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Molecular Cancer Therapeutics

Preclinical Development

Multiple Antigenic Peptides Based on H-2K^b–Restricted CTL Epitopes from Murine Heparanase Induce a Potent Antitumor Immune Response *In Vivo*

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Abstract

Accumulating research suggests that heparanase may be a universal tumor-associated antigen (TAA). Several heparanase T-cell epitopes from humans and mice have already been identified. However, because of low immunogenicity, polypeptide vaccines usually have difficulty inducing effective antitumor immune responses *in vivo*. In this study, to increase the immunogenicity of polypeptide vaccines, we designed and synthesized two four-branch multiple antigenic peptides (MAP) on the basis of mouse heparanase (mHpa) T-cell epitopes (mHpa398 and mHpa519). The dendritic cells (DC) from mice bone marrow loaded with above MAP vaccines from heparanase were used to evaluate immune response against various tumor cell lines, compared with immune response to their corresponding linear peptides, *ex vivo* and *in vivo*. We further assessed *IFN-γ* release both in CD4$^+$ T-cell–depleted and nondepleted mice. The results showed that effectors generated from DCs, loaded with MAP-vaccinated mice splenocytes, induced a stronger immune response against target cells expressing both heparanase and H-2K^b than did effectors generated from mice vaccinated with their corresponding linear peptides. Heparanase-specific CD8$^+$ T-cell responses induced by MAP and linear peptide vaccination required synergy of CD4$^+$ T cells. In addition, heparanse-derived MAP vaccines significantly inhibited the growth of B16 murine melanoma in C57BL/6 mice, while also increasing the survival rate of tumor-bearing mice. Our data suggest that MAP vaccines based on T-cell epitopes from heparanase are efficient immunogens for tumor immunotherapy. *Mol Cancer Ther; 11(5); 1183–92. ©2012 AACR.*

Introduction

Advanced malignant tumors are often characterized by invasion and metastasis (1). The prognosis of patients with tumor metastasis is very poor, and metastasis continues to be a major cause of mortality in patients with tumors. Once metastasis occurs, patients often lose the option of surgical treatment and must rely on chemotherapy and radiotherapy instead. Because of the severe adverse effects of these therapies, patients often do not tolerate these treatments well. Therefore, immunotherapies based on dendritic cells (DC), which have the advantages of strong immunogenicity and limited adverse effects, have become a topic of interest in the search for treatments for malignant tumors (2–4). DCs loaded with tumor-associated antigens (TAA) initiate antigen-specific immune responses and are usually used to induce antitumor responses (4, 5). However, all TAAs currently identified have tissue specificity; for example, MART-1 is only directed against melanoma, and prostate-specific antigen is only found in association with prostatic carcinoma. Therefore, immunotherapy targeting these TAAs is limited to the corresponding tumor, which leads to narrow therapeutic windows. Thus, it is important to search for a universal tumor-specific antigen (6).

Heparanase (Hpa) is a unique endogenous endoglycosidase that cleaves heparan sulfate proteoglycans, the main proteoglycan component of the extracellular matrix (7). Heparanase expression has been reported to be correlated with a poor prognosis for patients with cancer (8–10) in almost all advanced malignant tumors. Furthermore, inhibition of heparanase significantly reduced invasion and metastasis of tumor cells (3, 11–13), and several potent heparanase inhibitors have shown promising efficacy at the preclinical stage. One of representative heparanase inhibitors, PI-88, has been evaluated in a multicenter phase II or III clinical trial (1, 6), but due to the multiple biologic activities of heparanase inhibitors, the mechanism of their antitumor activity and their relation with heparanase inhibition are not clear; therefore, the

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application of heparanase inhibitors in the clinic may require further studies (14). Accumulating evidence suggests that heparanase can serve as a universal TAA for the immunotherapy of cancer (7, 15–20). In fact, several T-cell epitopes of heparanase have been identified (16–19).

Although promising results of tumor immunotherapy based on peptide vaccines have been shown from animal studies, limited objective clinical responses have been observed in patients with cancer (21, 22). An explanation may be related to the immunogenicity of the peptide vaccines. Synthetic peptide vaccines offer advantages for therapeutic use; they are easy to produce even at clinical grade, free from pathogen contamination, chemically stable, and they have minimal oncogenic potential. Unfortunately, due to their small molecular weight, weak immunogenicity, and rapid degradation, peptide vaccines cannot elicit an ideal immune response in the body. Therefore, their clinical application is limited. To solve this problem, the method of cross-linking epitopes to carrier proteins was often used to increase the immunogenicity of peptide vaccines. However, because carrier proteins are foreign antigens with high molecular weight, the induced antibody response is usually directly against carrier proteins rather than against the target polypeptides.

