

Efficient anti-tumor immunotherapy using tumor epitope-coated biodegradable nanoparticles combined with polyinosinic-polycytidylic acid and an anti-PD1 monoclonal antibody

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Research Article

Keywords: anti-PD1 monoclonal antibody, cytotoxic T lymphocytes, immunotherapy, polyinosinic-polycytidylic acid, tumor epitope nanoparticles, tumor peptide epitopes

Posted Date: September 16th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2043402/v1

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Abstract

Background Vaccination with tumor peptide epitopes associated with major histocompatibility complex class I molecules is an attractive approach directed at inducing tumor-specific cytotoxic T lymphocytes (CTLs). However, challenges remain in improving the therapeutic efficacy of peptide epitope vaccines, including the low immunogenicity of peptide epitopes and insufficient stimulation of innate immune components *in vivo*. To overcome this, we aimed to develop and test an innovative strategy that elicits potent CTL responses against tumor epitopes. The essential feature of this strategy is vaccination using tumor epitope-loaded nanoparticles (NPs) in combination with polyinosinic-polycytidylic acid (poly-IC) and anti-PD1 monoclonal antibody (mAb).

Methods Carboxylated NPs were prepared using poly(lactic-co-glycolic acid) and poly(ethylene/maleic anhydride), covalently conjugated with anti-H-2KbmAbs, and then attached to H-2Kb molecules isolated from the tumor mass (H-2b). Native peptides associated with the H-2Kbmolecules of H-2Kb-attached NPs were exchanged with tumor peptide epitopes. The tumor-specific CTL-inducing and anti-tumor activities of the tumor epitope-loaded NPs were examined in mice bearing EG7-OVA thymoma or B16-F10 melanoma. In addition, the anti-tumor therapeutic efficacy of the NPs was examined in combination with poly-IC, anti-PD1 mAb, or both.

Results Tumor peptide epitope-loaded NPs efficiently induced tumor-specific CTLs when used to immunize tumor-bearing mice as well as normal mice. This activity of the NPs significantly was increased when co-administered with poly-IC. Accordingly, the NPs exerted significant anti-tumor effects in mice implanted with EG7-OVA thymoma or B16-F10 melanoma, and the anti-tumor activity of the NPs was significantly increased when applied in combination with poly-IC. The most potent anti-tumor activity was observed when the NPs were co-administered with both poly-IC and anti-PD1 mAb.

Conclusions Immunization with tumor epitope-loaded NPs in combination with poly-IC and anti-PD1 mAb in tumor-bearing mice can be a powerful means to induce tumor-specific CTLs with therapeutic anti-tumor activity.

Background

In anti-tumor immunotherapy, tumor-specific cytotoxic T lymphocytes (CTLs) are central to eliminating tumor cells [1–4]. These cells must first be primed with and expanded in response to tumor epitopes, comprised of short peptides (~ 8–10 amino acids in length) and associated with the "peptide-binding groove" of major histocompatibility complex class I (MHC-I) molecules, before exerting tumor antigen-specific cytotoxic effects [5]. Tumor antigens are classified into two general categories, tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) [6]. TAAs are self-antigens that are either preferentially or abnormally overexpressed in tumors, whereas TSAs are expressed only in tumors, such as oncoviral antigens and neoantigens generated by somatic mutations [6, 7]. Peptide epitopes presented by MHC-I molecules (first signal) on antigen-presenting cells (APCs) are recognized by T cell receptors

(TCRs) on naïve CD8 T cells [1]. Following recognition of peptide epitope-MHC-I complexes, costimulatory molecules (second signal), such as CD80 and CD86, synergize with the first signal to promote T cell activation and expansion [8–10].

Vaccination with tumor epitopes associated with the peptide-binding groove of MHC-I molecules is an attractive approach to inducing tumor-specific CTLs [11–13]. In most studies, the peptide epitopes were synthetic peptides derived from TAAs or TSAs. Peptide epitope vaccines have several advantages over other vaccine types, including tumor-specific proteins, nucleic acids, and viral vectors. Peptide epitopes are easy to synthesize and purify, safe because they lack hypersensitivity-inducing or carcinogenic potential, and are highly specific in eliciting peptide-specific CTLs in vivo [14–17]. However, challenges remain in improving the therapeutic efficacy of peptide epitope vaccines. One major limitation is that peptide epitopes have low immunogenicity and inefficiently induce peptide-specific CTLs. For efficient naïve CD8 T cell priming, peptide epitopes must bind to MHC-I molecules on professional APCs expressing co-stimulatory molecules because efficient T cell activation requires at least two distinct APCderived signals, MHC-I-peptide complexes and co-stimulatory molecules [18, 19]. However, peptide epitopes can also bind to MHC-I molecules expressed on all nucleated cells that cannot perform professional APC functions. MHC-I-peptide complex presentation in the absence of co-stimulation induces peptide-specific immune tolerance [14, 20]. Thus, the binding of *in vivo*-administered peptide epitopes to MHC-I molecules expressed in non-APCs would critically limit the peptide epitope vaccine effectiveness. Other limitations include the short half-life of the peptides and insufficient stimulation of innate immune components in vivo [18].

Numerous strategies have been developed to overcome the limitations of peptide epitope vaccines. These include the combination of peptide epitopes with Toll-like receptor (TLR) agonists, such as polyinosinic-polycytidylic acid (poly-IC) and CpG oligomers, which induce the upregulation of co-stimulatory molecules and pro-inflammatory cytokines through APC activation, and peptide epitopes nano-encapsulation with a biodegradable polymer [21, 22]. Peptide epitopes Encapsulation with a biodegradable polymer [21, 22]. Peptide epitopes Encapsulation with a biodegradable polymer [21, 22]. Peptide epitopes Encapsulation with a biodegradable polymer [21, 23]. The peptide epitopes that also could deliver the peptide epitopes preferentially to phagocytic cells, including dendritic cells (DCs), via phagocytosis [21, 23]. The combination of peptide epitope vaccines with an immune checkpoint inhibitor (ICI), such as anti-PD1 monoclonal antibody (mAb), anti-PD-L1 mAb, and anti-CTLA-4 mAb, has received intense research recently [17, 24–26]. By interrupting co-inhibitory signaling pathways, ICIs reinvigorate exhausted CTLs to regain their function and eradicate tumor cells [27, 28]. However, ICI immunotherapy is only effective for cancer patients producing tumor-specific CTLs that recognize tumor antigens because ICI would not work in a microenvironment with no tumor-specific CTLs. Thus, for "immune desert cancers" with no tumor-specific CTLs, a strategy to induce tumor-specific CTLs would be the first objective before ICI use.

