ABSTRACT

Tumor peptides associated with MHC class I molecules or their synthetic variants have attracted great attention for their potential use as vaccines to induce tumor-specific CTLs. However, the outcome of clinical trials of peptide-based tumor vaccines has been disappointing. There are various reasons for this lack of success, such as difficulties in delivering the peptides specifically to professional Ag-presenting cells, short peptide half-life in vivo, and limited peptide immunogenicity. We report here a novel peptide vaccination strategy that efficiently induces peptide-specific CTLs. Nanoparticles (NPs) were fabricated from a biodegradable polymer, poly(D,L-lactic-co-glycolic acid), attached to H-2Kb molecules, and then the natural peptide epitopes associated with the H-2Kb molecules were exchanged with a model tumor peptide, SIINFEKL (OVA257-268). These NPs were efficiently phagocytosed by immature dendritic cells (DCs), inducing DC maturation and activation. In addition, the DCs that phagocytosed SIINFEKL-pulsed NPs potently activated SIINFEKL-H-2Kb complex-specific CD8+ T cells via cross-presentation of SIINFEKL. In vivo studies showed that intravenous administration of SIINFEKL-pulsed NPs effectively generated SIINFEKL-specific CD8+ T cells in both normal and tumor-bearing mice. Furthermore, intravenous administration of SIINFEKL-pulsed NPs into EG7.OVA tumor-bearing mice almost completely inhibited the tumor growth. These results demonstrate that vaccination with polymeric NPs coated with tumor peptide-MHC-I complexes is a novel strategy for efficient induction of tumor-specific CTLs.

Keywords: Polymeric nanoparticle; Tumor peptide; Peptide-MHC-I complex; Tumor vaccine; Cytotoxic T lymphocyte; Anti-tumor activity
INTRODUCTION

Many tumors express peptide antigens, which are typically 8 to 10 amino acids long and are presented in association with MHC-I molecules (1). Mounting evidence indicates that the induction of tumor peptide-specific CTLs is a critical factor for successful anti-tumor immunotherapy (2). Tumor peptides have been classified into two categories: tumor-associated Ags, which are over-expressed in tumors compared to normal tissues, and tumor-specific Ags, which are exclusively expressed in tumors and not in non-malignant tissues (3-6).

The tumor peptides associated with MHC-I molecules or their synthetic variants have attracted great attention for their potential use in inducing tumor-specific CTLs (7-10). However, the results of clinical trials using peptide vaccines have been disappointing (11). Currently, more than 66 clinical trials have been registered in ClinicalTrials.gov, yet no peptide-based vaccines have been approved by the Food and Drug Administration of the United States. One of the major limitations of peptide vaccines is that the peptide itself has limited immunogenicity and generates weak cellular responses (12). To increase the immunogenicity of peptide vaccines, strategies in which the peptides are combined with adjuvants, such as TLR agonist and anti-CD40 mAb, have been developed with some success (8,12-15). Yet another critical limitation is that upon in vivo administration, the peptides may indiscriminately bind to MHC-I molecules expressed on the surface of numerous types of cells apart from the professional Ag-presenting cells (APCs). Activation of naïve CD8+ T cells requires at least two distinct signals delivered from professional APCs. The first signal is generated by the antigenic peptide-MHC-I complexes that interact with TCRs, and the second, by costimulatory molecules such as CD80 and CD86 that interact with CD28 on T cells (16-20). Interaction between peptide-MHC-I complexes and TCR in the absence of the second signal induces T cell anergy or apoptosis, rather than stimulation (18). Thus, the binding of in vivo-administered peptides to MHC-I molecules expressed in non-APCs would critically limit the effectiveness of the peptide vaccine.

