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Delivery of foreign cytotoxic T lymphocyte epitopes to tumor tissues for effective antitumor immunotherapy against pre-established solid tumors in mice

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Abstract

Cytotoxic T lymphocyte (CTL) can have remarkable abilities to kill tumor cells. However, the establishment of successful CTL-based anticancer therapy has met with many challenges. Within tumor cells, there exist subpopulations with low or no expression of the targeted antigen (termed as antigen-loss variants). In addition, tumor cells can downregulate the levels of major histocompatibility complex class I (MHC-I) molecules on cell surface due to immune pressure. As a result, some tumor cells can escape the immune pressure bestowed by CTLs, resulting in treatment failure. To address these difficulties, a new approach is developed to deliver foreign high-affinity CTL epitopes to tumor tissues utilizing pH-responsive “smart” microparticles (MPs). These MPs could encapsulate CTL peptide epitope, release the peptide under acidic condition encountered in tumor tissues and enhance CTL activation. Mice bearing pre-established tumor as “antigen-loss variant” solid tumor models were administered intratumorally with MPs containing the CTL peptide, which showed 100% survival following the treatment. In contrast, all control mice died from tumor. Significant protection from tumor-induced death was also observed with systemic administration of CTL peptide-MPs. The therapeutic efficacy can be attributed to enhanced delivery of the epitope to tumor tissues, presentation of the epitope by tumor cells as well as tumor stromal cells and/or generation of epitope-specific CTLs by the peptide-containing MPs. These findings offer a promising new direction for treating established solid tumor using CTL therapy.

Keywords

Cytotoxic T cells; Epitope delivery; Immunotherapy; Microparticles; Solid tumor

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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Introduction

Cytotoxic T lymphocytes (CTLs) are a key component of antitumor immunity. Anticancer immunotherapies invoking CTLs are being actively pursued [1, 2]. However, clinical successes have been limited so far with multiple challenges to overcome. CTLs eliminate target cells through binding with specific peptide epitopes presented by the major histocompatibility complex class I (MHC-I) on the target cell surface. Since most of tumor antigens are self-antigens, it is often very difficult to generate highly active CTLs targeting tumor-associated antigens, as these CTLs are generally eliminated during development due to negative selection [3]. In addition, with high heterogeneities of tumor cells, subpopulations of the cells may not express the cognate tumor-associated antigen [4]. These antigen-loss variants (ALVs) are thus not subjected to the immune surveillance bestowed by the specific CTLs and can continue to grow, resulting in failure of the therapy. Moreover, due to the genetic instability, some tumor cells can downregulate their MHC-I expression levels under the selection pressure [5, 6], a phenomenon termed immune-editing [7]. The low abundance of MHC-I/antigen complexes on the cell surface may lead to insufficient tumor recognition by CTLs and eventual tumor escape [8]. To address these challenges, we report a strategy to selectively deliver a CTL peptide epitope (CTLp) by pH-sensitive microparticles to tumor tissues not endogenously expressing such an epitope and to effectively protect mice from pre-established ALV solid tumor models in a therapeutic setting.

Compared to tumor-associated antigens, foreign CTLp can be advantageous as high-affinity CTLs recognizing these epitopes are not subjected to negative selection and may be more readily elicited via vaccination. If the high-affinity CTLp can be delivered to tumor tissues in vaccinated hosts, they can potentially outcompete endogenous epitopes for binding with the limited number of MHC-I molecules, sensitizing tumor cells toward CTL killing. In addition, tumor stromal cells are known to provide the critical extracellular microenvironment supporting tumor growth [9]. As stromal cells are derived from the host, they are genetically more stable and less prone to mutation. CTLp delivered to tumor tissues can result in epitope presentation by stromal cells and their subsequent killing by CTLs [10]. The elimination of tumor stromal cells removes the favorable microenvironment supporting tumor growth and facilitates tumor death by the bystander effect [11, 12].