Here, the aim was to develop and test an efficient anti-tumor immunotherapy strategy using tumor epitope-loaded nanoparticles (NPs) in combination with poly-IC and anti-PD1 mAb. NPs attached to MHC-I molecules were first fabricated with a biodegradable polymer, poly(lactic-co-glycolic acid) (PLGA); then,

the native peptides associated with MHC-I molecules were exchanged with tumor epitopes using a peptide exchange reaction. The tumor epitope-loaded NPs were examined for their capacity to induce tumor-specific CTLs in tumor-bearing mice as well as normal mice. Furthermore, the anti-tumor therapeutic potential of the tumor epitope-loaded NPs alone and in combination with poly-IC, anti-PD1 mAb, or both was examined in tumor-bearing mice. Results showed that immunizing tumor-bearing mice with tumor epitope-loaded NPs combined with poly-IC and anti-PD1 mAb is a powerful strategy to treat tumors.

Materials And Methods

Mice and cell lines

Female C57BL/6 mice (8–12 weeks old) were purchased from Kosa Bio, Inc. (Seongnam, South Korea). All experimental animal procedures were performed in accordance with guidelines and regulations approved by the Institutional Animal Care and Use Committee of Chungbuk National University (CBNUA-1490-21-02). EG7-OVA cells, a mouse EL4 lymphoma cell line expressing OVA (H-2^b), were purchased from the American Type Culture Collection (Manassas, VA, USA). B16-F10 mouse melanoma cells were purchased from the Korean Cell Line Bank (Seoul, South Korea).

Peptides, antibodies, and reagents

OVA_{257 - 264} peptide (SIINFEKL), TRP2_{180 - 188} peptide (SVYDFFVWL), FITC-labeled TRP2_{180 - 188} peptide (FITC-TRP2_{180 - 188}), and dipeptide glycyl-cyclohexylalanine (GCha) were purchased from Peptron (Daejeon, South Korea). Dipeptide glycyl-leucine (GL) and glycyl-methionine (GM) were from Tokyo Chemical Industry (Tokyo, Japan) and BLD Pharmatech (Shanghai, China), respectively. mAbs specific to mouse H-2K^b (clone Y-3) and mouse PD1 (clone RMP1-14) were from BioXCell (West Lebanon, NH, USA). Fluorescence-labeled mAbs specific to mouse H-2K^b-OVA_{257 - 264} complex (clone 25-D1.16) and mouse CD8α (clone KT15) were from BioLegend (San Diego, CA, USA) and Invitrogen (San Diego, CA, USA), respectively. Fluorescence-labeled H-2K^b-OVA_{257 - 264} tetramers and H-2K^b-TRP2_{180 - 188} tetramers were from MBL (Tokyo, Japan). Poly-IC was from InvivoGen. Poly-IC stabilized with poly-L-lysine and carboxy-methylcellulose (poly-ICLC) was provided by Dr. Hyun-II Cho (ViGenCell Inc., Seoul, South Korea).

Preparation of carboxylated nanoparticles (NPs)

Carboxylated NPs were prepared using PLGA (lactide:glycolide 50:50, Mw 24,000–38,000, acid terminated; Evonik Industries, Essen, Germany) and poly(ethylene/maleic anhydride) (PEMA; Polysciences Inc., Warrington, PA, USA), as described previously [29]. Detailed methods are described in online supplemental methods. Briefly, 200 mg of PLGA was dissolved in 4 mL of ethyl acetate and combined with 8 mL of 1% PEMA. The mixture was emulsified using a homogenizer (HG-15D Homogenizer, DAIHAN Scientific, Seoul, South Korea) at 14,000 rpm for 4 min to form a water-in-oil (w/o) emulsion, which was transferred to a beaker containing a 0.2% PEMA and 0.1% polyvinyl alcohol (Sigma-Aldrich, St. Louis, MS, USA) mixture and stirred at room temperature for 18 h. The resulting NPs were

centrifuged at 3,500 × *g* for 20 min at 4°C and resuspended in 10 mL of sterile distilled water. The mean size and ζ-potential of the carboxylated NPs were assessed using a particle size analyzer (ELS-Z, Otsuka, Japan). The NPs were counted using qNano Gold (IZON Science, Christchurch, New Zealand).

Isolation of tumor-derived membrane protein

Tumor-derived membrane proteins were isolated from the EG7-OVA tumor mass as described previously [29]. Briefly, 1 g of the tissue was dissociated with 10 mL of extraction buffer containing 1X protease inhibitor cocktail (Sigma-Aldrich) using a gentlMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell debris and the lipid layer were removed by centrifugation for 5 min at $350 \times g$, and the supernatant was ultracentrifuged for 45 min at $80,000 \times g$ at 4°C (Beckman Coulter, Brea, CA, USA). After ultracentrifugation, the pellet was resuspended in extraction buffer (0.5 mL) and homogenized using a tissue grinder (SHS-30E, SciLab, Seoul, South Korea). The homogenate was mixed with solubilization buffer (0.5 mL) and solubilized for 90 min at 4°C. The homogenate containing solubilized membrane proteins was transferred to a new conical tube. The amount of isolated membrane protein was determined using a microbicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation of anti-mouse H-2K^b mAb-conjugated NPs and H-2K^b-attached NPs (NP-K^b)

Anti-mouse H-2K^b mAbs were conjugated to the surface of carboxylated NPs using a modified carbodiimide coupling method, and the NPs conjugated with mAbs were attached with H-2K^b molecules, as described previously [29]. Briefly, 10 mg of carboxylated NPs was resuspended in 1.2 mL of 50 mM activation buffer, added to 4 μ L of 300 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Thermo Fisher Scientific) and 8 μ L of 300 mM N-hydroxysuccinimide (Sigma-Aldrich), and incubated for 1 h at room temperature. EDC-activated NPs were washed twice with 50 mM activation buffer, resuspended in 850 μ L of 50 mM activation buffer, added to 150 μ L of 4 mg/mL anti-mouse H-2K^b mAbs, and incubated for 18 h at room temperature. To block non-specific conjugation, mAb-conjugated NPs were treated with 30 μ L of ethanolamine (Sigma-Aldrich) for 30 min and washed twice with phosphate buffered saline (PBS). The NPs conjugated with mAbs were incubated NPs (NP-K^b) were washed twice with 5 mg of tumor-derived membrane proteins for 24 h at 4°C. The resulting H-2K^b-attached NPs (NP-K^b) were washed twice with PBS and resuspended in 1 mL PBS.