We report here a novel peptide vaccination strategy to efficiently induce peptide-specific CTLs in vivo. The essence of this vaccine strategy is the fabrication of biodegradable nanoparticles (NPs) coated with MHC-I-binding peptide epitopes. The peptides attached to the NPs are delivered specifically to phagocytic cells, including DCs, which are cross-presented to CD8+ T cells to efficiently induce peptide-specific CTLs. We believe this peptide vaccination strategy involving NPs could be a novel means to overcome the several limitations encountered with free-form peptide vaccination, such as the binding of the peptides to MHC-I molecules expressed in non-professional APCs, poor immunogenicity, rapid degradation of peptides by tissue and serum peptidases, and poor DC activation.

MATERIALS AND METHODS

Animals
Female C57BL/6 mice (8-12 weeks old) were purchased from KosaBio Inc. (Seongnam, Korea). All experimental procedures involving animals were approved by the Animal Care Committee of Chungbuk National University (CBNUA-1490-21-02), and performed in accordance with the guidelines and regulations.
Preparation of carboxylated NPs

Carboxylated NPs were prepared using a solvent evaporation method. Briefly, 8 ml of 1% poly(ethylene/maleic anhydride) (PEMA; Polysciences Inc., Warrington, PA, USA) aqueous solution was mixed with 4 ml of 5% poly(D, L-lactic-co-glycolic acid) (PLGA; Evonik Industries, Essen, Germany) dissolved in ethyl acetate. The mixture was then homogenized at 14,000 rpm for 4 min to form a water-in-oil (W/O) emulsion. To solidify the NPs, the W/O emulsion was transferred to a beaker containing 0.2% PEMA and 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MS, USA) and then stirred for approximately 18–24 h. The resulting NPs were centrifuged at 3,500×g for 20 min at 4°C, washed twice with distilled water, and resuspended in 10 ml of sterile distilled water. Fluorescence-labeled NPs were prepared by adding FITC (Sigma-Aldrich) to the ethyl acetate.

Isolation of membrane proteins containing H-2Kb

Membrane proteins containing H-2Kb (Kb) were isolated from an EG7.OVA tumor mass. Briefly, 1 g of EG7.OVA tumor mass was dissociated with 10 ml of extraction buffer containing 0.15 M NaCl, 0.05 M sodium phosphate buffer (pH 7.0), and 1X protease inhibitor cocktail (Sigma-Aldrich) using gentiMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). After removing cell debris and lipids, the supernatant was ultra-centrifuged at 80,000 ×g for 45 min at 4°C using Ultracentrifuge (Beckman Coulter, Brea, CA, USA). The pellet was homogenized in 0.5 ml of extraction buffer using Tissue Grinder (SHS-30E; SciLab, Seoul, Korea). The homogenate was then mixed with 0.5 ml of solubilization buffer containing 2% n-dodecyl β-D-maltoside and 0.4% 3-[3-cholamidopropyl]dimethyl ammonio]-1-propanesulfonate hydrate (Sigma-Aldrich) in extraction buffer and solubilized for 90 min at 4°C. The supernatant containing membrane proteins was obtained by centrifugation at 17,000 rpm for 30 min at 4°C. The amount of isolated membrane proteins was determined using a microbicinchoninic acid assay kit (ThermoFisher Scientific, Rockford, IL, USA).

Preparation of anti-mouse Kβ mAb-conjugated NPs and tumor-derived Kβ-attached NPs

Anti-Kβ mAbs were conjugated to the carboxylated NPs by using the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; ThermoFisher Scientific)/N-hydroxysuccinimide (NHS; Sigma-Aldrich) coupling method. Briefly, 10 mg of carboxylated NPs was resuspended in 1.2 ml of 50 mM activation buffer (4-morpholineethanesulfonic acid buffer, pH 6.0, Sigma-Aldrich) and treated with 4 μL of 300 mM EDC and 8 μL of 300 mM NHS for 1 h at 24–26°C. The EDC-activated NPs were washed twice with 50 mM activation buffer and then resuspended in 1 ml of 50 mM activation buffer containing 600 μg/ml anti-mouse Kβ mAbs (Clone Y-3; BioXcell, Lebanon, NH, USA) for 18 h at room temperature. The Ab-conjugated NPs were treated with 30 μL of ethanolamine (Sigma-Aldrich) for 30 min to quench the reaction of free EDC-activated carboxyl residues and washed twice with PBS. To attach Kβ, Ab-conjugated NPs were incubated with tumor-derived membrane proteins for 24 h at 4°C. The Kβ-attached NPs were washed twice with PBS and resuspended in 1 ml of PBS.

