Tumor growth inhibition by mSTEAP peptide nanovaccine inducing augmented CD8⁺ T cell immune responses

Qiuqiang Chen^{1,2} · Ying Bao¹ · Danielle Burner³ · Sharmeela Kaushal³ · Yu Zhang^{4,5} · Theresa Mendoza³ · Michael Bouvet³ · Cengiz Ozkan⁴ · Boris Minev^{3,6} · Wenxue Ma^{3,2}

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Abstract

Poly(lactic-co-glycolic) acid (PLGA) has been successfully used in drug delivery and biomaterial applications, but very little attention has been directed towards the potential in vivo effects of peptide-loaded PLGA nanoparticles (NPs), specifically the potency of intravenous (IV) STEAP peptide-loaded PLGA-NP (nanovaccine) dosing and whether STEAP-specific CD8⁺ T cells directly play a key role in tumor inhibition. To address these concerns, syngeneic prostate cancer mouse models were established and treated with either mSTEAP peptide emulsified in incomplete Freund's adjuvant (IFA) via subcutaneous (SC) injection or mSTEAP peptide nanovaccine containing the same amount of peptide via IV or SC injection. Meanwhile, mice were treated with either CD8b mAb followed by nanovaccine treatment, free mSTEAP peptide, or empty PLGA-NPs. Immune responses in these mice were examined using cytotoxicity assays at 14 days after treatment. Tumor size and survival in various treatment groups were measured and monitored. The results demonstrated that mSTEAP peptide nanovaccine resulted in tumor inhibition by eliciting a significantly stronger CD8⁺ T cell immune response when compared with the controls. Moreover, the survival periods of mice treated with mSTEAP nanovaccine were significantly longer than those of mice treated with mSTEAP peptide emulsified in IFA or the treatment controls. Additionally, it was observed that the peptide nanovaccine was mainly distributed in the mouse liver and lungs after IV injection. These findings suggest that the peptide nanovaccine is a promising immunotherapeutic approach and offers a new opportunity for prostate cancer therapies.

Keywords Antigenic peptide · Poly(lactide-co-glycolide) acid · Nanovaccine · Antigen-presenting cells · Cancer immunotherapy

✉ Boris Minev
bminev@ucsd.edu

✉ Wenxue Ma
wma@ucsd.edu

¹ Key Laboratory for Translational Medicine, The First Affiliated Hospital of Huzhou University School of Medicine, Huzhou 313000, Zhejiang, China

² Department of Clinical Medicine, Huzhou University School of Medicine, Huzhou 313000, Zhejiang, China

³ Department of Medicine and Moores Cancer Center, University of California San Diego, La Jolla, CA 92093, USA

⁴ Materials Science and Engineering Program, Department of Mechanical Engineering, University of California Riverside, Riverside, CA 92521, USA

⁵ Mechanical and Automotive Engineering, School of Engineering, RMIT University, Melbourne, Victoria 3083, Australia

⁶ Calidi Biotherapeutics, San Diego, CA 92121, USA

Abbreviations

APC	Antigen-presenting cells
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
IFA	Incomplete Freund's adjuvant
IP	Intraperitoneal injection
IV	Intravenous injection
MHC	Major histocompatibility complex
mSTEAP	Mouse six-transmembrane epithelial antigen of the prostate
NP	Nanoparticle
PLGA	Poly(lactic-co-glycolic) acid
SC	Subcutaneous injection
SEM	Scanning electron microscope
TRAMP	Transgenic adenocarcinoma of the mouse prostate

Introduction

Prostate cancer is the most common type of cancer in American men. According to the American Cancer Society, it is estimated that there will be 174,650 new cases of prostate cancer in 2019, and approximately 31,620 patients will die from this disease (<https://www.cancer.org/cancer/prostate-cancer/about/key-statistics.html>). Prostate cancer treatment options are dependent on several factors, including the cancer type, stage, potential benefits of treatment, side effects of treatment, the patient's overall health, and the patient's treatment preference. Typically, an overall treatment strategy that combines local and systemic treatments is pursued.

Among the systemic treatments available for prostate cancer, one option is docetaxel chemotherapy in combination with prednisone which has been available since 2004 [1]. Another option is tumor antigen-loaded dendritic cells (DC-vaccine), a promising treatment that has been applied to various malignancies [2]. The DC-vaccine first became available to prostate cancer patients when the FDA approved sipuleucel-T (Provenge) in 2010, which activates T cells to kill prostate cancer [3, 4]. This method has been so effective that autologous DC-based immunotherapy is a treatment option for men with advanced asymptomatic, or minimally symptomatic, metastatic castrate-resistant prostate cancer and has achieved clinical success by improving overall survival rates for prostate cancer patients [5, 6]. However, considerable challenges still remain because Provenge failed to show improvements in disease-specific realms, namely disease response (PSA or radiographic) and time to progression [6]. In addition, this treatment option does not readily lend itself to large-scale production and worldwide distribution. Therefore, a more feasible treatment option is greatly needed. Nevertheless, administration of the DC-vaccine is still currently being investigated in clinical trials [7, 8].