To achieve tumor selectivity in CTLp delivery, we resorted to pH-responsive “smart” particles [13] that are stable at neutral pH. Due to the hyperpermeability of blood vessels and the defective lymphatic system in tumor tissues, nanoparticles/microparticles (MPs) can selectively accumulate in these tissues with slow clearance due to the enhanced permeability and retention effect [14, 15]. The CTLp can be released in the acidic tumor microenvironment [16] or intracellularly for MHC-I presentation by both tumor and stromal cells sensitizing them toward CTL killing.

Materials and methods

Preparation of CTLp-Ace-Dex-MPs

The CTLp SIINFEKL and PDTRPAP were synthesized through solid-phase peptide synthesis using Fmoc chemistry. Their cLogP values were calculated using ChemBio-Draw Ultra (version 14). CTLp-Ace-Dex-MPs were prepared following a reported emulsion evaporation method [17].

Hydrolysis of CTLp-Ace-Dex-MPs and CTLp release from CTLp-Ace-Dex-MPs

Solutions of MPs (4 mg/ml) were prepared in pH = 7.4, 6.5, 6.0 or 5.5 PBS buffer, respectively. The stock solutions were kept under room temperature or in 37 °C water bath. UV-Vis absorption at 600 nm were measured every 1–4 h over 72 h. In order to measure CTLp release rates, CTLp-Ace-Dex-MPs were dissolved in 4 ml of buffer. Each stock solution was divided into 4 Eppendorf tubes (1 ml each) and incubated at 37 °C. At each time point (6, 12, 24, 48 h), one Eppendorf tube from each group was taken out and centrifuged (10,000 revolutions per minute, 15 min). The pellets were collected and dissolved in 0.5 ml H₂O/trifluoroacetic acid (pH = 1), which were followed by high-performance liquid chromatography (HPLC) quantification.

Animals and cell lines

Pathogen-free female C57BL/6 mice aged 6–10 weeks were obtained from Charles River. EL4 lymphoma and MC38 colon adenocarcinoma cells were generously donated by Dr. Sandra Gendler (Mayo clinic). EL4 cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% FBS, 1% L-glutamine and 1% streptomycin penicillin. MC38 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 1% streptomycin penicillin. B3Z cells were generously donated by Dr. Nilabh Shastri (University of California, Berkeley). These cells were cultured in RPMI-1640 supplemented with 10% FBS, 1 mM pyruvate, 50 µM 2-mercaptoethanol, 1% L-glutamine and 1% streptomycin penicillin. All cell lines were grown at 37 °C in a 5% CO₂/air incubator.

Detection of CTLp presentation on MHC-I of tumor cells by fluorescence-activated cell sorting (FACS)

EL4 or MC38 cells were pulsed with increasing concentrations of either free CTLp or CTLp-Ace-Dex-MPs and incubated at 37 °C in a 5% CO₂/air incubator for 1 h. Cells were washed three times with FACS buffer (1% FBS, 0.5% NaN₃ in PBS), stained with either anti-mouse H-2K^b bound to SIINFEKL (Clone 25-D1.16) or anti-mouse IgG1K (isotype control) for 30 min in FACS buffer on ice and then washed with FACS buffer three times followed by FACS analysis. Control mAb showed little cellular staining over background.

B3Z T cell activation assay

2 × 10⁴ EL4 cells were cultured overnight in a 96-well plate and subsequently incubated with CTLp-Ace-Dex-MPs or free CTLp at increasing concentrations. After 6 h, the cells

were washed and 1×10^5 B3Z cells were added to the EL4 cells and co-cultured for an additional 16 h. The culture media was removed, and 100 μ L of chlorophenol red- β -D-galactopyranoside (CPRG) buffer (9.1 mg of CPRG, 0.125 mg of NP40 detergent and 90 mg MgCl_2 in 100 mL of PBS) was added to each well. After 6 h, the absorbance at 595 nm was measured using a microplate reader. The results were presented as a mean quadruplicate.