Characterization of carboxylated NPs and Kβ-attached NPs

The mean size and ζ-potential of carboxylated NPs were measured using a particle size analyzer (ELS-Z, Otsuka, Japan). The morphology of carboxylated NPs was visualized by scanning electron microscopy (LEO-1530, Carl Zeiss, Germany). The particle number of NPs was counted using qNano Gold (IZON Science, Christchurch, New Zealand). Kβ-attached NPs were stained with anti-mIgG (BioLegend, San Diego, CA, USA) and anti-Kβ (BD Biosciences,
San Jose, CA, USA) mAbs for 30 min and analyzed using flow cytometry (FACS Canto II, BD Biosciences). Flow cytometry data was analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

**Preparation of bone marrow-derived DC (BMDC)**
DCs were generated as described previously (21). Briefly, the bone marrow cells obtained from mouse femurs were cultured in 6-well plates (5×10^6 cells/well) in a culture medium with 40 ng/ml GM-CSF and 20 ng/ml IL-4 (Creagene, Seongnam, Korea). On days 3 and 4, non-adherent cells were removed by gently shaking the dish, and the medium was replaced. The cells, referred to as immature BMDC, were harvested on day-6 by gentle pipetting and used for further experiments.

**Phagocytosis analysis**
The FITC-labeled NPs (2×10^9 particles/well) were added to a mouse DC cell line, DC2.4 cells (2×10^6 cells/well), in 6-well plate. After 2 h, the non-phagocytosed NPs were removed by washing twice with pre-warmed PBS. The cells were harvested, fixed with 1% paraformaldehyde in PBS, and analyzed using flow cytometry. For confocal analysis, 50 nM LysoTracker Red DND-99 (Thermo Fisher Scientific) together with the FITC-labeled NPs (5×10^8 particles/well) were added to the DC2.4 cells (5×10^5 cells/well) in 6-well plate. After 2 h, the non-phagocytosed NPs were removed by washing twice with pre-warmed PBS. The cells were fixed with 4% paraformaldehyde in PBS, mounted with Antifade Mounting Medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA), and visualized using a confocal microscope (K1-fluo; Nanoscope Systems Inc., Daejeon, Korea).

**Phenotype analysis**
Immature BMDCs were seeded in 24-well plates (1×10^6 cells/well) and stimulated with 100 ng/ml LPS or treated with the NPs (1×10^9 particles/well) for 24 h. BMDCs were harvested and stained with mAbs to detect the mouse cell surface markers (CD11c, H-2K^b, I-A^b, CD80, and CD86) and isotype-matched control Abs (BD Biosciences), as described previously (21).

**Cytokine production analysis**
Immature BMDCs were seeded in 24-well plates (1×10^6 cells/well) and stimulated with 100 ng/ml LPS or treated with the NPs (1×10^9 particles/well). After 24 h, the culture supernatants were harvested, and the amounts of IL-1β, IL-6, and TNF-α were measured using commercial ELISA kits (BD Biosciences).

**Peptide exchange reaction in K^b-attached NPs**
To exchange the native peptide with SIINFEKL (Peptron, Daejeon, Korea), 5 mg of K^b-attached NPs was incubated with 20 µM or 100 µM SIINFEKL for 18 h at 37°C, washed twice with PBS, and resuspended in 0.5 ml of PBS. The level of SIINFEKL bound to the K^b-attached NPs was analyzed with mAbs recognizing the SIINFEKL-K^b complex (25-D1.16; BioLegend).