The combination of nanotechnology with medicine resulted in the development of a nanovaccine that has been used for disease treatment and prevention [9]. Nanomaterials, as exemplified by the FDA-approved poly(D,L-lactic-co-glycolic acid) (PLGA), have been used as nanocarriers for cancer therapy for decades [10–12]. This material has a characteristic controlled release, and a single dose of a nanomedicine usually exhibits a good therapeutic index [13], improved bioavailability, and reduced drug toxicity [14] in addition to inducing potent cytotoxic T lymphocyte (CTL) responses [15, 16]. Nanovaccines overcome the challenges of traditional drug delivery systems, especially water-insoluble drugs such as paclitaxel [12, 17], and easily degraded drugs such as peptides [18, 19]. In addition, synthetic free peptides (without a delivery system) have been shown to be ineffective because they are rapidly degraded by proteases and have poor immunogenicity [20].

Among the multiple tumor-associated antigens (TAA) in prostate cancer, six-transmembrane epithelial antigen of the

prostate (STEAP) is expressed predominantly in prostate tissue and is a target of CD8⁺ T cells [21, 22]. Thus, this expression profile renders STEAP an appealing candidate for immunotherapy in prostate cancer. Although therapeutic peptides are a promising and novel approach to treat many diseases including cancer [23], prostate cancer vaccines to date have only demonstrated modest efficacy and low immunogenicity [24]. To solve this issue, PLGA has been used as a carrier for STEAP peptide delivery, but few studies have explored this further. In addition to our own research on the STEAP peptide nanovaccine [25], Herrmann et al. immunized HLA-A*0201 transgenic mice with STEAP1 peptide encapsulated in PLGA microspheres (MS) and found that the STEAP1 peptide nanovaccine could effectively cross-prime CTLs in vivo [26]. However, the authors did not disclose the size of the PLGA microsphere they used or the route of immunization. We have previously demonstrated that human DCs loaded with PLGA-NPs encapsulating peptide induced significantly stronger CTL cytotoxicity than those pulsed with free peptide because the former are capable of continuously presenting tumor antigens to T cells in a sustained manner [27]. Moreover, we also reported that immunization with PLGA-NPs encapsulating mSTEAP peptide via subcutaneous injection (SC) or intraperitoneal (IP) injection elicited a stronger immune response [25]. Antigens encapsulated in PLGA-NPs can be presented by both MHC class I and class II molecules on the surface of DCs, with the final outcome of simultaneous activation of both CD8⁺ and CD4⁺ T cells [28]. Importantly, little research has been done on the effect of an intravenous (IV) dosing route of tumor antigenic peptide-loaded PLGA-NPs and whether STEAP-specific CD8⁺ T cells directly play a key role in tumor inhibition. Therefore, further studies are still necessary.

In an attempt to improve STEAP vaccines in order to achieve a better treatment result, we established syngeneic mouse models of prostate cancer and used mouse STEAP (mSTEAP) peptide that was formulated with either incomplete Freund's adjuvant (IFA) or PLGA-NPs in combination with different dosing routes including SC and IV injections. The aims of this study were to explore whether IV dosing has the same therapeutic effect as SC injection and whether mSTEAP-specific CD8⁺ T cells directly affect tumor inhibition. In addition, we also investigated the distribution of peptide nanovaccine in the body and organs including the brain, liver, lungs, and spleen at 30 min after IV dosing.

Materials and methods

Synthesis of PLGA-NPs encapsulating mSTEAP peptide

Peptide-loaded PLGA-NPs (MW 23,000, with an LA/GA ratio of 50:50, Birmingham Polymers, Inc., Birmingham, AL, USA) were formulated as previously described [25, 27].

Briefly, the primary emulsion was made using 30 mg PLGA, 0.75 mg mSTEAP_{326–335} peptide (DVSKINRTEM, GenScript Corp., Piscataway, NJ, USA), and 100 µg coumarin 6 fluorescent dye (Polyscience, Inc., Warrington, PA, USA) in 1 ml of chloroform. These reagents were gently sonicated on ice using a microtip probe sonicator at an output power of 55 W (SSE-1, Branson Digital Sonifier, Cleveland, OH, USA). The secondary emulsion was made by mixing the primary emulsion in 6 ml of 2% polyvinyl alcohol (PVA, average MW 30,000–70,000, Polyscience, Inc., Warrington, PA, USA) with sonication on ice. The peptide-loaded PLGA-NPs were harvested by high-speed centrifugation at 30,000 rpm for 30 min at 4 °C and then lyophilized at –80 °C for 48 h (Virtis Company, Freeze Dryer, Gardiner, NY, USA). The supernatant was saved for peptide loading efficiency examination. To characterize the surface morphology of the mSTEAP peptide-loaded PLGA-NPs, samples were imaged using a scanning electron microscope (SEM, Philips XL30, FEI, OR, USA). The size and zeta potential of the PLGA-NPs were analyzed using the Zetasizer® Nano ZS90 (Malvern Instruments, Worcestershire, UK) and the ZetaPlus™ instrument (Brookhaven Instruments Corporation, Holtsville, NY, USA). In addition, the peptide loading efficiency was determined by HPLC as previously described [29].

