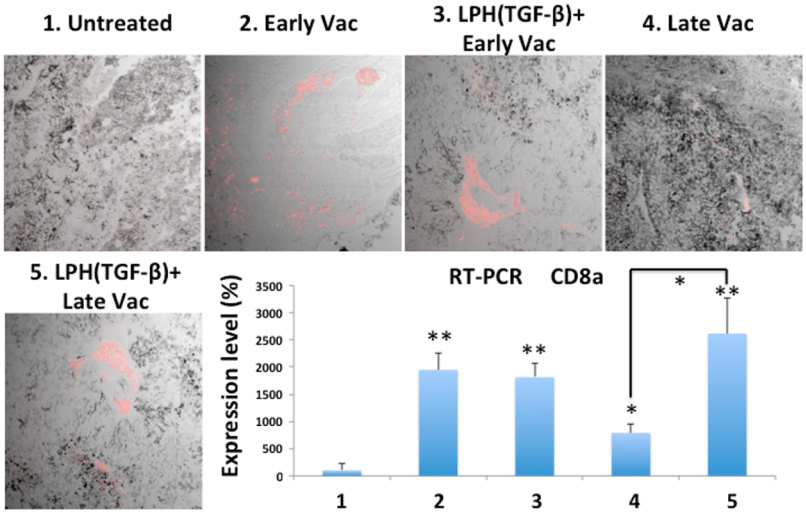


**Figure 7. Tumor infiltration of CD8+ T cells after treatment. B16F10 bearing C57BL/6 mice were treated with LCP vaccine and anti-TGF-$\beta$ siRNA loaded LPH NP as indicated. Mice were sacrificed on day 18, and tumor tissues were assayed for CD8+ T cells (red) detection with immunostaining and RT-PCR analysis. The silencing of TGF-$\beta$ in the late stage tumor reversed the immunosuppressive tumor microenvironment and increased the level of CD8+ T cells. $n = 5$, $^*P < 0.05$, $^{**}P < 0.001$. Statistical analyses were done by comparing with the untreated group unless specified with markings.**

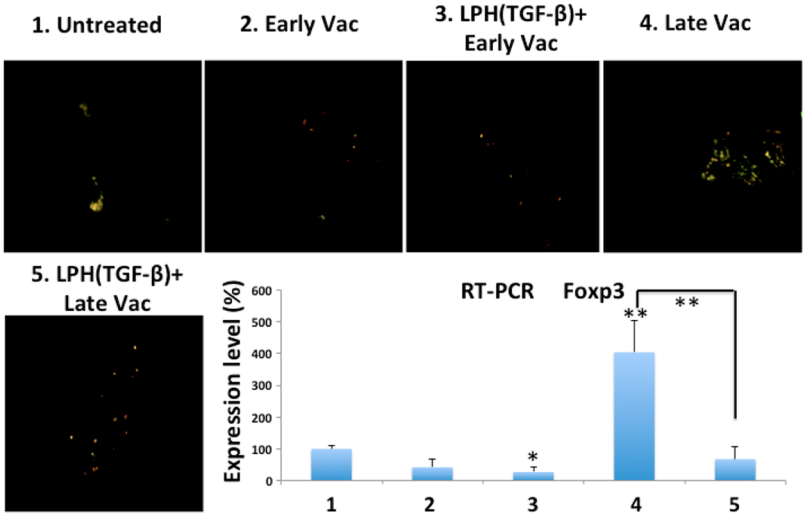


**Figure 8. Tumor infiltration of regulatory T cells after treatment. B16F10 bearing C57BL/6 mice were treated with LCP vaccine and anti-TGF-$\beta$ siRNA loaded LPH NP as indicated. Mice were sacrificed on day 18, and tumor tissues were assayed for CD4+ (green)/Foxp3+ (red) regulatory T cells (yellow) detection with immunostaining and RT-PCR analysis. Silencing of TGF-$\beta$ prohibited the infiltration of regulatory T cells in the late stage tumor microenvironment. $n = 5$, $^*P < 0.05$, $^{**}P < 0.001$. Statistical analyses were done by comparing with the untreated group unless specified with markings.**

shows an adverse effect on antitumor immunity and significantly inhibits tumor immune surveillance.[30] We therefore examined immune cell populations within the tumor microenvironment to uncover a possible mechanism governing the enhanced vaccine effects after tumor-specific silencing of TGF-$\beta$. Cell-specific markers were selected and utilized in both immunostaining and quantification with RT-PCR.