Generation of bone marrow-derived DCs (BMDCs) from bone marrow cells

BMDCs were generated from mouse bone marrow cells using GM-CSF and IL-4, as described previously [29]. Briefly, bone marrow cells from mouse femurs were cultured in 6-well plates (5 × 10⁶ cells/well) in a culture medium with 40 ng/mL GM-CSF and 20 ng/mL IL-4 (both from Creagene, Seongnam, South Korea). After 3 days, non-adherent cells were removed by gently shaking the plate and replacing the medium. On day 4, non-adherent cells were removed using the same method. On day 6, half of the culture

medium was replaced with a fresh medium. To induce maturation, BMDCs were exposed to 100 ng/mL LPS (Sigma-Aldrich) for 24 h. BMDCs were harvested by gentle pipetting on day 7.

Peptide exchange reaction on H-2K^b-attached NPs

Native peptides associated with the H-2K^b molecules of NP-K^b were exchanged with $OVA_{257-264}$ by incubating 5 mg of NP-K^b with 100 μ M $OVA_{257-264}$ peptide with or without 20 mM dipeptide GL for 18 h at 37°C. $OVA_{257-264}$ -pulsed NP-K^b was stained with mAbs recognizing H-2K^b-OVA_{257-264} complexes (clone 25-D1.16) and analyzed by flow cytometry. In some experiments, native peptides associated with H-2K^b molecules of NP-K^b were exchanged with TRP2₁₈₀₋₁₈₈ by incubating 5 mg of NP-K^b with 100 μ M TRP2₁₈₀₋₁₈₈ with or without 20 mM dipeptide GM for 18 h at 37°C, washed twice with PBS, and resuspended in PBS.

Competitive blocking assay for peptide epitope bindings

NP-K^b (1 mg) was incubated with a fixed concentration of FITC-TRP2_{180 - 188} (0.1 μ M) and various concentrations of unlabeled TRP2_{180 - 188} (0–100 μ M) with 20 mM dipeptide GM for 18 h at 37°C, washed twice with PBS, and resuspended in PBS. Levels of FITC-TRP2_{180 - 188} binding to NP-K^b were analyzed using flow cytometry.

In vivo generation of peptide-specific CD8⁺ T cells

C57BL/6 mice were immunized intravenously with PBS or the indicated NPs (1×10^9 particles/mouse) on days 0, 7, and 14. In poly-IC-treated mice, the NPs were mixed with poly-IC (50 µg/mouse) before intravenous injection. On days 5 and 19, whole blood cells were collected from the facial vein, stained with the H-2K^b-OVA_{257 - 264} tetramers and mAbs against mouse CD8a, and analyzed using flow cytometry.

Measurement of peptide-specific CTL activity

To administer the NPs exchanged with the TRP2_{180 - 188} peptide, C57BL/6 mice were intravenously immunized with PBS or the indicated NPs (1×10^9 particles/mouse) on days 0 and 5. In poly-ICLC-treated mice, the NPs were mixed with poly-ICLC (50 µg/mouse) before intravenous injection. On day 12, TRP2_{180 - 188}-specific CTL activity was assessed using an *in vivo* CTL assay.

Anti-tumor activity

The right flanks of C57BL/6 mice were shaved and subcutaneously inoculated with EG7-OVA cells (5 × 10^5 cells/mouse). The mice were intravenously administered with PBS or the indicated NPs (1 × 10^9 particles/mouse) on days 2, 9, and 16. In poly-IC-treated mice, the NPs were mixed with poly-IC (50 µg/mouse) before intravenous injection. For combination therapy, anti-PD1 mAbs (100 µg/mouse) were intraperitoneally administered six times before or 2 days after each NP immunization. The mice were euthanized when the tumor volume reached 2,500 mm³. Tumor volume was calculated using the following formula: tumor volume [mm³] = 0.52 × (long diameter [mm]) × (short diameter [mm])². Spleen or lymph node cells were isolated from tumor-bearing mice, stained with H-2K^b-OVA_{257 - 264} tetramers and mAbs against mouse CD8 α , and analyzed using flow cytometry according to the manufacturer's instructions.

For the B16-F10 melanoma model, the right flanks of C57BL/6 mice were shaved and subcutaneously inoculated with B16-F10 cells (1×10^5 cells/mouse). The mice were intravenously administered with PBS or the indicated NPs (1×10^9 NPs/mouse) on days 2, 7, and 12. In poly-ICLC-treated mice, the NPs were mixed with poly-ICLC (50 µg/mouse) before intravenous injection. For combination therapy, anti-PD1 mAbs (100μ g/mouse) were intraperitoneally administered six times before or 1 day after NP immunization. Spleen cells were isolated from tumor-bearing mice, stained with H-2K^b-TRP2_{180 - 188} tetramers and mAbs against mouse CD8 α , and analyzed using flow cytometry according to the manufacturer's instructions.

Restimulation of splenocytes isolated from tumor-bearing mice

Splenocytes (2 × 10⁶ cells/well) isolated from tumor-bearing mice were co-cultured with peptide epitopepulsed BMDCs (2 × 10⁵ cells/well) in 24-well plates for 3 days. Peptide epitope-pulsed BMDCs were prepared by pulsing LPS-stimulated BMDCs with 10 μ M peptide epitopes for 1 h at 37°C. IFN- γ levels in the culture supernatant were measured using commercial ELISA kits (BD Biosciences, San Jose, CA, USA).

Anti-metastatic activity

C57BL/6 mice were intravenously inoculated with B16-F10 cells (1×10^5 cells/mouse) into the tail vein. Mice were intravenously administered with PBS or the indicated NPs (1×10^9 particles/mouse) on days 1, 6, and 11. In poly-ICLC-treated mice, the NPs were mixed with poly-ICLC (50 µg/mouse) before intravenous injection. For combination therapy, anti-PD1 mAbs ($100 \mu g/mouse$) were intraperitoneally administered six times before or 1 day after NP immunization. Mice were euthanized on day 17 after tumor cell injection, and lungs were harvested and fixed in Bouin's solution (BioGnost, Zagreb, Croatia). Metastatic nodules on the lung surface were counted.

Statistical analysis

A two-tailed paired Student's *t*-test was performed for a single comparison of two groups after a normality evaluation. One-way or two-way ANOVA with *post-hoc* Tukey's test was performed to compare the significance of multiple groups; $p \le 0.05$ was considered statistically significant.