**MHC-I-restricted cross-presentation assay**
DC2.4 cells (1×10^5 cells/well) were incubated with the NPs (1×10^8 particles/well) in 96-well plate for 2 h, washed with pre-warmed PBS, fixed with 1% paraformaldehyde, and washed again with PBS. The SIINFEKL-specific CD8^+ T cell stimulatory capacity of the DC2.4 cells was measured using SIINFEKL-specific CD8^+ T cell hybridoma CD8OVA cells (2×10^5 cells/well), which recognize SIINFEKL-K^b complexes and secrete IL-2 in response, as previously described (22).
In vivo generation of SIINFEKL-specific CD8+ T cells
C57BL/6 mice were intravenously immunized with PBS or the NPs (1×10^9 particles/mouse) on days 0 and 7. On days 5 and 12, whole blood samples were collected from the facial vein, stained with T-Select H-2Kb OVA Tetramer (MBL, Tokyo, Japan) and mouse CD8a mAb (Clone KT15; BioLegend), and analyzed using flow cytometry, according to the manufacturer’s instructions.

In vivo SIINFEKL-specific CTL assay
C57BL/6 mice were intravenously immunized with PBS or the NPs (1×10^9 particles/mouse) on days 0 and 7. On day 14, SIINFEKL-specific CTL activity was assessed by an in vivo CTL assay, as described previously (21). Target cells were syngeneic splenocytes pulsed with 1 μM SIINFEKL and labeled with 25 μM CFSE. Syngeneic splenocytes labeled with 5 μM CFSE without SIINFEKL pulsing were used as control target cells. The specific killing of SIINFEKL-pulsed target cells was determined by analyzing spleen cells and lymph node cells isolated from each recipient mouse via flow cytometry.

Anti-tumor activity
The right flank of each C57BL/6 mouse was shaved and subcutaneously inoculated with EG7.OVA cells (3×10^5 cells/mouse). Mice were then intravenously administered the indicated NP preparations (1×10^9 particles/mouse) on days 2, 9, and 16. The tumor was measured every 2 days using calipers, and the tumor volume was calculated using the following formula: tumor volume [mm^3] = 0.52 × (long diameter [mm]) × (short diameter [mm])^2.

Statistical analysis
A two-tailed paired Student’s t-test was performed for single comparisons of two groups after an evaluation for normality. One-way or two-way ANOVA with a post-hoc Tukey’s test were performed to determine significant differences among multiple groups; p ≤ 0.05 was considered statistically significant.

RESULTS
Fabrication and characterization of Kb-attached NPs
Carboxylated NPs were fabricated with PLGA and PEMA using the W/O emulsion solvent evaporation method. The mean size of the carboxylated NPs was 767.0±79.9 nm (Fig. 1A), which falls within the optimal size range for DC uptake (23,24). The average polydispersity index and ζ-potential of the carboxylated NPs were 0.258±0.021 and −38.61±0.35 mV, respectively (Fig. 1A, Supplementary Table 1). The carboxylated NPs had a spherical shape with a relatively homogeneous size distribution, as shown by scanning microphotography (Fig. 1B).

The carboxylated NPs (bare NPs) were then covalently coupled with anti-Kb mAbs (isotype mIgG2b) by carbodiimide conjugation. The Ab-conjugated NPs (NP-Ab) were incubated with solubilized membrane proteins isolated from the EG7.OVA tumor mass (H-2^b) to prepare Kb-attached NPs (NP-Ab-Kb). Attachment of Ab and Kb molecules to NPs was confirmed by flow cytometry after staining with PE-labeled anti-mIgG and FITC-labeled anti-Kb mAb. The NPs were successfully coated with anti-Kb (98.2%) and Kb (83.9%) (Fig. 1C). An increase in the average size (767.0±79.9 to 903.1±32.2 nm) and a decrease in the ζ-potential (−38.61±0.35 to −18.68±3.64 mV) indicated that Ab and Kb were immobilized on the NP surface in expense of carboxyl residues (Supplementary Table 1).

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NP-Ab-Kb in PBS was stable for at least 9 days, when stored at 4°C, with respect to average particle size, \( \zeta \) -potential, and the attachment of both Ab and Kb molecules (Fig. 1D, E, and F). The number of Kb molecules immobilized per NP was estimated to be \( 1.56 \times 10^4 \), as determined using NP-Ab-Kb which were prepared with the same methods used for the fabrication of NP-Ab-Kb (Supplementary Table 2).