Tumor protection studies against EL4 tumor

C57BL/6 wild-type mice were immunized subcutaneously under the scruff on day 0 with 0.1 mL of ovalbumin (50 μ g) as an emulsion in complete Freund's adjuvant. Boosters were given on days 14 and 28 with emulsion in incomplete Freund's adjuvant. The mice were inoculated with subcutaneous tumor (2×10^5 EL4 or 5×10^5 MC38 cells were injected) on the left flank on day 35. When the tumor size was about 70–90 mm^3 , intratumoral injection of either CTLp-Ace-Dex-MPs, free CTLp, empty Ace-Dex-MPs, or PBS was performed every other day for a total of three injections (10 mice per study group). For the groups where treatment was via intravenous injection, mice were treated when tumor size was about 20–30 mm^3 (usually on day 3 following tumor inoculation). The amounts of injected CTLp were normalized to 7 μ g for intratumoral injection and 20 μ g for intravenous injection. Tumor volume was determined using the formula: $\text{volume (cm)}^3 = 1/2(L \times W \times H)$ [19]. Tumor growth was monitored by measuring the tumor volume every other day. Mice were euthanized when the tumor volume was over 1.6 cm^3 or ulceration of tumor was observed.

In vivo CTL activity assay

To evaluate CTL activities induced by MP, naïve mice were injected intravenously through tail vein with 1 mg CTLp-Ace-Dex-MP (with 20 μ g OVA peptide encapsulated), 1 mg MP (empty) + 20 μ g CTLp, or 20 μ g CTLp, respectively, every other day with a total of three injections. On day 7 after the final injection, a mixture of CFSE^{lo}- and CTLp-pulsed CFSE^{hi} splenic cells (0.2 mL, 2 million cells) was injected through tail vein to mice. The excess cell mixture was stored in culture media under 37 °C as a “before injection” sample. Mice were euthanized after 24 h. Spleen cells were collected and then suspended in 3 mL FACS buffer containing 30 μ g/mL 7-aminoactinomycin D (7-AAD). Three to four lymph nodes were collected from each mouse. Lymph nodes were set in same Petri dish, smashed and filtered to create lymph node cell samples. Cells were washed twice with PBS buffer and suspended in 1 mL FACS buffer containing 10 μ g/mL 7-AAD. The changes of ratio of CFSE^{hi} to CFSE^{lo} before and after injection were determined from FITC-SSC plot to calculate target cell viability.

CTLp distribution in organs and quantification of CD8⁺ T cell infiltration in tumor

C57BL/6 mice with established subcutaneous MC38 tumor ($\sim 300 \text{ mm}^3$) were injected with CTLp-Ace-Dex-MPs (20 μ g CTLp) or PBS. Animals were euthanized 9 h post-injection. Organs (spleen, liver, lung and tumor) were collected from each mouse and digested into single-cell suspension with collagenase IV (1 mg/mL in DMEM-F12 media) followed by washing with PBS buffer twice. Cells were stained with anti-mouse H-2K^b bound to SIINFEKL (Clone 25-D1.16) or anti-mouse IgG1K (isotype control) for 30 min in FACS buffer on ice and then washed with FACS buffer three times followed by FACS analysis. Mean fluorescence intensity ratio of 25-D1.16 mAb-stained group versus isotype control

group was recorded for each organ. In addition, single-cell suspension of tumor from PBS group was stained with phycoerythrin (PE)–anti-mouse CD8a mAb or PE–anti-IgGa Ab (isotype control). FACS analysis was performed to determine the percentage of CD8⁺ T cells in tumor.

Results

Synthesis and characterization of the CTLp-Ace-Dex-MPs

As a first-generation approach, acetalated dextran (Ace-Dex) [18] was utilized to construct the pH-responsive particles due to its high biocompatibility. The Ace-Dex was prepared by treating a mixture of dextran (MW: 9–11 kDa) and 2-methoxypropene in DMSO with pyridinium *p*-toluenesulfonate (Fig. 1). Two CTL peptide epitopes, one from human mucin-1 with the sequence of PDTRPAP [20] and the other from ovalbumin with the sequence of SIINFEKL, were synthesized through solid-phase peptide synthesis using fluorenylmethyloxycarbonyl (Fmoc) chemistry (Supplementary Figures S1,2). Ace-Dex was treated with an emulsion evaporation method [17] to form MPs encapsulating the peptide. Of the two peptides, SIINFEKL was encapsulated at a much higher efficiency (40%) compared to mucin-1 (<5%) and was used in subsequent studies. The SIINFEKL is a well-known CTLp capable of binding strongly with MHC-I H-2K^b with a K_d value of 3 nM [21]. The higher encapsulation efficiency of SIINFEKL peptide is presumably due to its higher lipophilicity (The value of cLogP, a common measure of lipophilicity of a compound, of SIINFEKL is calculated to be –2.2, while that for MUC1 PDTRPAP sequence is –5.1). The CTLp-Ace-Dex-MPs had average diameters of 597 nm (Supplementary Figure S3) with a zeta potential of –5.3 mV.