Results

Fabrication and characterization of NP-K^b pulsed with OVA_{257 - 264}

Carboxylated NPs were prepared using PLGA and PEMA using the w/o emulsion solvent evaporation method. The carboxylated NPs were covalently conjugated with anti-H-2K^b mAbs (isotype mlgG2b) via carbodiimide conjugation and then attached to H-2K^b molecules by incubating the mAb-conjugated NPs with solubilized membrane proteins isolated from the EG7-OVA tumor mass (H-2^b) to generate H-2K^b- attached NPs (NP-K^b). Native peptides associated with the H-2K^b molecules of NP-K^b were exchanged with a model tumor-specific peptide, $OVA_{257-264}$, via a peptide exchange reaction (Fig. 1A). The peptide exchange reaction was maximized by optimizing the reaction conditions, such as the use of dipeptides, GL, GM, and GCha, which are known to enhance the peptide exchange reaction [30], and the concentrations of $OVA_{257-264}$. The peptide exchange efficiency was maximal when NP-K^b was treated with 100 µM $OVA_{257-264}$ in the presence of 20 mM GL, whereas GM and GCha were ineffective (Supplemental Fig. 1). The relative degree of H-2K^b-OVA_{257-264} complexes on NP-K^b was 2.8-fold higher when NP-K^b was treated with 100 µM $OVA_{257-264}$ only (NP-SIIN^{Lo}) (Fig. 1B). The H-2K^b-OVA_{257-264} complexes on NP-K^b was treated with 100 µM $OVA_{257-264}$ only (NP-SIIN^{Lo}) (Fig. 1B). The H-2K^b-OVA_{257-264} complexes on NPs remained stable for up to 5 days (Fig. 1C). The average NP-SIIN^{Hi} size was 1032.1 ± 44.8 nm, and the average ζ -potential was – 19.48 ± 1.61 mV.

NPs loaded with higher amounts of $OVA_{257 - 264}$ induce $OVA_{257 - 264}$ -specific CTLs more efficiently in normal mice

To compare the $OVA_{257 - 264}$ -specific CTL-inducing activity of NP-SIIN^{Lo} and NP-SIIN^{Hi}, mice were intravenously injected with the NPs (1 × 10⁹ particles/mouse) three times at 7-day intervals. Control mice were intravenously injected with PBS or NP-K^b. Five days after the first and last immunizations, $OVA_{257 - 264}$ -specific CTLs in peripheral blood were analyzed using H-2K^b-OVA_{257 - 264} tetramers. After priming, the percentage of $OVA_{257 - 264}$ -specific CTLs among CD8⁺ T cells reached 1.28% and 1.71% in mice treated with NP-SIIN^{Lo} and NP-SIIN^{Hi}, respectively (Fig. 2A, B). After boosting, the percentage of $OVA_{257 - 264}^{-1}$ specific CTLs among CD8⁺ T cells increased to 3.36% and 5.63% in mice treated with NP-SIIN^{Lo} and NP-SIIN^{Hi}, respectively (Fig. 2C, D). Seven days after the last immunization, $OVA_{257 - 264}^{-1}$ -specific CTL activity in the spleen and lymph nodes was analyzed using an *in vivo* CTL assay. Representative histograms are shown in Fig. 2E and 2G, respectively. Immunization with NP-SIIN^{Hi} induced $OVA_{257 - 264}^{-1}$ -specific CTL activity more efficiently (specific target cell killing of 57.2% in the spleen and 53.6% in the lymph node) than immunization with NP-SIIN^{Lo} (specific target cell killing of 34.7% in the spleen and 36.8% in the lymph node) (Fig. 2F, H). These results showed that immunization with NPs loaded with high amounts of $OVA_{257 - 264}^{-1}$ efficiently induced potent CTL responses.

Combining NP-SIIN^{Hi} with poly-IC further increases OVA₂₅₇ $_{-264}$ -specific CTL induction in normal mice

The OVA_{257 - 264}-specific CTL-inducing activity of NP-SIIN^{Hi}, in combination with poly-IC, was examined in normal mice. In this experiment, mice were intravenously injected three times with NP-SIIN^{Hi} (1 × 10⁹ particles/mouse) alone or with poly-IC at 7-day intervals. Five days after the first and last immunizations, $OVA_{257 - 264}$ -specific CTLs in peripheral blood were analyzed using H-2K^b-OVA_{257 - 264}-tetramers. In primed mice, the combination of NP-SIIN^{Hi} with poly-IC did not induce discernible increases in $OVA_{257 - 264}$ -specific CTLs among CD8⁺ T cells compared to that with NP-SIIN^{Hi} alone (1.98% vs. 1.61%) (Fig. 2I, J). However, after boosting, the combination of NP-SIIN^{Hi} with poly-IC significantly increased the percentage of $OVA_{257 - 264}$ -specific CTLs among CD8⁺ T cells compared to that with NP-SIIN^{Hi} alone (8.79% vs. 5.66%) (Fig. 2K, L). These results suggest that the adjuvant activity of poly-IC is prominent during boosting with NP-SIIN^{Hi}, but not during priming.

Seven days after the last immunization, $OVA_{257-264}$ -specific CTL activity in the spleen and lymph nodes was analyzed using an *in vivo* CTL assay. Representative histograms are shown in Fig. 2M and 20, respectively. In accordance with tetramer analysis results, immunization with NP-SIIN^{Hi} combined with poly-IC potently induced $OVA_{257-264}$ -specific CTL activity (specific target cell killing of 84.7% in the spleen and 75.3% in the lymph node), compared to that upon immunization with NP-SIIN^{Hi} alone (specific target cell killing of 56.2% in the spleen and 53.4% in the lymph node) (Fig. 2N, P).

NP-SIIN ^{Hi} and poly-IC combination exerts potent antitumor effects in EG7-OVA tumor-bearing mice with increased production of OVA_{257 - 264}-specific CTLs

The anti-tumor therapeutic potential of NP-SIIN^{Hi} combined with poly-IC was examined with EG7-OVA tumor-bearing mice. Mice were subcutaneously inoculated with EG7-OVA tumor cells, and 2 days later,

they were intravenously injected three times with PBS, NP-SIIN^{Hi}, poly-IC, or NP-SIIN^{Hi} plus poly-IC at 7-day intervals. The combination of NP-SIIN^{Hi} and poly-IC exerted much greater anti-tumor effects than NP-SIIN^{Hi} alone (Fig. 3A). Poly-IC alone did not show anti-tumor activity. The anti-tumor therapeutic potential of NP-SIIN^{Hi} plus poly-IC was also confirmed by the survival rate (Fig. 3B).