Among all the lymphocytes, CD8+ T cells are considered to be critical effector cells in tumor inhibition following immunotherapy. It is reported that TGF-$\beta$ can suppress CD8+ T cells by inhibiting clonal expansion and repressing expression of cytotoxic genes.[31] We stained for CD8+ T cells (Figure 7) and found that despite a similar CTL response was induced after either vaccination (Figure 3), significantly fewer CD8+ T cells were observed in tumor tissues after late vaccination compared with the early one. Silencing of TGF-$\beta$ in tumor tissue was able to restore CD8+ T cell numbers to the same level seen with early vaccination (LPH (TGF-$\beta$) + Late vac compared with Early vac, $P > 0.05$).

Regulatory T (Treg) cells are an important regulator for maintenance of immunological tolerance by inhibiting T cell-mediated immune responses and suppressing autoreactive T cells.[32] Tumor Treg cells contribute to tumor growth by inhibiting tumor-primed CD4+ cell

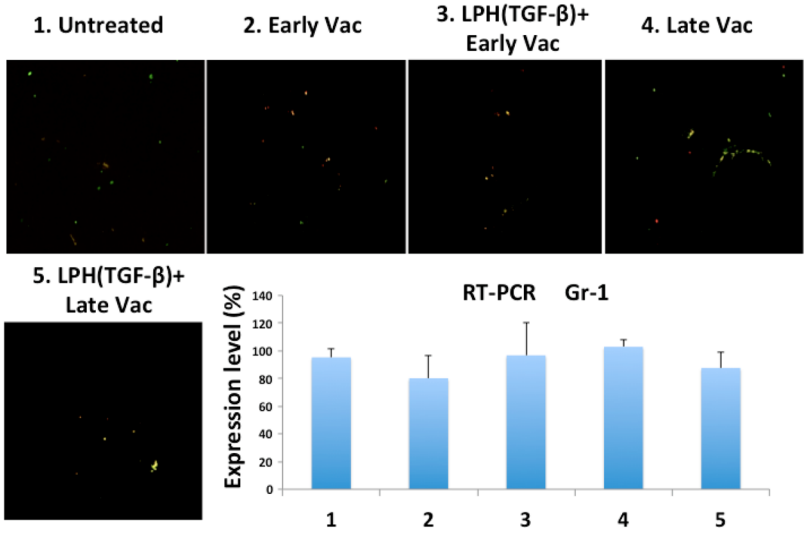


**Figure 9. Tumor infiltration of MDSCs after treatment. B16F10 bearing C57BL/6 mice were treated with LCP vaccine and anti-TGF-$\beta$ siRNA loaded LPH NP as indicated. Mice were sacrificed on day 18, and tumor tissues were assayed for CD11b+ (green)/Gr-1+ (red) MDSCs (yellow) detection with immunostaining and RT-PCR analysis. Silencing of TGF-$\beta$ had negligible impact on the infiltration of MDSCs in the late stage tumor microenvironment. $n$ = 5. Statistical analyses were done by comparing with the untreated group unless specified with markings.**

activity, suppressing CD8+ T cell activation and hindering DC function.[33] TGF-$\beta$ could induce Foxp3 and generate induced Treg cells.[34] Indeed, an elevated Treg cell level was seen in the late vaccination group (Figure 8), correlating with aggressive tumor growth (Figures 2 and 4). Inhibition of TGF-$\beta$ depleted the Treg cells in tumor tissues significantly (Figure 8).

In addition to the lymphoid cells, TGF-$\beta$ also correlates with the function of some myeloid cells. Myeloid-derived suppressor cells (MDSCs) suppress antigen-specific T-cell responses by several distinct mechanisms, resulting in tumor's escape from immune surveillance.[35] Therefore, we are interested in revealing the effect of TGF-$\beta$ inhibition on MDSCs. Both immunostaining and RT-PCR quantification results indicate that there was no significant change in CD11b+Gr-1+ cell level after treatment (Figure 9). Thus, MDSCs were not responsible for the enhanced therapy effect by TGF-$\beta$ siRNA in late vaccination.

Besides the tumor cells, CD11b+Gr-1+ cells, MDSCs are considered as another major source of immune suppressive cytokines such as IL-10 and TGF-$\beta$. For future studies, it will be interesting to test whether down-regulation of TGF-$\beta$ in both tumor cells and MDSCs using nanoparticles will generate a more significant effect compared with present data. Since TGF-$\beta$ has multiple functions on multiple cell populations, it will be also interesting to test whether and how the specific depleting TGF-$\beta$ receptors on a single cell population, such as Treg cells, with targeted nanoparticles would affect the tumor microenvironment.