To test the pH responsiveness, the Ace-Dex-MPs were incubated in PBS buffers with pH values of 7.4, 6.5, 6.0 and 5.5 at room temperature and 37 °C, respectively. The scattering of light by the MP suspension was monitored by UV–Vis at 600 nm. At pH 7.4, 6.5 and 6.0, there were very little absorbance changes over 80 h at either room temperature or 37 °C (Fig. 2a). At pH 5.5, the MPs gradually dissolved in solution over 40 h due to the cleavage of acetal linkages under the acidic condition. The rate of peptide release from the MPs was evaluated with the amounts of CTLp discharged to solution determined by HPLC analysis. At pH 7.4, the majority of CTLp remained within the MPs over 24 h, suggesting despite the small size of the CTLp, the spontaneous release rate from the MPs was slow (Fig. 2b). The release rate was significantly enhanced at pH 5.5 with a half-life of about 4 h. Based on HPLC quantification (Supplementary Figure S4), the CTLp-Ace-Dex-MPs contained 20 µg CTLp/mg MP and 100% of the peptide could be released from the MPs at pH 5.5.

CTLp delivered by the MPs could be loaded on MHC-I of target cells and activate T cells in vitro

In order to elicit a CTL response, the CTLp needs to be presented on MHC-I molecules of target cells. The abilities of the MPs to deliver CTLp to cells were evaluated next in vitro using EL4 cells. EL4 cells do not naturally express the CTLp SIINFEKL, thus serving as a model of ALV cells. CTLp-MPs were incubated with EL4 cells at 37 °C. To detect the presentation of SIINFEKL peptide by MHC-I through flow cytometry, fluorescently labeled

monoclonal antibody (mAb) (clone 25-D1.16), which recognizes the complex formed by SIINFEKL with MHC-I but not the free peptide itself, was utilized. A dose-dependent increase of cellular fluorescence intensity was observed upon increasing the amounts of CTLp added (Supplementary Figure S5). Even with a short incubation time of 15 min with the CTLp-MPs (containing 54 ng of CTLp), significant amounts of CTLp/MHC-I complexes were detected on cell surface (Supplementary Figure S6a). The highest fluorescence intensity was observed after 6 h of incubation. These results suggest that the exogenous CTLp delivered by CTLp-MPs could compete with endogenous CTLp to be presented by MHC-I molecules on tumor cells.

The duration of CTLp SIINFEKL presented on tumor cells was evaluated next. After incubation of EL4 cells with CTLp-MP or free CTLp (54 ng) for 1 h, cells were centrifuged to remove the culture media containing the unbound MP and peptide. Fresh media was then added to the cells, and the amounts of CTLp/MHC-I complexes remained on cell surface were monitored. While free CTLp was presented on cell surface quicker than encapsulated CTLp (0–3 h) (Supplementary Figure S6b), more CTLp/MHC-I complexes were observed 6 h after cells were pulsed with CTLp-MPs, hinting the gradual release of encapsulated CTLp inside the cells. After 24 h, there were about 15% of CTLp/MHC-I remaining on cell surface.

To assess whether the CTLp presented by EL4 tumor cells could be recognized by CTLs, in vitro CTL activation assay was carried out using B3Z CD8⁺ T cells [22]. B3Z cells express T cell receptors specific to the SIINFEKL epitope presented on H-2K^b MHC-I molecules. Upon engagement of its T cell receptors by CTLp/H-2K^b complexes, B3Z cells are activated to secrete β -galactosidase, which cleaves its substrate CPRG to yield a colorimetric readout. EL4 cells were pulsed with either the free CTLp or CTLp-MPs for 6 h and then co-cultured with B3Z cells for an additional 16 h followed by addition of CPRG. Incubation of EL4 tumor cells with CTLp-MPs led to much higher activation of B3Z cells than the free peptide at the equivalent peptide concentrations (Fig. 3). These results suggest CTLp-Ace-Dex-MPs can potentially be highly effective against tumors.