Upon completion of the anti-tumor experiment (day 25 of Fig. 3A), the induction degree of $OVA_{257 - 264}^{-1}$ specific CTLs was measured in the spleen and lymph nodes isolated from EG7-OVA tumor-bearing mice. The percentage of $OVA_{257 - 264}^{-1}$ -specific CTLs in the spleen was increased 2.1-fold in mice immunized with NP-SIIN^{Hi} plus poly-IC (12.9%) compared to that in mice immunized with NP-SIIN^{Hi} alone (6.18%) (Fig. 3C, D). The percentage of $OVA_{257 - 264}^{-1}$ -specific CTLs in the lymph node was also increased 1.8-fold in mice immunized with NP-SIIN^{Hi} plus poly-IC (2.04%) compared to that in mice immunized with NP-SIIN^{Hi} alone (1.13%) (Fig. 3E, F). IFN- γ production by CTLs is a major characteristic of activated CTLs [31, 32]. To confirm tumor-specific CTL functional activation, spleen cells isolated from EG7-OVA tumor-bearing mice were co-cultured with $OVA_{257 - 264}^{-1}$ -pulsed mature BMDCs, and IFN- γ levels in the culture supernatants were measured using ELISA. Spleen cells isolated from mice immunized with NP-SIIN^{Hi} plus poly-IC produced more IFN- γ (2.8-fold) than those immunized with NP-SIIN^{Hi} alone (Fig. 3G). Mice immunized with PBS or poly-IC did not show $OVA_{257 - 264}^{-1}$ -specific CTL induction (Fig. 3D, F) or IFN- γ production from re-stimulated spleen cells (Fig. 3G).

Combination of NP-SIIN^{Hi} with poly-IC and anti-PD1 mAb further increases anti-tumor activity in EG7-OVA tumorbearing mice

The anti-tumor therapeutic potential of NP-SIIN^{Hi} in combination with both poly-IC and anti-PD1 mAbs was examined in EG7-OVA tumor-bearing mice. Mice were subcutaneously inoculated with EG7-OVA tumor cells, and 2 days later, the mice were intravenously injected three times with PBS, NP-SIIN^{Hi}, or NP-SIIN^{Hi} plus poly-IC at 7-day intervals with or without anti-PD1 mAbs. In groups treated with anti-PD1 mAbs, anti-PD1 mAbs were intraperitoneally administered six times before or 2 days after intravenous injection with PBS, NP-SIIN^{Hi}, or NP-SIIN^{Hi} plus poly-IC (Fig. 4A). Immunization with NP-SIIN^{Hi} plus poly-IC significantly inhibited tumor growth, and its combination with anti-PD1 mAbs increased the anti-tumor activity of NP-SIIN^{Hi} plus poly-IC (Fig. 4B).

On the last day of the experiment, $OVA_{257 - 264}$ -specific CTLs were measured in the spleen and lymph nodes. NP-SIIN^{Hi} and poly-IC combination significantly increased the percentage of $OVA_{257 - 264}$ -specific CTLs among CD8⁺ T cells compared to that with NP-SIIN^{Hi} alone in the spleens (10.3% vs 1.57%) (Fig. 4C, D) and lymph nodes (3.17% vs 1.44%) (Fig. 4E, F). However, the combination of anti-PD1 mAbs with NP-SIIN^{Hi} or NP-SIIN^{Hi} plus poly-IC did not further increase the percentage of $OVA_{257 - 264}$ -specific CTLs, although the addition of anti-PD1 mAbs significantly increased the anti-tumor activity (Fig. 4C-F). The combination with anti-PD1 mAbs did not affect the number of CTLs or the functional CTL activity. The IFN-γ-producing capacity of spleen cells re-stimulated with OVA_{257 - 264}-pulsed mature BMDCs was not increased by combination therapy with anti-PD1 mAbs, compared to that without anti-PD1 mAbs (Fig. 4G). These results suggest that the increased anti-tumor activity with the anti-PD1 mAb combination was due to the blockade of inhibitory signals. In fact, we found that EG7-OVA tumor cells expressed PD-L1, and the expression of PD-L1 was further increased by IFN-γ treatment (Supplemental Fig. 2A).

NPs loaded with melanoma TRP2 $_{180-188}$ peptide (NP-SVYD) in combination with poly-ICLC potently induces TRP2₁₈₀₋₁₈₈-specific CTLs in normal mice

The therapeutic efficacy of the tumor epitope-loaded NPs was extended to a melanoma mouse model. NP-K^b was first prepared using the methods described, and then, native peptides associated with H-2K^b molecules were exchanged with a melanoma-associated peptide, TRP2₁₈₀₋₁₈₈. Because a mAb recognizing the H-2K^b-TRP2₁₈₀₋₁₈₈ complex was not available, FITC-labeled TRP2₁₈₀₋₁₈₈ (FITC-TRP2₁₈₀-188) was used to optimize reactions, and the degree of FITC-TRP2₁₈₀₋₁₈₈ exchange was analyzed using flow cytometry. The peptide exchange efficiency was maximal when NP-K^b was treated with 100 µM FITC-TRP2₁₈₀₋₁₈₈ in the presence of 20 mM GM (Supplemental Fig. 3). The binding of unlabeled TRP2₁₈₀₋₁₈₈ to NP-K^b was confirmed by competitively blocking the binding of FITC-TRP2₁₈₀₋₁₈₈ to NP-K^b with unlabeled TRP2₁₈₀₋₁₈₈ (Fig. 5A).

The TRP2_{180 - 188}-specific CTL-inducing activity of NP-SVYD in combination with poly-ICLC, a synthetic derivative that stabilizes poly-IC through the addition of poly-L-lysine and carboxymethyl cellulose [33], was examined in mice. In this experiment, mice were intravenously injected twice with NP-K^b or NP-SVYD (1×10^9 particles/mouse) together with poly-ICLC at 5-day intervals. Seven days after the last immunization, TRP2_{180 - 188}-specific CTL activity in the spleen was analyzed using an *in vivo* CTL assay. Representative histograms are shown in Fig. 5B. Immunization with NP-SVYD plus poly-ICLC efficiently induced TRP2_{180 - 188}-specific CTL activity (specific target cell killing of 39.5%) compared to that with NP-K^b plus poly-ICLC immunization (specific target cell killing of 8.2%) (Fig. 5B, C).