Establishment of prostate cancer mouse models

Eight-week-old male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in a pathogen-free mouse facility at the Moores Cancer Center, University of California San Diego (UC San Diego). All animal handling and experimental procedures adhered to the Laboratory Animal Care Guidelines and were approved by the IACUC of UC San Diego. One million TRAMP-C2 (transgenic adenocarcinoma of mouse prostate cancer, ATCC, Manassas, VA, USA) cells were suspended in 200 µl of a 50:50 PBS and Matrigel solution (BD Biosciences, San Jose, CA, USA) and then injected subcutaneously at the left flank of C57BL/6 mice. Tumor size was measured twice a week for mice in the tumor inhibition study using a digital caliper (Thermo Fisher Scientific, Pittsburgh, PA, USA), but not for mice in the survival study. Tumor volume was calculated using the formula, tumor volume (mm³) = (length [mm]) × (width [mm])² × 0.52 [30].

In vivo distribution of mSTEAP peptide nanovaccine

Five C57BL/6 mice were IV injected with 200 µl PBS containing 500 µg or 1000 µg mSTEAP peptide nanovaccine loaded with coumarin 6 fluorescent dye, while a separate group was IV injected with 200 µl PBS as blank controls. The mice were sacrificed using CO₂ at 30 min after IV

injection. Then, whole-body imaging was performed with an Olympus OV100 imaging system (Olympus Corp, Shinjuku, Japan) using an exposure time of 1000 ms and a range of 63 × 47 mm (0.14×). The major organs including the brain, liver, lungs, and spleen were removed and also imaged using an exposure time of 120 ms and a range of 12 × 16 mm (0.56×). To further investigate the distribution of mSTEAP peptide nanovaccine after IV injection, the organs were embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA), cryosectioned at a thickness of 12 µm, and imaged with an inverted Nikon DE 30 microscope using an exposure time of 1000 ms.

LI-COR analysis

Fluorescence signal from images of whole mouse bodies, organs, and tissue sections was quantified using Image Studio Analysis Software version 4.0 (Lincoln, NE, USA). All images were imported to Image Studio Analysis Software for fluorescence quantification according to the Image Studio tutorial guide. Quantitative fluorescence signal mapping was used to analyze the distribution of nanovaccine in the body.

Treatment of prostate cancer mouse models and survival period monitoring

Forty prostate cancer mouse models were randomized into the following 5 groups: (1) free mSTEAP peptide group, treated by IV injection; (2) empty PLGA-NP group, treated by IV injection; (3) mSTEAP peptide emulsified in IFA group, treated by SC injection; (4) mSTEAP peptide nanovaccine group, treated by IV injection; and (5) mSTEAP peptide nanovaccine group, treated by SC injection. Mice in each group were treated only once, 3 days after tumor implantation. Tumor size was measured twice a week. Three mice in each group were sacrificed for immune response assays 14 days after their corresponding treatment, and the remaining mice in each group were kept for further tumor size measurement until day 44 or until the humane endpoint for the survival monitoring group.

Cytotoxicity assays

Three mice in each group were sacrificed 14 days after treatment. Splenocytes collected from mouse spleens were used for restimulation with the mSTEAP peptide at a concentration of 5 µg/ml. Cytolytic activity of the CTLs induced in each group was assayed 6 days after restimulation using a LDH cytotoxicity assay (Promega Corp., Madison, WI, USA) with target cells from both mSTEAP peptide-pulsed EL4 cells (ATCC, Manassas, VA, USA) and TRAMP-C2 cells.

CD8⁺ T cell depletion and tumor size monitoring in prostate cancer-bearing mice

To validate CD8⁺ T cell depletion and whether mSTEAP-specific CD8⁺ T cells result in tumor inhibition, another set of TRAMP-C2 mouse models was established by SC injection of 200 μ l of a 50:50 PBS and Matrigel solution containing 1.25×10^6 TRAMP-C2 cells into the right flank of male C57BL/6 mice. Tumor size was measured twice a week using a digital caliper. Three mice were included in each of the following groups: (1) anti-mouse CD8b mAb by IP combined with mSTEAP nanovaccine by IV injection, (2) empty PLGA-NPs by IV injection, and (3) mSTEAP nanovaccine by IV injection. All nanovaccine treatments including mSTEAP nanovaccine and empty PLGA-NPs were conducted on day 0. To deplete CD8⁺ T cells in the control groups, 100 μ g of anti-mouse CD8b mAb was IP injected at the following time points: (1) three days prior to mSTEAP peptide nanovaccine treatment, (2) the same day but prior to mSTEAP peptide nanovaccine treatment, (3) three days after mSTEAP peptide nanovaccine treatment, and (4) seven days after mSTEAP peptide nanovaccine treatment. Depletion of CD8⁺ T cells in the peripheral blood was assessed by flow cytometric analysis at 48 h after the last anti-mouse CD8b mAb treatment. Peripheral blood was collected retro-orbitally under general anesthesia. Red blood cells in each sample were lysed with ACK lysis buffer and split in two, followed by staining with either anti-CD8a mAb (clone 53-6.7) or mouse isotype control IgG2a, κ mAb (clone, MOPC-173, BioLegend, San Diego, CA). The samples were analyzed on a MACSQuant Analyzer (Miltenyi Biotec Inc.), and the flow cytometry data was analyzed by FlowJo software (FlowJo, LLC, Ashland, Oregon, USA).