CTLp-Ace-Dex-MPs protected mice from tumor-induced death under a therapeutic setting

With the abilities of MPs delivering CTLp to cells and activating CTLs established in vitro, tests were carried out to determine if CTLp-MPs can offer protection against tumor growth in vivo. To expand CTLs against the SIINFEKL epitope, C57BL/6 mice were immunized subcutaneously under the scruff on day 0 with 50 μ g of ovalbumin protein containing the SIINFEKL sequence as an emulsion in complete Freund's adjuvant [23]. Booster injections were given on days 14 and 28 with ovalbumin and incomplete Freund's adjuvant. To confirm the generation of SIINFEKL-specific CTLs, an in vivo CTL assay based on carboxyfluorescein succinimidyl ester (CFSE) labeling was carried out by injecting CFSE-labeled cells loaded with the CTLp to ovalbumin-immunized mice [24]. The viabilities of cells pulsed with the CTLp were significantly reduced compared to those not incubated with the epitope (Supplementary Figure S7), suggesting the successful production of SIINFEKL-specific CTLs in mice through immunization.

To establish tumor in mice, EL4 cells (2×10^5 cells) were injected subcutaneously into the left flank of ovalbumin-immunized mice (day 0) [25]. When the tumor sizes reached 90 mm³, mice were injected intratumorally with free SIINFEKL CTLp (7 µg), CTLp-Ace-Dex-MPs (equivalent to 7 µg of peptide), empty Ace-Dex-MPs or PBS, respectively. Each mouse received an injection every other day for a total of three injections, and tumor growth was continuously monitored. As EL4 cells do not endogenously express the SIINFEKL epitope, ovalbumin immunization may not have much direct beneficial effects on mouse survival. Although there were CD8⁺ T cells present in the tumor (Supplementary Figure S8), tumor in the group receiving PBS only grew rapidly and all mice died from tumor on day 20 (Fig. 4a, b). The group administered with empty Ace-Dex-MPs showed similar tumor growth and mortality rates as the PBS group, suggesting empty Ace-Dex-MPs did not bestow any protections. The injection of free peptide slowed down the growth of tumor and protected 20% of the mice from tumor-induced death. In contrast, the group receiving CTLp-Ace-Dex-MPs showed 100% survival and the sizes of the tumor were significantly smaller (Fig. 4a, b). The superiority of CTLp-Ace-Dex-MPs compared to free peptide in tumor protection may be partially due to the more efficient CTL activation by tumor cells incubated with the CTLp-MPs compared to those with the free peptide (Fig. 3).

With the efficacy of local intratumoral delivery of CTLp-MPs established, systemic administration was tested next. The subcutaneous EL4 tumor-bearing ovalbumin-immunized mice were injected intravenously via tail vein with CTLp-Ace-Dex-MPs (equivalent to 20 µg of CTLp), free CTLp (20 µg), empty Ace-Dex-MPs or PBS, respectively. A total of three injections were administered on days 3, 5 and 7 as in the intratumoral study. All mice in the PBS and empty Ace-Dex-MP study groups died within 15 days (Fig. 4c, d). The free CTLp had little protection as all mice died by day 18. The CTLp-Ace-Dex-MPs, however, provided significant protection with 60% of the mice surviving the tumor challenge (Fig. 4d). No adverse autoimmunity complications were observed in all mice.

To test the generality of the approach, another subcutaneous tumor model was examined using MC38 colon adenocarcinoma cells. The subcutaneous MC38 tumor-bearing ovalbumin-immunized mice were treated with PBS, free CTLp, empty Ace-Dex-MPs or CTLp-Ace-Dex-MPs following a similar schedule as that for the EL4 model. The tumor sizes of CTLp-Ace-Dex-MPs-treated groups were significantly smaller than other groups (Supplementary Figure S9). In addition, a group of non-immunized mice was grafted with MC38 cells and treated with CTLp-Ace-Dex-MPs under the same schedule to probe the protective effect of CTLp-Ace-Dex-MPs without pre-immunization. Interestingly, even without prior ovalbumin immunization, these mice exhibited reduced tumor growth rates as pre-immunized mice.