NP-SVYD in combination with poly-ICLC and anti-PD1 mAb exerts potent anti-tumor activity in B16-F10 tumor-bearing mice

The anti-tumor therapeutic potential of NP-SVYD in combination with poly-ICLC, anti-PD1 mAb, or both was examined in B16-F10 tumor-bearing mice. Mice were subcutaneously inoculated with B16-F10 tumor cells, and 2 days later, the mice were intravenously injected with NP-SVYD plus poly-ICLC three times at 5-day intervals. Control mice were intravenously injected with PBS or NP-SVYD. In groups treated with anti-PD1 mAbs, anti-PD1 mAbs were intraperitoneally administered six times before or 1 day after intravenous injection with NP-SVYD or NP-SVYD plus poly-ICLC (Fig. 6A). Immunization with NP-SVYD significantly inhibited melanoma growth, and the anti-tumor activity of NP-SVYD was potently increased upon immunization in combination with poly-ICLC or anti-PD1 mAb. The most potent anti-tumor activity of NP-SVYD was observed when used in combination with both poly-ICLC and anti-PD1 mAb (Fig. 6B, C).

On the last day of the experiment, the degree of TRP2_{180 - 188}-specific CTL induction was measured in the spleen. The combination of NP-SVYD with poly-ICLC significantly increased the percentage of TRP2_{180 - 188}-specific CTLs among CD8⁺ T cells compared to that with NP-SVYD alone (2.68% vs 1.53%) (Fig. 6D). However, the combination of anti-PD1 mAbs with NP-SVYD or NP-SVYD plus poly-ICLC did not further increase the percentage of TRP2_{180 - 188}-specific CTLs, although the addition of anti-PD1 mAbs significantly increased anti-tumor activity (Fig. 6B, C). The combination of anti-PD1 mAbs did not affect the number of CTLs or the functional CTL activity. The IFN- γ -producing capacity of spleen cells restimulated with TRP2_{180 - 188}-pulsed mature BMDCs was not increased by the combination therapy with anti-PD1 mAbs, compared to that without anti-PD1 mAbs (Fig. 6E). These results suggest that the increased anti-tumor activity mediated by the anti-PD1 mAb combination is due to the blockade of inhibitory signals. In fact, we found that B16-F10 tumor cells expressed PD-L1, which was further increased by IFN- γ treatment (Supplemental Fig. 2B).

Combined NP-SVYD with poly-ICLC and anti-PD1 mAb potently reduces B16-F10 melanoma lung metastasis

The anti-metastatic activity of NP-SVYD combined with poly-ICLC and anti-PD1 mAb was examined in a mouse B16-F10 melanoma model. Mice were intravenously injected with B16-F10 tumor cells, and 1 day later the mice were intravenously injected with NP-SVYD alone or in combination with poly-ICLC three times at 5-day intervals. In mice treated with NP-SVYD plus poly-ICLC together with anti-PD1 mAbs, anti-PD1 mAbs were intraperitoneally administered six times before or 2 days after intravenous injection with NP-SVYD plus poly-ICLC (Fig. 7A). NP-SVYD Immunization significantly reduced the number of lung metastatic nodules, and the combination of NP-SVYD with poly-ICLC further reduced this number (Fig. 7B, C). The most potent anti-metastatic activity was observed in mice treated with NP-SVYD combined with poly-ICLC and anti-PD1 mAb.

Discussion

One of the major findings of the present study is that tumor peptide epitope-loaded NPs can efficiently induce tumor-specific CTLs when used to immunize tumor-bearing mice as well as normal mice. The CTL-mediated anti-tumor activity of the tumor epitope-loaded NPs was confirmed with two mouse tumor

models, EG7-OVA lymphoma and B16-F10 melanoma. Moreover, the combination of tumor epitopeloaded NPs with poly-IC, a TLR3 agonist, dramatically increased tumor-specific CTL-mediated anti-tumor activity. Furthermore, we showed that the combination of tumor epitope-loaded NPs with both poly-IC and anti-PD1 mAb elicited the most potent anti-tumor activity, although anti-PD1 mAb alone did not increase the generation of tumor-specific CTLs. The tumor epitopes used in this study were synthetic peptides that bind to the antigen-binding cleft of the MHC-I molecule, H-2K^b.

Tumor epitope-based vaccination strategies, aiming to elicit tumor-specific CTLs using synthetic tumor peptides binding to MHC-I molecules, could be an attractive means of tumor immunotherapy [17, 34], because short peptides have several advantages such as their low toxicity, high specificity, straightforward chemical synthesis, and cost-effectiveness, as compared to other immunotherapies [35]. However, most peptide epitope-based vaccines have demonstrated only a modest capacity to elicit tumor-specific CTLs in patients with limited therapeutic efficacy and disappointing clinical outcomes [36, 37]. The most critical drawback of MHC-I-binding epitope vaccines is that the epitopes could bind to a variety of cells expressing MHC-I molecules [14, 38]. For efficient CTL generation, peptide epitopes must be presented to CD8 T cells by professional APCs expressing co-stimulatory molecules, such as CD80 and CD86 [1, 9]. Further, the presentation of peptide epitopes in the absence of co-stimulatory signals can even induce T cell anergy or apoptosis [14]. Accordingly, the strategy developed in the present study would provide a novel approach that circumvents this issue (i.e., prompting the uptake of peptide epitope-loaded NPs by professional APCs is advantageous because peptide epitopes can be delivered specifically to professional APCs). In addition, peptide epitope pre-binding to H-2K^b in the NP-K^b formulation prevents rapid degradation of the peptide epitopes mediated by tissue and serum peptidases.

Immunization using short peptide epitopes typically generates unsatisfactory immune responses owing to the insufficient stimulation of innate immune components [18]. Hence, suitable delivery systems and additional stimuli, such as immune adjuvants, are necessary to efficiently induce innate immune responses against peptide epitopes [18, 23]. In this regard, our vaccination strategy using NPs coated with peptide epitopes has distinctive merits in that they can stimulate innate immune components. Our previous studies showed that NP phagocytosis itself induces the activation of innate immune cells, including DCs, as demonstrated by the increased expression of co-stimulatory molecules and cytokine production [39]. Furthermore, the NPs fabricated here can be phagocytosed specifically by cells expressing Fc receptors, because some of the Abs on NPs might be conjugated via the Ag-binding site, leaving the Fc region outside the NPs. Indeed, we have shown that Ab-conjugated NPs are much more efficient in upregulating co-stimulatory molecule expression and inducing pro-inflammatory cytokines in DCs than NPs unconjugated with Abs [29].

Immune adjuvants, such as poly-IC, activate innate immune components, including the optimization of antigen presentation, recruitment of DCs, and creation of a cytokine environment that supports the generation of CTLs [14]. Poly-IC induces DC activation leading to the upregulation of co-stimulatory signals required for the generation of tumor-specific CTLs [40]. We examined the CTL-inducing activity of the tumor epitope-loaded NPs in combination with poly-IC and showed that this combination could

further inhibit tumor growth by efficiently enhancing tumor-specific CTLs, compared to that with the NPs alone. Notably, poly-ICLC, instead of poly-IC, was used in the melanoma model which used NPs loaded with TRP2_{180 - 188}. Poly-ICLC is a synthetic derivative of poly-IC that prevents poly-IC degradation by RNases, thereby sustaining and enhancing its activity [33, 41].