Statistical analysis

The data were representative of three independent experiments and statistically analyzed by GraphPad Prism 8.0. The differences between the experimental groups were considered to be significant when $p < 0.05$, as determined by Student's t test or one-way ANOVA when appropriate. Data were shown as the mean $\pm$ SEM. Kaplan-Meier survival curves were analyzed using a log-rank test and one-way ANOVA.

Results

Preparation of mSTEAP peptide nanovaccine

Biodegradable and biocompatible PLGA-NPs encapsulating mSTEAP peptides were prepared using the method as previously described [25, 27, 29]. To characterize the nanovaccine, the lyophilized nanovaccine powder was sputter coated with

gold/palladium and imaged using a scanning electron microscope (SEM) at a voltage of 10 kV (Fig. 1a, b). The unfractionated nanovaccine demonstrated a peak size of 297 nm in diameter and an average size of 369 nm in diameter (Fig. 1c). The mSTEAP peptide nanovaccine exhibited spherical morphologies under SEM (Fig. 1a, b). The zeta potential was analyzed using the Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). The results showed that the zeta potential of the peptide nanovaccine was -16.3 ± 0.56 mV at pH 7.4 (Fig. 1d, $n = 3$) and the polydispersity index (PDI) was 0.296 ± 0.03 ($n = 3$).

Peptide loading capacity and encapsulation efficiency in mSTEAP peptide nanovaccine

To measure the peptide loading capacity (LC) and encapsulation efficiency (EE) of the peptide nanovaccine, we defined peptide LC and EE as previously described [25]. The LC and EE were calculated using the following formulas: $LC = (A - B / C) \times 100\%$ and $EE = (A - B / A) \times 100\%$, where A is the total peptide amount, B is the free peptide amount, and C is the quantified nanoparticle weight [31]. Based on these definitions and our HPLC-analyzed results, the mSTEAP peptide LC in the mSTEAP peptide nanovaccine was $3.08 \pm 0.15\%$ ($n = 3$) and the peptide EE was $83.33 \pm 5.56\%$ ($n = 3$).

In vivo distribution of the peptide nanovaccine after IV injection

To investigate in vivo distribution of the peptide-loaded nanovaccine, we prepared mSTEAP peptide nanovaccine containing coumarin 6. Some C57BL/6 mice were intravenously injected with 200 μ l PBS containing 500 μ g or 1000 μ g coumarin 6-loaded mSTEAP peptide nanovaccine, while other mice were IV injected with only 200 μ l PBS as the blank control. These mice were sacrificed 30 min after injection. Images of mouse bodies (Fig. 2a), organs (Fig. 2b), and organ cryosections (Fig. 2c) were taken using an Olympus OV100 imaging system. The results showed that weak auto fluorescent signals were observed in the paws of blank control mice (Fig. 2a). Fluorescent signals were observed in the bodies of mice that were IV injected with 500 μ g or 1000 μ g of mSTEAP peptide nanovaccine (Fig. 2a). The major organs including the brain, liver, lungs, and spleen were collected and imaged. The results showed that fluorescent signals were found in the brain, liver, and lungs from mice injected with 1000 μ g PLGA-NPs (right column, Fig. 2b).

To further investigate the distribution of the peptide nanovaccine after IV injection, these organs were embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA) for cryosectioning at a thickness of 12 μ m. The images were taken with an Olympus OV100 imaging system. The results showed that scattered fluorescent

Fig. 1 Preparation of mSTEAP peptide nanovaccine. Peptide-loaded PLGA nanovaccine was prepared using a double emulsification solvent evaporation method. The physical characteristics of the nanovaccine including morphology, nanoparticle size, and zeta potential were examined. mSTEAP peptide nanovaccine was sputter coated with gold/palladium using a Cressington 108 auto sputter-coater (Cressington, Watford, UK) for 100 s. Images were taken using a scanning electron microscope (SEM) at 10 kV. **a** SEM image of mSTEAP peptide-loaded nanovaccine

(magnification 13,890×, scale bar = 2 μm). **b** SEM image of mSTEAP peptide nanovaccine (magnification 27,780×, scale bar = 1 μm). **c** The peptide nanovaccine size was measured using the Zetasizer Nano ZS90. The results indicated that PLGA-NP had a peak size of 297 nm in diameter and an average size of 369 nm in diameter. **d** The zeta potential of the peptide nanovaccine was -16.3 ± 0.56 mV in HEPES buffer (0.001 M, pH = 7.4) measured using ZetaPlus

signals were mainly observed in the liver and lungs collected from mice injected with either 500 μg or 1000 μg of the peptide nanovaccine (Fig. 2c).