CTLp-Ace-Dex-MPs delivered the CTLp into tumor tissues

To better understand the protective effects of CTLp-Ace-Dex-MPs via systemic administration, the delivery of CTLp to tumor environment was evaluated. Naïve mice were inoculated with EL4 tumors. When the tumor size grew to 300 mm³, CTLp-Ace-Dex-MPs were administered intravenously via tail vein. The mice were euthanized 9 h post-injection with the tumors harvested. Single-cell suspension was generated from the tumor tissues by

enzymatic digestion, stained with mAb 25-D1.16 to detect the presentation of CTLp by MHC-I on cell surface, and the cells were subjected to flow cytometry analysis (Supplementary Figure S10). Significant enhancement in cellular fluorescence was observed, suggesting the successful epitope delivery and MHC-I presentation by cells in tumor tissues. Similar enhancement of CTLp in tumor tissues was also observed in the MC38 model (Supplementary Figure S11). The presence of CTLp in other organs was examined in CTLp-Ace-Dex-MP-treated mice. There were negligible changes of CTLp presented by MHC-I in spleens and lungs, while higher mean level of CTLp was found in livers compared to PBS-treated mice (Supplementary Figure S11).

Further confirmation of CTLp presentation in tumor was obtained by histopathology staining of the tumor tissues. Tumor was sliced and stained for both CTLp SIINFEKL bound to MHC-I and a stromal cell marker CD11b, which was subsequently analyzed by confocal microscopy. The images revealed significant staining for CTLp/MHC-I complexes in tumor tissues (Fig. 5a) compared to slices stained with isotype control antibodies. The co-localization of the red (CTLp/MHC-I) and green (CD11b) colors (Fig. 5c) indicated that CTLp was presented by tumor stromal cells as well. In contrast, tumor obtained from mice injected with free CTLp gave little staining by the mAb 25-D1.16 (Fig. 5d).

Besides delivery of CTLp to tumor tissues, it is possible that CTLp-MPs may elicit CTL responses directly in vivo. To test this possibility, CTLp-MPs, free CTLp, CTLp admixed with MPs were injected intravenously into naïve mice at equivalent amounts of 20 µg peptide. Mice immunized with ovalbumin were used as a positive control. CTL activities were evaluated in these mice via the in vivo CFSE CTL assay. Interestingly, even without any exogenous adjuvant, CTLp-MPs were able to induce SIINFEKL-specific CTL responses 7 days after intravenous injection leading to reduced viabilities of CTLp-bearing cells to a similar extent as the positive control (Fig. 6). By day 14, the CTL activities waned. In comparison, mice receiving free CTLp or empty MPs admixed with free CTLp without encapsulation did not elicit much CTLp-specific responses. These results suggest that empty Ace-Dex-MPs do not non-specifically boost immune responses. The superior protection bestowed by CTLp-Ace-Dex-MPs compared to free CTLp could be attributed to enhanced CTLp delivery to tumor tissues and/or the additional CTLp-specific CTLs generated by CTLp-Ace-Dex-MPs.

Discussion

Development of novel constructs that can provide powerful immune protection against tumor development is of high current interests. While particle-based antitumor vaccine constructs have been investigated [26], they have been primarily aimed at targeting professional antigen-presenting cells. At the same time, most of the studies on introducing foreign antigenic epitopes to tumor did not include a delivery system and therefore required administration of antigens locally to tumor [27–29]. Recently, a novel hyaluronic acid–OVA protein conjugate was shown to transport OVA epitopes to tumor cells upon systemic administration [30]. In comparison, our approach delivers foreign epitopes to tumor cells as well as stromal cells utilizing pH-responsive MPs.