**DCs efficiently phagocytose NP-Ab-Kb**

To examine the efficiency of NP-Ab-Kb uptake by DCs, we prepared carboxylated NPs containing FITC (NP[FITC]) and then generated NP[FITC]-Ab and NP[FITC]-Ab-Kb. Each type of NP was added to cultures of DC2.4 cells for 2 h and washed with PBS, and the degree of phagocytosis was analyzed using flow cytometry. DC2.4 cells phagocytosed NP[FITC]-Ab and NP[FITC]-Ab-Kb much more efficiently compared to NP[FITC] phagocytosis (Fig. 2A). The MFI values of DC2.4 cells treated with NP[FITC]-Ab and NP[FITC]-Ab-Kb were 15.2-fold and 11.6-fold higher, respectively, than that of DC2.4 cells treated with NP[FITC] control (Fig. 2B). It is reasonable to speculate that the increased phagocytosis of NP[FITC]-Ab and NP[FITC]-Ab-Kb is due to the opsonization effect of the Ab. The NP[FITC]-Ab was fabricated so that the Fc fragment of Ab attached to the NP, leaving the Ag-binding site exposed outside (25). However, some of the Abs could be conjugated via the Ag-binding site, leaving the Fc region exposed outside. The relative decrease in the phagocytosis of NP[FITC]-Ab-Kb compared to that of NP[FITC]-Ab might be explained by the steric hindrance of the Fc by the Kb attached nearby.

Internalization of the NPs was confirmed by confocal imaging. The colocalization of NPs and lysosomes appeared as yellow fluorescence upon overlaying green fluorescent NPs and red fluorescent lysosomes (Fig. 2C).

**Figure 1.** Characterization of NP-Ab-Kb. (A) The average size and \( \zeta \) -potential of bare NPs, as determined by a particle size analyzer, were 767.0±79.9 nm and -38.61±0.35 mV, respectively. (B) Scanning electron microscopy image showing bare NPs with spherical shapes and a relatively homogeneous size distribution. (C) Attachment of Ab and Kb molecules to NPs was confirmed by flow cytometry after staining with fluorochrome-labeled anti-mIgG and anti-Kb. Stability of NP-Ab-Kb with respect to: (D) average particle size, (E) \( \zeta \) -potential, and (F) the attachment of both Ab and Kb molecules, for at least 9 days. In E and F, the data are presented as mean ± SD of three independent experiments.
Phagocytosis of NP-Ab-K\textsuperscript{b} induces maturation and activation of immature DCs

To investigate the effects of NP-Ab-K\textsuperscript{b} on the maturation and cytokine production of DCs, immature BMDCs were exposed to NP-Ab-K\textsuperscript{b} for 24 h. Control DCs were treated with PBS, bare NP, or NP-Ab, or matured with LPS. Phenotypic analysis of the DCs showed that NP-Ab-K\textsuperscript{b}-treated BMDCs expressed much higher levels of CD80, CD86, and MHC-II (I-A\textsuperscript{b}) molecules, all of which are associated with Ag-specific T cell priming, than PBS-treated immature BMDCs (Fig. 3A). The phenotypic maturation-inducing activity appeared to be stronger in the order of bare NP, NP-Ab-K\textsuperscript{b}, NP-Ab, and LPS. BMDCs treated with NP-Ab-K\textsuperscript{b} produced much higher amounts of the pro-inflammatory cytokines IL-1\textbeta, IL-6, and TNF-\alpha than PBS-treated immature BMDCs (Fig. 3B). The cytokine-inducing activity of NP-Ab-K\textsuperscript{b} was higher than that of bare NP, but significantly lower than that of NP-Ab. These results align with the NP phagocytic activity of the DCs (Fig. 2). As expected, NP-Ab-K\textsuperscript{b}, which was fabricated with PLGA and PEMA, did not exert cytotoxic effects on DCs. Conversely, NP-Ab-K\textsuperscript{b} induced DC proliferation when added to the cultures for 24 h (Supplementary Fig. 1).