Single-dose treatment with mSTEAP peptide nanovaccine resulted in a significant tumor growth inhibition and a significant longer survival period

To determine whether prostate cancer cells in vivo could be recognized and eliminated by mSTEAP nanovaccine-induced tumor antigen-specific CTLs, mouse prostate cancer models were established by introducing syngeneic TRAMP-C2 cells subcutaneously into male C57BL/6 mice. Three days after implantation, these mouse models were randomized into the following groups for treatment: (1) free mSTEAP peptide by IV injection, (2) empty PLGA-NPs by IV injection, (3) mSTEAP peptide emulsified in IFA by SC injection, (4) mSTEAP peptide nanovaccine by IV

injection, and (5) mSTEAP peptide nanovaccine by SC injection. All the mice were treated only once, and the tumor size was measured twice a week. The results showed that tumor growth in the group that received mSTEAP peptide nanovaccine treatment via IV injection (red line, Fig. 3a) was significantly inhibited at 28 days after treatment when compared with the free mSTEAP peptide, empty PLGA-NPs, and mSTEAP peptide emulsified in IFA treatment conditions (Fig. 3a, $**p < 0.01$, $***p < 0.001$, unpaired *t* test, one-way ANOVA). Meanwhile, tumor growth in the group that received mSTEAP peptide nanovaccine treatment via SC injection (black line) was also significantly inhibited (Fig. 3a, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, unpaired *t* test, one-way ANOVA).

To investigate whether treatment with mSTEAP peptide nanovaccine increased the survival periods of tumor-bearing mice, the survival time of mice in each group was closely monitored. The results demonstrated

Fig. 2 In vivo distribution of mSTEAP peptide nanovaccine. C57BL/6 mice were IV injected with 200 µl PBS (blank control) and 200 µl PBS containing 500 µg or 1000 µg of mSTEAP peptide nanovaccine containing coumarin 6 fluorescent dye. Mice were sacrificed 30 min after IV injection. **a** Whole-body images were taken using an Olympus OV100 imaging system. **b** Organ images including the brain, liver, lungs, and spleen were taken using the same imaging machine (scale bar = 2 mm). **c** OCT-embedded brain, liver, and lung organs were sectioned at a thickness of 12 µm and imaged with an Olympus OV100 imaging system.

that the survival periods of mice treated with mSTEAP peptide nanovaccine via IV injection were significantly longer than those of mice treated with the free mSTEAP peptide and empty PLGA-NP controls (Fig. 3b, $**p < 0.01$, log-rank test, ANOVA), and they were significantly longer than those for mice treated with mSTEAP peptide emulsified in IFA (Fig. 3b, $*p < 0.05$, log-rank test, one-way ANOVA). Similarly, the survival periods of mice treated with mSTEAP peptide nanovaccine via SC injection were also significantly longer than those of mice treated with free mSTEAP peptide, empty PLGA-NPs, or mSTEAP peptide emulsified in IFA (Fig. 3b, $*p < 0.05$, log-rank test, ANOVA). Meanwhile, the results also revealed that the survival periods of mice treated with mSTEAP peptide emulsified in IFA were also significantly longer than those of mice treated with empty PLGA-NPs or free mSTEAP peptide by IV injection (Fig. 3b, $*p < 0.05$, log-rank test, one-way ANOVA).

Scattered fluorescent signals (peptide nanovaccine) were found in both the liver and lungs for doses of 500 µg and 1000 µg of mSTEAP peptide nanovaccine. In order to quantify the distribution of nanovaccine, the fluorescence signal was analyzed using Image Studio Analysis Software version 4.0 (Lincoln, NE, USA). Quantitative fluorescence signal mapping was used to analyze the distribution of nanovaccine in the body and organs. Bar graphs displaying fluorescence intensity in different organs for each condition are included to the right of the images

mSTEAP peptide nanovaccine treatment elicited a significantly stronger immune response

To investigate whether the mSTEAP peptide nanovaccine treatment could elicit a significantly greater immune response compared with other treatments, a cytotoxicity assay was used to compare the tumor antigen-specific CTL responses among mice that were treated with the mSTEAP peptide nanovaccine via IV or SC injections, mSTEAP peptide emulsified in IFA via SC injection, free mSTEAP peptide via IV injection, and empty PLGA-NPs via IV injection. Three mice in each group were sacrificed 14 days after their corresponding treatment, and splenocytes were harvested and restimulated with mSTEAP peptide at 5 µg/ml for 6 days. The cytolytic activity of the CTLs was measured using a cytotoxicity assay with either TRAMP-C2 cells (Fig. 4a) or EL4 cells pulsed with mSTEAP peptide (Fig. 4b) as the target cells.