ICI immunotherapy, which uses mAbs specific to co-inhibitory molecules, has emerged as one of the most promising approaches to invigorate anti-tumor immune responses [28]. However, anti-tumor immunotherapy using ICIs has some clinical limitations. Some patients exhibit complete and durable responses that prolong survival, whereas others do not respond to ICI immunotherapy at all [42]. Because ICIs block the co-inhibitory signaling induced by tumors, ICI immunotherapy will not work in patients without sufficient levels of pre-existing tumor-specific CTLs. For such patients, strategies to generate tumor-specific CTLs must be applied before ICI use [27]. Our vaccination strategy using tumor epitope-loaded NPs could overcome the shortcomings of anti-tumor immunotherapy using ICIs. Our results showed that the anti-tumor activity of tumor epitope-loaded NPs could be further enhanced when combined with anti-PD1 mAbs. However, the combination with anti-PD1 mAbs did not increase the generation of tumor-specific CTLs in tumor-bearing mice. This suggests that the anti-PD1 mAb used in combination with the NPs exerted its effect by blocking the inhibitory signals delivered by PD-L1 in tumors. We confirmed that tumor cells indeed expressed PD-L1.

PLGA is an extensively investigated biocompatible and biodegradable polymer approved by the US Food and Drug Administration for clinical applications in humans [43]. It is degraded via hydrolysis of the ester bonds into lactic and glycolic acid and is commonly used in the production of NPs that are investigated for the delivery of drugs and/or vaccine antigens [44, 45]. NPs prepared using PLGA, for which the particle size ranges from 500 nm to 2 μ m, are efficiently internalized by the phagocytosis of professional APCs [22, 46]. PLGA NPs with a size range below 200 nm are inappropriate for the delivery of antigens to APCs because they are minimally taken up through phagocytic recognition [47]. The average size of tumor epitope-loaded NPs fabricated in the present study was approximately 1,032 nm, which can be taken up by professional APCs. The average number of tumor epitopes loaded onto the NPs was not measured exactly; however, it could be estimated as a maximum of 1.56×10^4 /NP. In our previous study, we measured the average number of MHC-I molecules attached to NPs fabricated using the same methods described in this study and found that the number of MHC-I molecules immobilized per NP was 1.56×10^4 molecules/NP [29].

Conclusions

The vaccination strategy described here would provide a novel approach that circumvents most of the drawbacks of peptide epitope-based vaccines such as difficulties in delivering the peptides specifically to professional APCs, short peptide half-life *in vivo*, and limited peptide immunogenicity. This study comprises the development and testing of a novel strategy to elicit potent CTL responses against tumor epitopes, based on vaccination using tumor epitope-loaded NPs combined with poly-IC and an anti-PD1 mAb. The present study demonstrates that vaccination with tumor epitope-loaded NPs combined with

poly-IC and an anti-PD1 mAb is a novel strategy for efficient induction of tumor-specific CTLs for tumor immunotherapy.

Vaccination strategies using tumor peptide epitopes have attracted great attention as vaccines to induce tumor-specific CTLs, but there are

Abbreviations

APC antigen-presenting cell BMDC bone marrow-derived dendritic cell CFSE carboxyfluorescein succinimidyl ester CTL cytotoxic T lymphocyte DC dendritic cell EDC 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride GCha glycyl-cyclohexylalanine GL glycyl-leucine GM glycyl-methionine ICI immune checkpoint inhibitor mAb monoclonal antibody MHC-I major histocompatibility complex class I NP nanoparticle PBS phosphate buffered saline PEMA poly(ethylene/maleic anhydride) PLGA poly(lactic-co-glycolic acid) poly-IC polyinosinic-polycytidylic acid poly-ICLC poly-IC stabilized with poly-L-lysine and carboxy-methylcellulose TAA tumor-associated antigen

TCR T cell receptor

TLR Toll-like receptor

TSA tumor-specific antigen

Declarations

Acknowledgments

The authors would like to thank Jonathan Yewdell, M.D., Ph.D., Cellular Biology Section Laboratory of Viral Diseases NIAID, NIH, MD, USA, for his critical and productive comments.

Authors' contributions

Conception and design: S-HK and C-KL. Development of methodology: S-HK, J-KJ and C-KL. Performance of experiments: S-HK, J-HP, S-JL, and H-SL. Analysis and interpretation of data: S-HK, J-HP, and C-KL. Writing, review, and/or revision of the manuscript: S-HK, Y-RL, H-IC, J-KK, KK, C-SP, and C-KL. Technical, or material support: Y-RL and H-IC. Guarantors: C-SP, and C-KL. Study supervision: C-KL.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (NRF-2020R1A2C1009484, MRC-2017R1A5A2015541).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental animal procedures were performed in accordance with guidelines and regulations approved by the Institutional Animal Care and Use Committee of Chungbuk National University (CBNUA-1490-21-02).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Fig. 1



Comparison of the OVA257–264-loaded degree between NP-SIINLo and NP-SIINHi and the stability of H-2Kb-OVA257–264 complexes on the NP-SIINHi. **A** NP-Kbwas incubated with 100 μ M OVA257–264 in the presence or absence of 20 mM GL over 18 h at 37°C. **B** H-2Kb-OVA257–264 complexes on the NPs were stained using a mAb (Clone 25-D1.16) and analyzed by flow cytometry. NP-Kb loaded with OVA257–264 in the absence or presence of GL was denoted as NP-SIINLo or NP-SIINHi, respectively. **C** NP-SIINHiwas stored in PBS at 4°C. At indicated time points, H-2Kb-OVA257–264 complexes on the NP were stained using a mAb (Clone 25-D1.16) and analyzed by flow cytometry. The data are presented as the mean ± SD of at least three independent experiments. **p*<0.05, ****p*<0.001.