The Tam laboratory proposed the design of multiple antigen peptides (MAP) in 1988 (ref. 23; in which lysine with small molecular weight and weak immunogenicity was considered as the core matrix). Several strips (generally 4 or 8) of epitope monomers are coupled together to form a branch-like structure. This design strengthens the specificity of the peptide chain structure of the epitope and, at the same time, increases the molecular weight of the epitope peptide. The MAP structure simulates the conformation of the target protein and induces strong immune response without the use of a carrier protein. The attribution of increased *in vivo* stability of epitope peptides allows peptide molecules to be used for the specific targeting of pathologic markers and pathogens (24). It has great prospects for application in vaccine research and development, as well as in tumor immunotherapy (25–27). The design principle of MAP vaccines can be used for B- and T-cell epitopes (24, 25). Recently, Yang and colleagues synthesized a MAP vaccine with B-cell epitopes from heparanase *in vitro* with MAP vaccine theory (28). *In vitro* studies showed that this MAP vaccine induced heparanase-specific antibodies, produced heparanase-specific antigen–antibody reactions, and inhibited the proliferation of liver cancer cells.

To investigate the *in vivo* immune response elicited by heparanase CTL epitopes, we generated two 4-branched MAP vaccines on the basis of previously described H-2K^b–restricted CTL epitopes, derived from murine heparanase (mHpa; ref. 17). Our results show that MAP vaccinations elicited stronger immune responses than did vaccinations with their corresponding linear peptides *in vitro* and *in vivo*. Notably, development of tumor xenografts of B16 melanoma cells was markedly inhibited in mice immunized with MAP vaccines compared with development in mice immunized with their corresponding linear peptide vaccines; this result was observed with both immunoprotection and immunotherapeutic approaches. Moreover, MAP vaccination also prolongs the survival rate of tumor-bearing mice. These data suggest that MAP vaccines that are based on CTL epitopes of heparanase can increase the immunogenicity of peptide vaccines and can be used as efficient immunogens in tumor immunotherapy.

Materials and Methods

Mice and cell lines

Eight-week-old female C57BL/6 (H-2K^b) mice were purchased from the Third Military Medical University (Chongqing, PR China). Animals were housed in a specific pathogen-free environment. Animal experiments were approved by the local ethics committee of the Third Military Medical University. Murine-originated cell lines, including EL-4 lymphoma cell line, Lewis lung cancer cell line, B16 melanoma cell line, and P815 mastocytoma cell line, were purchased from Cell Bank of Shanghai (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, PR China) and used at fewer than 10 passages. No additional authentication of these cell lines was conducted by the authors. All above mentioned cells were cultured in RPMI-1640 media containing 10% fetal calf serum (FCS), streptomycin (100 μg/mL), and penicillin (200 units/mL). Cells were kept at 37°C at 5% CO_2 and medium was refreshed every 2 days.

Expression of heparanase and H-2K^b in target cells

Expression of heparanase in target cells was determined by reverse transcription PCR (RT-PCR) and Western blot analysis as previously described (17). Briefly, we isolated the total RNA of target cells by TriPure RNA Isolation Reagent (Roche), according to manufacturer's instructions. Conditions used for amplification were as described in our previous article (17). For Western blot assay, proteins in the target cells were separated by SDS-PAGE through an 8% PAGE, before being transferred onto a nitrocellulose membrane. Membranes were incubated with 5% nonfat milk in PBS and were later incubated with anti-mouse heparanase polyclonal antibody (Ab) for 2 hours at 37°C. After washing, membranes were incubated with an alkaline phosphatase–conjugated goat anti-rabbit IgG antibody (Amersham Biosciences) for 1 hour at 20°C. Immunoreactive bands were detected with the ECL Western Blot Analysis System (Amersham Biosciences).

Expression of H-2K^b on the surface of target cells was determined by flow cytometry (17). Lewis lung cancer cells, EL-4 lymphoma cells, B16 melanoma cells, and P815 mastocytoma cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-H-2K^b monoclonal Ab or FITC-conjugated immunoglobulin G (IgG) isotype control antibody (eBioscience) for 1 hour. Next, cells were washed twice with fluorescence-activated cell-sorting

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(FACS) buffer (PBS with 0.1% FBS) and examined with a FACScan (Becton, Dickinson and Company).