Fig. 3 mSTEAP peptide nanovaccine treatment resulted in significantly improved tumor growth inhibition and a significant longer survival period. Three days after SC implantation of 1×10^6 TRAMP-C2 cells mixed with the Matrigel matrix, prostate cancer-bearing mice were randomized into the different groups including free mSTEAP peptide (blue line), empty PLGA-NPs (orange line), mSTEAP peptide emulsified in IFA (green line), mSTEAP peptide nanovaccine by IV (red line), and mSTEAP peptide nanovaccine by SC (black line). Mice in each group were treated only once, and tumor size was monitored twice a week. **a** Significant tumor growth inhibition was observed in groups treated with the IV and SC mSTEAP peptide nanovaccine when compared with the mSTEAP peptide emulsified in IFA, empty PLGA-NPs, and free mSTEAP peptide treatment controls (mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA). Significant tumor growth inhibition was also observed in the mSTEAP peptide emulsified in IFA (SC) treatment group when compared with the empty PLGA-NPs and free mSTEAP peptide treatment controls (mean $\pm$ SEM, * $p < 0.05$, one-way ANOVA). **b** The survival periods of mice treated with the mSTEAP peptide nanovaccine (both IV and SC injections) were significantly longer than those of the control groups treated with mSTEAP peptide emulsified in IFA, empty PLGA-NPs, or free mSTEAP peptide (* $p < 0.05$, log-rank test, ANOVA). In addition, the survival periods of mice treated with mSTEAP peptide emulsified in IFA were also significantly longer than those of mice treated with empty PLGA-NPs and free mSTEAP peptide ($p < 0.05$, log-rank test, one-way ANOVA)

Fig. 4 Peptide nanovaccine treatment elicited a significantly stronger immune response. When the effector cell (E) to target cell (T) ratio was 50:1, mSTEAP-specific CTLs derived from mice treated with mSTEAP peptide nanovaccine (IV and SC) lysed a significantly higher percentage of TRAMP-C2 cells (**a**) (mean $\pm$ SEM, $p < 0.0001$, one-way ANOVA) and EL4 cells pulsed with the mSTEAP peptide (**b**) (mean $\pm$ SEM, $p < 0.0001$, one-way ANOVA) when compared with mSTEAP-specific CTLs derived from mice treated with mSTEAP peptide emulsified in IFA, free mSTEAP peptide, or empty PLGA-NPs

The results showed that mSTEAP-specific CTLs derived from mice treated with mSTEAP peptide nanovaccine via IV injection (green line, Fig. 4a) and SC injection (red line, Fig. 5a) lysed a significantly higher percentage of TRAMP-C2 cells when compared with those derived from mice treated with mSTEAP peptides emulsified in IFA via SC injection (blue line, Fig. 4a, *** $p < 0.001$), free mSTEAP peptide via IV injection (black line, Fig. 4a, **** $p < 0.0001$), and empty PLGA-NPs via IV injection (pink line, Fig. 4a, **** $p < 0.0001$). Meanwhile, mSTEAP-specific CTLs derived from mice treated with mSTEAP peptides emulsified in IFA via SC injection (blue line, Fig. 4a) also lysed a significantly higher percentage of TRAMP-C2 cells when compared with those derived from mice treated with free mSTEAP peptide via IV injection and empty

PLGA-NPs via IV injection (Fig. 4a, $***p < 0.001$). All statistics were performed using an unpaired *t* test and one-way ANOVA.

In repeat cytotoxicity assays, EL4 cells pulsed with mSTEAP peptide as the target cells were used. The results showed that mSTEAP-specific CTLs derived from mice treated with mSTEAP peptide nanovaccine via IV injection (green line, Fig. 4b) lysed a significantly higher percentage of the EL4 cells when compared with those derived from mice treated with mSTEAP peptide emulsified in IFA via SC injection (blue line, Fig. 4b, $***p < 0.001$), free mSTEAP peptide via IV injection (black line, Fig. 4b, $****p < 0.0001$), and empty PLGA-NPs via IV injection (pink line, Fig. 4b, $****p < 0.0001$).

Meanwhile, the mSTEAP-specific CTLs derived from mice treated with mSTEAP peptide nanovaccine via SC injection (orange line, Fig. 4b) also lysed a significantly higher percentage of the EL4 cells when compared with those derived from mice treated with mSTEAP peptide emulsified in IFA via SC injection (blue line, Fig. 4b, $**p < 0.01$) and lysed a significantly higher percentage of the EL4 cells when compared with those treated with free mSTEAP peptide via IV injection (black line, Fig. 4b, $****p < 0.0001$) or empty PLGA-NPs via IV injection (pink line, Fig. 4b, $****p < 0.0001$). All the statistics were performed using an unpaired *t* test and one-way ANOVA.

mSTEAP-specific CD8⁺ T cells inhibit tumor growth

To verify whether mSTEAP-specific CD8⁺ T cells resulted in tumor inhibition, anti-mouse CD8b mAb was used to deplete CD8⁺ T cells. Two days after the last anti-CD8b mAb treatment, peripheral blood from mice in each group was collected for analysis of CD8⁺ T lymphocyte depletion using flow cytometric analysis. Representative flow cytometric results showed that the frequency of CD8⁺ T cells in peripheral blood samples from the control group was only 0.88% (Fig. 5a) whereas the frequency of CD8⁺ T cells in peripheral blood from mice treated with empty PLGA-NPs was 8.12% (Fig. 5a). It is noteworthy that the frequency of CD8⁺ T cells in peripheral blood samples from mice treated with mSTEAP peptide nanovaccine was 18.9% (Fig. 5a).