SIINFEKL-pulsed NP-Ab-K\textsuperscript{b} efficiently induces MHC-I-restricted cross-presentation of SIINFEKL in DCs

The natural peptide epitopes associated within the K\textsuperscript{b} molecules of NP-Ab-K\textsuperscript{b} were exchanged with a model tumor-specific peptide, SIINFEKL. NP-Ab-K\textsuperscript{b} was incubated with 0, 20, or 100 \muM of SIINFEKL for 2 to 18 h at 37\degree C, and then the relative degree of the SIINFEKL exchange reaction was assessed using an mAb (Clone 25-D1.16) that recognizes the SIINFEKL-K\textsuperscript{b} complexes. The SIINFEKL exchange reaction was maximal when NP-Ab-K\textsuperscript{b} was incubated with 100 \muM SIINFEKL for 18 h (Fig. 4).
The cross-priming capacity of the DCs treated with SIINFEKL-pulsed NP-Ab-Kb was investigated using an MHC-I-restricted cross-presentation assay. DC2.4 cells were incubated with SIINFEKL-pulsed NP-Ab-Kb for 2 h, washed and fixed, and then co-cultured with SIINFEKL-specific CD8+ T cell hybridoma, CD8OVA cells, which recognize SIINFEKL-Kb complexes and secrete IL-2. DCs treated with SIINFEKL-pulsed NP-Ab-Kb potently activated CD8OVA cells, and the activation-inducing activity was dependent on the SIINFEKL concentration used for the peptide exchange reaction (Fig. 5A). DCs treated with SIINFEKL-unpulsed NP-Ab-Kb also activated CD8OVA cells, although the potency of activation was extremely low compared to that of SIINFEKL-pulsed NP-Ab-Kb (Fig. 5B). It is noteworthy that Kb molecules were isolated from EG7.OVA tumor tissue; thus, some of the Kb molecules were naturally complexed with SIINFEKL.

**SIINFEKL-pulsed NP-Ab-Kb induces SIINFEKL-specific CTLs in normal and tumor-bearing mice**

Specific CTL-inducing activity of SIINFEKL-pulsed NP-Ab-Kb was first examined in normal mice. The optimal particle number of SIINFEKL-pulsed NP-Ab-Kb for inducing SIINFEKL-specific CTLs was 1×10⁹ particles/mouse (Supplementary Fig. 2). Accordingly, mice were i.v. injected with SIINFEKL-pulsed NP-Ab-Kb (1×10⁹ particles/mouse) twice, with an interval of 7 days. Control mice were i.v. injected with PBS or bare NP. Five days after the last immunization, peripheral blood was collected from each mouse by cheek bleeding, and SIINFEKL-specific CTLs were analyzed using SIINFEKL-loaded Kb tetramers. Immunization of mice with SIINFEKL-pulsed NP-Ab-Kb potently induced SIINFEKL-specific CTLs (Fig. 6A). The percentage of SIINFEKL-specific CTLs among CD8+ T cells reached 1.8% when 20 μM SIINFEKL-pulsed
NP-Ab-Kb was injected and further increased to 2.5% when 100 μM SIINFEKL-pulsed NP-Ab-Kb was injected (Fig. 6B).

Seven days after the last immunization, SIINFEKL-specific CTL activity was assessed by an in vivo CTL assay using CFSE-labeled syngeneic target cells. Representative histograms are shown in Fig. 6C. The percent specific killing of SIINFEKL-pulsed target cells was 29.7%
when 20 μM SIINFEKL-pulsed NP-Ab-Kb was injected and further increased to 62.7% when 100 μM SIINFEKL-pulsed NP-Ab-Kb was injected (Fig. 6D).