A significant challenge in antitumor CTL therapy is the immune-editing of tumor cells rendering the immune system ineffective against ALVs of tumor cells. EL4 and MC38 cells serve as ALV models as they do not express the SIINFEKL epitope endogenously. These cells do not elicit SIINFEKL-specific CTLs, and anti-SIINFEKL immunity by itself did not have much effect on these tumors. However, treatment of mice with SIINFEKL containing MPs either intratumorally or systematically significantly reduced tumor growth (Fig. 4 and Supplementary Figure S9). This suggests epitope delivery by MPs can be an effective approach to combat ALVs.

CTLp-Ace-Dex-MPs can deliver CTLp to tumor microenvironment (Fig. 5 and Supplementary Figure S10). In contrast, systemic administration of free CTLp to tumor-bearing mice did not bestow any tumor protection with little CTLp found in tumor tissues. The inability of free CTLp to reach tumor was possibly due to unfavorable pharmacokinetics and limited half-life of the free peptide in vivo [31]. Through encapsulation, the CTLp-Ace-Dex-MPs could protect the CTLp from protease digestion and enhance epitope delivery and retention in tumor tissues through the enhanced permeability and retention effect [14, 15]. The MPs could release the free CTLp in the tumor microenvironment for loading onto MHC-I of tumor and stromal cells, rendering it possible to remove both tumor cells and the cells supporting tumor growth by anti-SIINFEKL CTLs. Alternatively, cells in tumor tissues including macrophages can uptake the MPs into acidic organelles such as lysosomes, which can cleave MPs and release the encapsulated CTLp for cross-presentation on MHC-I.

Besides improving epitope delivery to tumor tissues, MPs can enhance CTL activities. In vitro, CTLp-Ace-DEX-MPs showed greater-than-tenfold enhancement of B3Z activation upon target cell incubation compared to free CTLp (Fig. 3). This observation corroborated with the report from Frechet group that a protein encapsulated by MPs could activate CTLs in vitro [18]. As proteins would require protease processing to generate the desired epitope for MHC-I binding while CTLp could be directly loaded, much more protein (μg) was needed to induce strong B3Z activation versus the CTLp (70 pg). The CTLp-Ace-Dex-MPs could also induce CTL activation in vivo. Without ovalbumin pre-immunization or exogenous adjuvants, administration of CTLp encapsulating MPs to tumor-bearing mice provided significant protection from tumor growth compared to empty MPs or free CTLp. The exact mechanism for CTL activation by CTLp-Ace-Dex-MPs is not known and needs further study.

In addition to tumor tissue, an increase of CTLp levels in livers was observed upon intravenous injection of CTLp-Ace-Dex-MPs, while little CTLp was found in the spleen and the lung. This indicates that a major pathway for MP clearance is through the liver, which is common for many particle-based systems [32]. No autoimmune responses or liver toxicities were observed during the current study.

Innovative studies have been performed using Ace-DEX-MPs for cargo delivery [18, 33–35]. Our study is the first time that Ace-DEX-MPs were shown to be effective in delivering small peptides to tumor microenvironment to enhance antitumor immunotherapy. Compared to intratumoral injection, intravenous administration of CTLp-Ace-Dex-MPs did not provide complete protection of the mice from tumor-induced death. This may be due to lower

concentration of CTLp reaching the tumor tissue upon systematic administration, which could be improved by increasing the frequency of injections and optimization of the injection protocol. Furthermore, tumor tissue targeting ligands can be installed onto MPs to enhance tumor selectivity and reduce the amounts of CTLp reaching other organs such as liver.

SIINFEKL epitope from ovalbumin was selected as the CTLp due to its high affinity with MHC-I H-2K^b and efficient MP encapsulation. For potential future translations to human patients, as many people have been immunized against diseases such as papillomavirus and hepatitis B infection, high-affinity lipophilic CTL epitopes can be selected from these viruses [36, 37] to match MHC-I haplotypes of the subjects. Patients may already have memory CTLs [38] against these epitopes from prior immunizations enabling ready expansion of epitope-specific CTLs to combat tumor. In addition, antigen delivery to tumor tissues can be combined with other promising antitumor immunotherapy strategies to increase the protective efficiencies. For example, mAbs capable of blocking immune regulatory checkpoints [39] can enhance antitumor functions of CTLs. Combination of CTLp delivery and immune checkpoint inhibition can provide synergistic effects on tumor protection. These considerations suggest the potential for clinical translation using MPs for CTLp delivery.