## CONCLUSION

LCP NP-based vaccine elicited a potent systemic immune response regardless of the existence or stage of tumors. Targeted silencing of TGF-$\beta$ expression in the tumor microenvironment with LPH NP enhanced the efficacy of the LCP vaccine on an advanced melanoma model. Combination of these two nanoparticles offers a flexible and powerful platform for both mechanistic studies and immunotherapeutic strategy development.

## MATERIALS AND METHODS

**Reagents and Murine Cell Line.** 1,2-Dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), dioleoylphosphatydic acid (DOPA), and 1,2-distearoryl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol-2000)] ammonium salt (DSPE-PEG) were purchased from Avanti Polar Lipids. Mannose-PEG-DSPE was synthesized as described previously,[14] and the structure was confirmed using $^1$H NMR. DSPE-PEG-AA was synthesized according to the previously established protocol.[15] H-2K$^b$ restricted peptides Trp2 (SVYDFFVWL, MW 1175), OVA (SIINFEKL, MW 1773), and modified Trp2 peptide, p-Trp2 (pSpSSSVYDFFVWL, MW 1626) were purchased from Peptide 2.0 (Chantilly, VA). CpG ODN 1826 (5′-TCCATGACGTTCCTGACGTT-3′) and siRNA against TGF-$\beta$ (sense, GCAACAACGCCAUCUAUGA; antisense, UCAUAGAUGGCGUUGUUGC) were obtained from Sigma-Aldrich (St. Louis, MO). Cholesterol (Chol), hyaluronic acid (HA), protamine sulfate (fraction x from salmon) and all the other chemicals were purchased from Sigma Aldrich (St. Louis, MO), unless otherwise mentioned.

Murine melanoma cell line B16F10 (syngeneic with C57BL/6) was purchased from ATCC, and cultured in Dulbecco's modified Eagle medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin (Invitrogen), and 100 $\mu$g/mL streptomycin (Invitrogen).

**Preparation of LCP-Based Vaccine.** LCP-based vaccine was prepared as previously described.[14] The calcium phosphate (CaP) core was formed in a water-in-oil microemulsion. Briefly, 600 $\mu$L of 2.5 M $CaCl_2$ containing CpG ODN and peptide was added into

a 20 mL Cyclohexane/Igepal CO-520 (71:29, v/v) solution to form Ca phase. Meanwhile, 600 $\mu$L of 12.5 mM $Na_2HPO_4$ (pH = 9.0) and 200 $\mu$L of 20 mM DOPA were dispersed into a separate 20 mL oil phase to obtain the phosphate phase. The above two solutions were mixed and stirred at room temperature for 30 min. Equal volume of ethanol was added to break the microemulsion. CaP cores were collected with centrifugation at 10 000*g* and then washed three times with 20 mL of ethanol to remove cyclohexane and surfactant. The pellets were dissolved in chloroform for further use.

CaP cores were mixed with 200 $\mu$L of 20 mM DOTAP/Chol (1:1), and 20 $\mu$L of 20 mM DSPE-PEG-2000/DSPE-PEG-mannose (1:1) in chloroform. After evaporating the chloroform, LCP particles were formed by adding 100 $\mu$L of 5% glucose.

**Preparation of Liposome and LPH.** LPH-NP were prepared through a stepwise self-assembly process based on previously established protocol.[15] DOTAP and Chol (1:1, molar ratio) in chloroform were mixed, and the solvent was evaporated under a reduced pressure. Distilled water was added, and the liposomes were extruded sequentially through 400, 200, 100, and 50 nm polycarbonate membranes (Millipore, Billerica, MA) to form 80−100 nm unilamellar liposomes with a final concentration of 10 mM DOTAP and cholesterol. LPH cores were prepared by mixing 140 $\mu$L of solution A (36 $\mu$g protamine in 5% glucose) and 140 $\mu$L of solution B (24 $\mu$g HA and 24 $\mu$g siRNA in 5% glucose). After incubation at room temperature for 10 min, 60 $\mu$L of DOTAP/Chol liposome was added into the LPH cores. LPH NP was formed and PEGylated by adding 30 $\mu$L of DSPE-PEG (10 mg/mL) and 30 $\mu$L of DSPE-PEG-AA (10 mg/mL) at 50 °C for 15 min. Both final particles 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).

**Tumor Growth Inhibition.** For vaccination studies, 6−8 weeks old female C57BL/6 mice were subcutaneously inoculated with $2 \times 10^5$ B16F10 cells on their flank on day 0. In early vaccination group, LCP vaccine in 5% glucose was injected subcutaneously into the contralateral side of the inoculation site on day 4. For late vaccination group, LCP vaccine was given on day 13.