Fig. 2



NP-SIINHiefficiently induces OVA257–264-specific CTLs in normal mice. **A–H** PBS, NP-Kb, NP-SIINLo or NP-SIINHi (1 × 109 particles/mouse) was i.v. injected into C57BL/6 mice on days 0, 7, and 14. **A, B** Five days after priming, peripheral blood cells were stained with H-2Kb-OVA257–264tetramers and anti-mouse CD8α mAbs (Clone KT15) and analyzed using flow cytometry. **C, D** Five days after boosting, peripheral blood cells were stained anti-mouse CD8α mAbs. On day 21,

OVA257–264-specific CTL activity was assessed using an *in vivo* CTL assay. Target cells comprised a 1:1 mixture of syngeneic cells pulsed with OVA257–264 and then labeled with a high concentration of CFSE and syngeneic cells unpulsed and labeled with a low concentration of CFSE. Specific killing of the target cells was analyzed for the spleen (**E**, **F**) and lymph nodes (**G**, **H**). **I**–**P** NP-SIINHi (1 × 109 particles/mouse) with or without poly-IC was i.v. injected into C57BL/6 mice on days 0, 7, and 14. Five days after priming (**I**, **J**) and boosting (**K**, **L**), peripheral blood cells were stained with H-2Kb-OVA257–264 tetramer. On day 21, OVA257–264-specific CTL activity was assessed using an *in vivo* CTL assay. Specific killing of the target cells is shown for the spleen (**M**, **N**) and lymph node (**O**, **P**). Histograms are representatives of each experiment, and the bar data are presented as the mean \pm SD of at least three independent experiments (n=4–5 mice/group in each experiment). **p*<0.05, ***p*<0.01, ****p*<0.001.





Combination of NP-SIINHi and poly-IC exerts potent anti-tumor effects in EG7-OVA tumor-bearing mice with increased production of OVA257–264-specific CTLs. C57BL/6 mice were s.c. inoculated with EG7-OVA tumor cells (5 × 105 cells/mouse) on day 0. PBS, poly-IC, NP-SIINHi, or NP-SIINHi plus poly-IC (1 × 109 particles/mouse) was i.v. injected on days 2, 9, and 16. **A** Tumor volume was measured every 2 days. **B** Mice of each experimental group were sacrificed when the tumor volume reached 2,500 mm3, and

survival data were analyzed. The data are presented as the mean \pm SD of at least three independent experiments (n=6 mice/group in each experiment). Spleen cells (**C**, **D**) and lymph node cells (**E**, **F**) were stained with H-2Kb-OVA257–264 tetramers and anti-mouse CD8 α mAbs and analyzed using flow cytometry. **G** Spleen cells of each experimental group were also co-cultured with OVA257–264-pulsed mature BMDCs. After 3 days, IFN- γ production was analyzed using an ELISA. The data are presented as the mean \pm SD of at least three independent experiments (n=6 mice/group in each experiment). **p<0.01, ***p<0.001.





Combination of NP-SIINHi with poly-IC and anti-PD1 mAb further increases anti-tumor activity in EG7-OVA tumor-bearing mice. **A** C57BL/6 mice were s.c. inoculated with EG7-OVA tumor cells (5 × 105 cells/mouse) on day 0. PBS, NP-SIINHi or NP-SIINHi plus poly-IC (1 × 109 particles/mouse) was i.v. injected on days 2, 9, and 16. In groups treated together with anti-PD1 mAbs, anti-PD1 mAbs were i.p. administered six times before or 2 days after each NP immunization. **B** Tumor volume was measured every 2 days. Spleen cells (**C**, **D**) and lymph node cells (**E**, **F**) were stained with H-2Kb-OVA257–264 tetramers and anti-mouse CD8a mAbs and analyzed using flow cytometry. **G** Spleen cells of each experimental group were also co-cultured with OVA257–264-pulsed mature BMDCs. After 3 days, IFN-yproduction was analyzed using an ELISA. The data are presented as the mean ± SD of at least three independent experiments (n=6 mice/group in each experiment). **p*<0.05, ***p*<0.01, ****p*<0.001.



Figure 5

NP-SVYD in combination with poly-ICLC potently induces TRP2180–188-specific CTLs in normal mice. **A** Confirmation of the binding of TRP2180–188 to NP-Kb. NP-Kb was incubated with 0.1 μ M FITC-TRP2180–188 plus 0, 0.1, 1, 10, or 100 μ M unlabeled TRP2180–188 in the presence of 20 mM GM for 18 h at 37°C. The relative amounts of FITC-TRP2180–188 bound to NP-Kb were analyzed using flow cytometry. **B**, **C** PBS, NP-Kb or NP-SVYD plus poly-ICLC (1 × 109 particles/mouse) was i.v. injected into C57BL/6 mice on days 0, 5, and 10. On day 17, TRP2180–188-specific CTL activity was assessed using an *in vivo* CTL assay. The target cells comprised a 1:1 mixture of syngeneic cells pulsed with TRP2180-188 and then labeled with a high concentration of CFSE and syngeneic cells unpulsed and labeled with a low concentration of CFSE. The data are presented as the mean ± SD of at least three independent experiments (n=6 mice/group in each experiment). **p*<0.05, ***p*<0.01, ****p*<0.001.





NP-SVYD in combination with poly-ICLC and anti-PD1 mAb exerts potent anti-tumor effects in B16-F10 tumor-bearing mice. **A** C57BL/6 mice were s.c. inoculated with B16-F10 tumor cells (1 × 105 cells/mouse) on day 0. PBS, NP-SVYD or NP-SVYD plus poly-ICLC (1 × 109 particles/mouse) was i.v. injected on days 2, 7, and 12. In groups treated together with anti-PD1 mAbs, anti-PD1 mAbs were i.p. administered six times before or 2 days after each NP immunization. **B**, **C** Tumor volume was measured every 2 days. **D**Spleen cells were stained with H-2Kb-TRP2180–188 tetramers and anti-mouse CD8α mAbs and analyzed using flow cytometry. **E** Spleen cells of each experimental group were also co-cultured with TRP2180–188-pulsed mature BMDCs. After 3 days, IFN-γ production was analyzed using an ELISA. The data are presented as the mean ± SD of at least three independent experiments (n=6 mice/group in each experiment). **p*<0.05, ***p*<0.01, ****p*<0.001.

Fig. 7



Combination of NP-SVYD with poly-ICLC and anti-PD1 mAb potently reduces B16-F10 melanoma lung metastasis. **A** C57BL/6 mice were i.v. injected with B16-F10 tumor cells (1 × 105 cells/mouse) on day 0. NP-SVYD or NP-SVYD plus poly-ICLC (1 × 109 particles/mouse) was i.v. injected on days 1, 6, and 11. Anti-PD1 mAbs were i.p. administered six times before or 2 days after each NP immunization. **B** Representative images of the lungs from each group. **C** The number of metastatic nodules on the surface of the lung was counted on day 16. The data are presented as the mean ± SD of at least three independent experiments (n=8 mice/group in each experiment). ****p*<0.001.

Supplementary Files

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