Specific CTL-inducing activity of SIINFEKL-pulsed NP-Ab-Kb was also examined in tumor-bearing mice. Starting 2 days after s.c. inoculation with EG7.OVA tumor cells, the mice received three i.v. immunizations with SIINFEKL-pulsed NP-Ab-Kb at 7-day intervals. Control mice received PBS or bare NP. Five days after the first immunization (post-priming) and the last immunization (post-boosting), peripheral blood was collected from each mouse by cheek bleeding, and SIINFEKL-specific CTLs were analyzed using SIINFEKL-loaded Kb tetramers. Immunization of tumor-bearing mice with SIINFEKL-pulsed NP-Ab-Kb potently induced SIINFEKL-specific CTLs (Fig. 7A and C). In mice injected with 100 μM SIINFEKL-pulsed NP-Ab-Kb, the relative frequency of SIINFEKL-specific CTLs among CD8+ T cells was 1.8% in the post-priming group and further increased to 3.4% in the post-boosting group (Fig. 7B and D).

**SIINFEKL-pulsed NP-Ab-Kb exerts potent anti-tumor activity in mice bearing EG7.OVA tumors**

The anti-tumor therapeutic potential of SIINFEKL-pulsed NP-Ab-Kb was examined in EG7.OVA tumor-bearing mice. Starting 2 days after s.c. inoculation with EG7.OVA tumor cells, the mice received three i.v. injections of SIINFEKL-pulsed NP-Ab-Kb at 7-day intervals. Control mice received PBS or bare NP. Injection of mice with bare NP had no noticeable impact on tumor growth. In marked contrast, injection of mice with SIINFEKL-pulsed NP-Ab-Kb exerted potent anti-tumor activity (Fig. 8), resulting in almost complete tumor regression in mice injected with 100 μM SIINFEKL-pulsed NP-Ab-Kb.
DISCUSSION

MHC-I-binding tumor peptide vaccines have several advantages over other immunotherapies, such as immune checkpoint inhibitors and CAR T cell therapy, because short peptides are
easy to synthesize and apply in clinical practice, are cost-effective, and are unlikely to induce
Ag-induced anaphylaxis (8,12,26). However, most clinical studies using peptide vaccines have
been disappointing. The challenges with therapeutic peptide vaccines include their poor
immunogenicity and poor DC activation, binding to various cell types other than professional
APCs, rapid degradation by tissue and serum peptidases, and an immunosuppressive tumor
environment that prevents CTL induction (12,27). The vaccination strategy described here
would provide a novel approach that circumvents most of these issues.

Notably, following in vivo administration, the tumor peptide-pulsed NP-Ab-Kb were efficiently
phagocytosed by phagocytes including DCs, most probably due to opsonization by some of
the Abs. Although NP-Ab was fabricated to facilitate ‘tail-on’ attachment of the Ab to the
NP leaving the Ag-binding site (25), some of the Abs were conjugated via the Ag-binding
site outside of the Fc region, allowing uptake by Fc-receptor-positive DCs. Prompt uptake of
tumor peptide-pulsed NP-Ab-Kb by DCs is distinctively advantageous because tumor peptides
can be delivered specifically to DCs. In addition, pre-binding of the peptides to Kb in the NP-
Ab-Kb formulation prevents their binding to other Kb molecules expressed on non-APCs.

Phagocytosis of NPs can activate DC maturation and cytokine production (21,28). In line
with this, we showed that uptake of NP-Ab-Kb by immature DCs led to their maturation
accompanied by increased production of pro-inflammatory cytokines, such as IL-1β, IL-6,
and TNF-α. In addition, following phagocytosis of tumor peptide-pulsed NP-Ab-Kb, DCs
efficiently presented the tumor peptide in association with Kb molecules. It has been well-
documented that cross-presentation of tumor peptides by professional APCs, particularly
DCs, is critical for efficient generation of tumor peptide-specific CTLs (1,6,29-33). Direct
presentation of tumor peptide-MHC-I complexes by tumor cells does not efficiently
stimulate the CTLs, although it is crucial for tumor recognition by CTLs in the effector/
cytolytic phase (34,35).