At the end of the experiment (32 days after treatment), tumor size in the CD8⁺ T cell depletion control group was significantly larger when compared to those in the empty PLGA-NP (Fig. 5b, $**p < 0.0015$) and mSTEAP nanovaccine via IV injection treatment groups (Fig. 5b, $****p < 0.0001$). In addition, tumor size in the empty PLGA-NP treatment group was significantly larger when compared to that in the mSTEAP nanovaccine via IV injection treatment group (Fig. 5b, $***p = 0.0003$).

Discussion

Cancer vaccines are used to stimulate the immune system and to initiate an immune response against cancer cells. One of the

Fig. 5 Confirmation of CD8⁺ T lymphocyte depletion and tumor growth inhibition by CD8⁺ T cells. CD8⁺ T lymphocyte depletion was conducted using flow cytometric analysis of cells from peripheral blood, which was collected at 2 days after the last anti-CD8b mAb treatment. Peripheral blood samples were lysed with ACK lysis buffer and split in two, followed by staining with either anti-CD8a mAb (clone 53-6.7) or mouse isotype control IgG2a, κ mAb (clone, MOPC-173). The samples were analyzed on a MACSQuant Analyzer (Miltenyi Biotec Inc.). Results were shown on dot plots using a logarithmic scale. CD8⁺ T lymphocyte depletion is shown in the upper left column (a). Frequency of CD8⁺ T cells in the peripheral blood of mice in each group is shown in the top row, and their corresponding isotype controls are displayed in the bottom row (a). The tumor size in each group was closely observed and measured twice a week. The tumor size (mean ± SEM) in the CD8⁺ T lymphocyte depletion group was significantly larger than those in the empty PLGA-NP treatment group ($**p < 0.0015$, b) and the mSTEAP nanovaccine treatment group ($****p < 0.0001$, b). Additionally, the tumor size (mean ± SEM) in the empty PLGA-NP treatment group was also significantly larger than the tumor size in the mSTEAP nanovaccine treatment group ($***p = 0.0003$, b)

primary objectives of cancer immunotherapy is to successfully induce long-lasting T cell responses against cancer cells. However, it is difficult to generate strong antitumor responses

due to limited numbers of tumor antigens and the low immunogenicity of cancer cells.

Tumor antigen-loaded PLGA-NPs are a new rising class of vaccines and have a competitive advantage over other vaccines because they are biocompatible and biodegradable and the antigens encapsulated inside can be sustainably released [29]. In the past, PLGA-NPs have been used to deliver peptides but not without significant obstacles [25, 27, 29, 32]. Tumor antigenic peptide nanovaccines could serve as a better alternative to overcome the obstacles faced by other antigen delivery systems. The advantages of peptide nanovaccine formulated with PLGA include (1) sustained antigen release over a longer period [33], (2) antigen protection against proteolytic enzyme degradation [34], (3) formulations that can deliver a single dose that does not require additional booster doses [35], (4) improved cellular uptake of peptides by APC [27], and (5) enhanced adsorption due to the small size and large surface area of the PLGA nanovaccine when compared with other nanocarriers [36].

The size and potency of the PLGA nanovaccine are important parameters for drug delivery. In general, when a double emulsification solvent evaporation method is used to formulate the PLGA nanovaccine, PVA is a common stabilizer to prevent the aggregation of PLGA-NPs. The mean size of the nanovaccine decreases with increasing concentrations of PVA from 0.5 to 2.5% (w/v). The surfactant exerts its stabilizing effect by reducing the nanovaccine's interface, thus lessening the surface tension between the two phases, thereby preventing aggregation of nanovaccine and minimizing the size. In addition, zeta potential is another important parameter for the stability of the PLGA nanovaccine in a water suspension. The zeta potential of our nanovaccine was -16.3 ± 0.56 mV. The repulsion between negatively charged nanovaccine microspheres provides stability and prevents aggregation. Moreover, sonication is a necessary step to break down aggregation before using the PLGA nanovaccine.

Cancer immunotherapy relies on the identification and characterization of potential target antigens that can be recognized by effector cells of the immune system. Six-transmembrane epithelial antigen of the prostate (STEAP) is overexpressed in prostate cancer and is a target of CD8⁺ T cells [22]. This suggests that STEAP could function as a potential target antigen for prostate cancer therapies. Rodeberg et al. [37] reported that STEAP-292 peptide is presented in the major histocompatibility complex HLA-A2 in sufficient amounts and can induce CTL recognition of STEAP-containing tumors including prostate cancer. In addition to prostate cancer, STEAP is a therapeutic target in other cancer types including renal cell cancer, bladder cancer [38], and lung cancer [39].