For combined therapy, female C57BL/6 mice of age 6−8 weeks old were inoculated subcutaneously with $2 \times 10^5$ B16F10 cells on their lower back on day 0. Vaccination was given on day 4 (early vaccination) or day 13 (late vaccination). LPH NP containing siRNA (0.6 mg/kg) against TGF-$\beta$ was injected intravenously on day 13, day 15, and day 17 through tail vein.

Tumor size was measured every 2 to 3 days using digital calipers (Thermo Fisher Scientific, Pittsburgh, PA) and calculated as (0.5 × length × width × height). Body weight was also monitored. Humane sacrifice of mice was performed when tumor reached 20 mm in one dimension.

***In Vivo* Cytotoxic T Lymphocyte (CTL) Assay.** The *in vivo* CTL assay was performed on C57BL/6 mice seven days after the vaccination, according to the previous protocol with slight modifications.[36] Splenocytes from naïve C57BL/6 mice were collected and pulsed with either 10 $\mu$M Trp2 or Ova peptide in complete media at 37 °C for 1 to 2 h. Both pulsed cell populations were stained with 2 $\mu$M PKH-26 (Sigma-Aldirch, St. Louis, MO) following the manufacturer's instructions. Then, the Trp2 peptide-pulsed and Ova peptide-pulsed cells were labeled with 4 and 0.4 $\mu$M CFSE, respectively. Equal amount of $CFSE^{high}$ (Trp2 pulsed cells) and $CFSE^{low}$ (Ova pulsed cells) were mixed and injected intravenously into the control or immunized mice. After 18 h, splenocytes from these treated mice were collected and subjected to flow cytometry analysis. The number of $CFSE^{high}$ and $CFSE^{low}$ was calculated, and the *in vivo* Trp2 specific lysis percentage was enumerated according to a published equation.

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

$$\text{where } x = \frac{\text{Trp2}}{\text{Ova}} \text{ from naïve mice}$$

**Quantitative RT-PCR.** RNA from tissue samples were extracted with the RNeasy kit (Qiagen) and reverse-transcribed to cDNA using SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies). TaqMan Universal PCR Master Mix (Life Technologies) was mixed with 200 ng of cDNA, and the reactions were conducted using 7500 Real-Timer PCR System (Life Technologies). GAPDH was used as an endogenous control. All the primers for RT-PCR reactions were listed in Table 1. The data were analyzed with the 7500 Software. The RT-PCR was performed in triplicates.

**TABLE 1**

| primer | applied biosystems/ref |
|---|---|
| Mouse TGF-$\beta$1 | Mm01178820_m1 |
| Mouse IL6 | Mm00446190_m1 |
| Mouse IL10 | Mm00439614_m1 |
| Mouse Gr-1 | Mm00439154_m1 |
| Mouse Foxp3 | Mm00475162_m1 |
| Mouse CD8 | Mm01182107_g1 |
| Mouse TNF-$\alpha$ | Mm00443260_g1 |
| Mouse CCL2 | Mm00441242_m1 |
| Mouse GAPDH | Mm99999915_g1 |

**Western Blot Analysis.** Tumor tissues were collected and lysed with radioimmunoprecipitation assay (RIPA) buffer. Protein concentration of the tumor lysates was measured using the Bradford protein assay reagents following the manufacturer's instruction. Samples were diluted in 4× sample buffer with reducing reagent and heated at 95 °C for 5 min. After the electrophoresis on NuPAGE 4−12% Bis-Tris Gels (Invitrogen, Grand Island, NY), proteins were transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA) and probed with the primary antibody (anti-TGF-$\beta$1 mAb (Santa Cruz, Santa Cruz, CA) or anti-GAPDH mAb (Cell Signaling, Danvers, MA), respectively) and the horseradish peroxidase-conjugated secondary antibody. Signals were developed using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL).

**Immunofluorescence.** Immunofluorescence detection of different cell populations was performed using paraffin sections of tumor tissues (obtained from the UNC Tissue Procurement Core). The slides were deparaffinized, antigen recovered, permeabilized and fixed if necessary, and blocked with 1% BSA at room temperature for 1 h. Cell markers were detected with antibodies conjugated with fluorophores (Santa Cruz, Santa Cruz, CA) as indicated. Images were taken using fluorescence microscopy (Nikon, Tokyo, Japan).

**Statistical Analysis.** Data were analyzed statistically using a two-tailed Student's *t*-test by comparing with the control group unless specified with markings. Differences were considered statistically significant if the *p* value was less than 0.05.

*Conflict of Interest:* The authors declare no competing financial interest.

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