In conclusion, we have demonstrated that acid-responsive MPs can deliver foreign high-affinity CTLp to tumor tissues and release CTLp for presentation by MHC-I on both tumor and stromal cells. CTLp delivery by MPs to two models of ALV solid tumor led to significant reduction of tumor growth. The simplicity and versatility of the MP system coupled with its antitumor efficacy suggest that it can be a promising new direction to enhance the efficacy of CTL therapy against established solid tumor.

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Abbreviations

7-AAD	7-Aminoactinomycin D
ACE-Dex	Acetalated dextran
ALV	Antigen-loss variant
CFSE	Carboxyfluorescein succinimidyl ester
CRPG	Chlorophenol red- β -D-galactopyranoside
CTL	Cytotoxic T lymphocyte
CTLp	CTL peptide epitope
DCM	Dichloromethane

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fmoc	Fluorenylmethyloxycarbonyl
HBSS	Hank's balanced salt solution
mAb	Monoclonal antibody
MHC-I	Major histocompatibility complex class I
MP	Microparticle
PBS	Phosphate-buffered saline
PE	Phycoerythrin
RPMI	Roswell Park Memorial Institute

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Fig. 1.
Synthesis of CTLp-Ace-Dex-MPs

Fig. 2.

a Hydrolysis of Ace-Dex-MP at pH values of 7.4, 6.5, 6.0 and 5.5 at room temperature and 37 °C, respectively, in PBS buffer as monitored by UV–Vis at 600 nm. **b** CTLp release from CTLp-Ace-Dex-MPs at 37 °C in PBS buffer at pH 5.5, 6.0, 6.5 and 7.4

Fig. 3.

In vitro CTL activation assay of B3Z cells cultured with EL4 cells following incubation with either free CTLp or CTLp-Ace-Dex-MPs. The *t*-test was used for analysis, **** $p < 0.0001$. Absorbance was measured at 595 nm

Fig. 4. Protection efficacy of mice bearing established subcutaneous EL4 tumors through **a–b** intratumoral and **c–d** intravenous administration of PBS, Ace-Dex-MP, free CTLp or CTLp-Ace-Dex-MPs. Compounds were administered on days 3, 5 and 7. **a, c** Changes of tumor volume over time; **b, d** Kaplan–Meier survival curve. ** $p < 0.005$; **** $p < 0.0001$ compared to the group receiving free CTLp. The log-rank and the t -test methods were used for analysis

Fig. 5. Confocal microscopy images of tumor tissues from mice receiving intravenous injection of **a–c** CTLp-Ace-Dex-MPs and **d–f** free CTLp. **a, d** *Red channel* showing PE-labeled mAb 25-D1.16 detecting CTLp/MHC-I H-2K^b complexes; **b, e** *Green channel* showing FITC-labeled anti-CD11b, a marker for stromal cells; **c** Overlay of panels **a** and **b**; **f** Overlay of panels **d** and **e**. The *scale bar* is 10 μm. The significantly higher intensities of *red color in panel a* versus *d* indicate higher amounts of CTLp/MHC-I complexes in tumor tissues of mice receiving CTLp-Ace-Dex-MPs

Fig. 6.

In vivo CTL activity assays. Mice were injected with CTLp-Ace-Dex-MP, Ace-Dex-MP mixed with CTLp or free CTLp (20 μ g), respectively, every other day with a total of three injections. The positive control is ovalbumin-immunized mice. The time indicated is the number of days after the final injection. CFSE-labeled live CTLp-pulsed cells were then injected into the mice and isolated from lymph nodes and spleens 24 h after injection. Mice with high CTL activities led to lower relative survival rates of CFSE-labeled live CTLp-pulsed cells. Cells isolated from lymph nodes and spleens gave similar results that CTLp-Ace-Dex-MPs could induce SIINFEKL-specific CTLs, while Ace-Dex-MPs admixed with CTLp or free CTLp was ineffective