In this study, we extended the scope of our previous work to include IV injection of mSTEAP peptide-loaded nanovaccine. Our results demonstrated that mSTEAP peptide

nanovaccine was superior to an IFA-based vaccine with mSTEAP peptide. IV administration of mSTEAP peptide nanovaccine resulted in significant tumor inhibition when compared with the controls. To keep the errors consistent in this study, we used SEM instead of SD. In addition, before starting this *in vivo* experiment, we completed a pilot run on comparing the tumor growth and tumor size between the groups of TRAMP-C2 cells with and without Matrigel. We found that the tumor in the TRAMP-C2 group with Matrigel grew faster and also the tumor size was more uniform than that in the TRAMP-C2 group without Matrigel. This may be the reason why the error bars look smaller. The survival period of prostate cancer-bearing mice treated with mSTEAP peptide nanovaccine was significantly longer than those treated with mSTEAP peptide emulsified in IFA and the other two controls. These results were consistent not only with our previous study [25] but also with Herrmann et al.'s report [26]. Through similar methods, Hamdy et al. reported that vaccination of melanoma B16 tumor-bearing mice with tyrosinase-related protein 2 (TRP2)-containing PLGA-NPs induced a therapeutic anti-tumor effect with activated TRP2-specific CD8⁺ T cells [40]. These related studies strongly support the potential use of PLGA-NPs as competent carriers for cancer vaccine formulations in translational medicine.

Recently, there has been an increased interest in cancer vaccine development to induce potent CD8⁺ T cell-mediated cytotoxic responses against cancer cells. This requires a detailed understanding of how naive CD8⁺ T cells are activated and differentiated into effector cells. In this study, we hypothesized that tumor growth inhibition in prostate cancer is caused by a significantly enhanced CD8⁺ T cell immune response induced by mSTEAP peptide nanovaccine. In order to prove this hypothesis, *in vivo* depletion of CD8⁺ T cells was utilized as a means of studying the role of specific CD8⁺ T cell subpopulations in the initiation and effector phases of particular *in vivo* immune responses. Our results demonstrated that the tumor size in the CD8⁺ T cell depletion control group was significantly larger when compared with those in groups that received empty PLGA-NP treatment (Fig. 5, $**p < 0.0015$) or mSTEAP nanovaccine treatment (Fig. 5, $***p < 0.0001$). Based on these results, we have demonstrated that mSTEAP-specific CD8⁺ T cells play a key role in the eradication of prostate cancer cells.

In addition to these results, we also noticed that the frequency of CD8⁺ T cells in the mouse group receiving mSTEAP peptide nanovaccine treatment (18.9%) was much higher than the frequency of CD8⁺ T cells in the mouse group receiving empty PLGA-NP treatment (8.12%). In general, the frequency of CD8⁺ T cells in mouse peripheral blood is 7–10%. The higher frequency of CD8⁺ T cells in mice receiving the mSTEAP peptide nanovaccine was most likely caused by stimulation of mSTEAP peptide released from the nanovaccine, because a number of immune cells including

macrophages and APC presented mSTEAP antigens to naïve CD8⁺ T cells.

Florescence imaging showed that the peptide nanovaccine was mainly distributed in the mouse liver and lungs after IV injection. This distribution pattern of the peptide nanovaccine in the body is interesting. Although the peptide distributed mainly in the liver and lungs, no side effects from the mSTEAP peptide nanovaccine were observed based on the health of the mice in this study. In addition, PLGA is nontoxic and degraded by hydrolysis of its ester linkages in the presence of water. The time required for degradation of PLGA is related to the monomer ratio used in the PLGA product. The monomer ratio of PLGA used in this study was 50:50 lactic acid and glycolic acid. This type of PLGA has the fastest degradation time (approximately 2 months). Theoretically, the PLGA nanovaccine dosage should be tolerable if it does not impact liver and lung function. However, the maximum dosage of PLGA nanovaccine has not been determined and will be another research topic.

Kupffer cells account for approximately 15% of the cellular content of the liver and constitute 80–90% of the tissue-resident macrophages including alveolar, splenic, and peritoneal macrophages in the body [41]. As the resident macrophages of the liver, Kupffer cells have immune clearance function in the liver [42]. Alveolar macrophages (dust cells) in the lungs form 90–95% of the cellular content within the alveoli under normal conditions, making them the natural “gatekeepers” of the respiratory system [43]. Kupffer cells in the liver and alveoli cells in the lungs might have contributed to mSTEAP antigen presentation by eliciting immune responses.

Targeted cancer therapies are aimed at reducing side effects while retaining their anti-cancer efficacy. In future studies, to minimize the possible side effects of nanovaccines by IV administration, nanovaccines can be optimized by adding a targeted molecule such as a transferrin ligand, as shown in our previous study [12]. Transferrin receptor is abnormally expressed in various cancers including brain, breast, liver, ovarian, prostate, and lung and leukemia [44]. Because the transferrin receptor (also known as CD71) is ubiquitously expressed at low levels in most human tissues and higher expression of transferrin receptor in malignant cells correlates with tumor progression, the transferrin receptor is an attractive target for delivery of nanotherapeutics for multiple cancer types.

Overall, our findings demonstrated that the peptide nanovaccine is a promising immunotherapeutic approach for treating cancer by inducing a more effective antitumor immune response. Consequently, a tumor antigen peptide nanovaccine offers a simple, safe, and robust strategy to boost anti-tumor immunity for cancer immunotherapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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