PLGA is a biocompatible and biodegradable polymer that is hydrolyzed to lactic and glycolic
acids in the body and has been broadly used in pharmaceutical products as a drug carrier
(36,37). PLGA-NPs are generally prepared using PVA as a particle stabilizer (38). In the
present study, we substituted PVA with PEMA to generate carboxylated NPs. Next, anti-Kb
mAb was covalently coupled to the NPs using a carbodiimide coupling reaction between the
hydrophilic carboxyl side chains of NPs and the primary amine side chains of Abs (38,39).
This NP-Ab was then incubated with solubilized membrane proteins isolated from a tumor
mass (H-2b) to produce NP-Ab-Kb. These NPs were stable for at least 9 days at 4°C with
respect to particle size, ζ-potential, and the attachment of both the Ab and Kb molecules.
The stability of NP-Ab-Kb is crucial for its clinical application, as it allows for advanced
conjugation of NPs with anti-HLA-I Abs and storage at 4°C while the patient’s tumor tissue
is processed for isolation of HLA-I molecules. Once the patient’s tumor mass is obtained, it
takes just 2 days to prepare NP-Ab-HLA-I using solubilized membrane proteins isolated from
the tumor and pulse it with the appropriate tumor peptides via peptide exchange reaction.

Injection of mice with SIINFEKL-pulsed NP-Ab-Kb exerted potent anti-tumor activity,
resulting in almost complete tumor regression in mice implanted with EG7.OVA tumor
cells. Because Kb molecules were isolated from EG7.OVA tumor cells, which are mouse
thymoma EL4 cells stably transfected with the cDNA of OVA and thus express OVA epitopes
in association with Kb, we expected that injection of mice with SIINFEKL-unpulsed NP-Ab-Kb
also exerts anti-tumor activity. However, mice injected with SIINFEKL-unpulsed NP-Ab-Kb

did not exert discernable anti-tumor activity. This indicates that injection of NPs attached with endogenous SIINFEKL-Kb complexes are not enough to inhibit tumor growth.

This study was performed in only one tumor model using an immunogenic peptide as vaccine candidate. Future studies are warranted to test this vaccine strategy using more diverse peptide antigens in less immunogenic tumor models. Nevertheless, the peptide vaccination strategy described in this study may be applicable for other purposes, such as virus-specific CTL induction using a viral peptide, as long as MHC-I binding peptide epitopes are identified. We believe that this study lays the groundwork for efficient peptide-specific CTL induction in vivo.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Summary of the characteristics of the NPs
Click here to view

Supplementary Table 2
Determination of the number of immobilized H-2Kd molecules per NP
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Supplementary Figure 1
Effects of NP-Ab-Kb on DC viability. (A) DC2.4 cells (5 × 10^4 cells/well) were incubated with bare NP or NP-Ab-Kb (6.25×10^5, 1.25×10^6, 2.5×10^6, or 5×10^6 particles/well) in 96-well plate for 24 h. The plate was washed twice with pre-warmed PBS, and 100 μL/well of complete culture medium without 2-ME was added. The cells were then incubated with 10 μL/well of WST-8 solution for 4 h. Absorbance was measured at 450 nm using a microplate reader. (B) BMDCs (5×10^5 cells/well) were incubated with bare NP or NP-Ab-Kb (6.25×10^5, 1.25×10^6, 2.5×10^6, or 5×10^6 particles/well) in a 96-well plate for 24 h. Cell viability was measured as described in (A).
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Supplementary Figure 2
Determination of optimal dose of NP-Ab-Kb. C57BL/6 mice were i.v. immunized with PBS or SIINFEKL-pulsed NP-Ab-Kb (4×10^7, 2×10^7, or 1×10^7 particles/mouse) on days 0 and 7. On day 5 (A) and on day-12 (B), whole blood samples were collected, stained with SIINFEKL-Kb tetramer and anti-mouse CD8a mAb, and analyzed using flow cytometry. The dot plot shows the relative frequency of SIINFEKL-specific CD8+ T cells in each experimental group. The data are presented as the mean ± SD of at least two independent experiments (n=5 mice/group in each experiment).
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