Synthetic polypeptides

Two H-2K^b–restricted CTL epitope peptides from murine heparanase, mHpa398 (LSLLFKKL), and mHpa519 (FSYGFFVI), and their corresponding 4-branched polypeptides (MAP4-mHpa398 and MAP4-mHpa519) were synthesized by the Chinese Peptide Company (Fig. 1). An octapeptide derived from human influenza virus (NYKHCFEI) was synthesized as a negative control. On the basis of selected amino acid sequences, MAPs were produced by solid-phase peptide synthesis (SPPS) with Fmoc chemistry onto a 4-branched lysine core, resulting in four 8-mer peptides presented on one structure (29). The purity of the peptides was confirmed by analytic high-performance liquid chromatography (HPLC), and their identity was confirmed by mass spectrographic analysis.

DC polypeptide pulsing

We generated DCs from bone marrow of C57BL/6 mice. The method of generating DCs from mouse bone marrow was conducted as previously described (17). Murine bone marrow–derived DCs were cultured in 1 mL of RPMI-1640 containing 10% FCS, 50 μmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The concentration of peptide pulsing is 30 μmol/L. The different MAP peptides and their corresponding linear peptide and DCs were incubated at 37°C for 3 hours, respectively. DCs were then washed by normal saline twice and used for further experiments.

Induction of memory T cells *in vivo*

C57BL/6 mice were immunized by s.c. injection of 1×10^6 of the above peptide-pulsed DCs for 3 times, once a week. As a control, mice were vaccinated with the same procedure, but with negative peptide–pulsed DCs as described previously (17, 19).

Prepare heparanase-specific CTLs

Mice were sacrificed 7 days after the third vaccination and their spleens were removed. Ficoll-purified lymphocytes were incubated (4×10^5/mL) with different peptides (30 μmol/L) in 6-well plates in RPMI-1640 containing interleukin (IL)-2 (50 U/mL). After 5 days of incubation, the lymphocytes from C57BL/6 spleen were collected for standard 4-hour ^{51}Cr-release assay (17, 19).

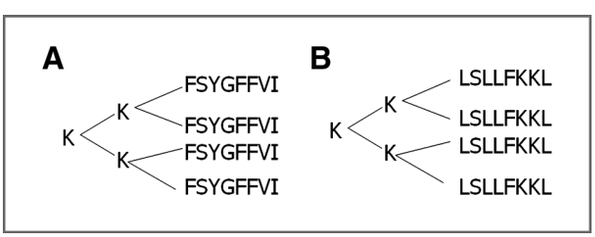

Figure 1. Analogue structure of 4-branched peptides based on H-2K^b–restricted CTL epitopes from murine heparanase. A, MAP4-mHpa519 and B, MAP4-mHpa398.

Cytotoxicity assays

Standard 4-hour ^{51}Cr-release assays were used to evaluate the ability of effectors to lyse target cells. The experiment was conducted as we described previously (16–19). Briefly, target cells were labeled by ^{51}Cr (PerkinElmer). Then target cells were resuspended in RPMI-1640, 10^4 per well in 96-well plate, the effector cells were added to target cells at effector-to-target (E/T) ratios of 10:1, 20:1, 40:1, or 80:1. Target cells and effector cells were incubated for 4 hours in 37°C. After 4-hour incubation, 100 μL supernatants of each well were harvested and detected by γ counter (FM-1000 γ counter). The specific lysis rate was calculated according to the following formula:

$$\text{Specific lysis}(\%) = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Enzyme-linked immunospot assay for IFN-γ

IFN-γ–producing cells in effectors were tested by enzyme-linked immunospot (ELISPOT) assay as described previously (16,17). Briefly, C57BL/6 mice were immunized by s.c. injection of 1×10^6 of MAP4-mHpa398-, MAP4-mHpa519-, mHpa398-, and mHpa519-pulsed DCs 3 times, once a week. One week after the last immunization, the spleen cells from mice were used as CTLs. Ficoll-purified spleen CD8$^+$ T lymphocytes (CD8$^+$ T-cell isolation kit; Miltenyi Biotec) were isolated and cultured in antibody-coated 96-well nitrocellulose plates at a final concentration of 1×10^5 cells per well. Effectors were stimulated with candidate peptides at a final concentration of 30 μmol/L for 20 hours. Phytohemaglutinnin was used as positive control. Plates were processed with a biotin-labeled anti-mouse IFN-γ antibody and then incubated with alkaline phosphatase–conjugated streptavidin. Next, freshly prepared developer was added, and plates were incubated in the dark at 37°C for 8 minutes (Quick Spot Mouse IFN-γ Precoated ELISPOT Kit, DAKEWE). Spots were quantified by the ELISPOT reader (BioReader 4000 Pro-X, BIOSYS). ELISPOT assays were repeated after we depleted CD4$^+$ cells from C57BL/6 mice. The depletion of CD4$^+$ cells was conducted according to the method reported previously (30).

Immunoprotection and immunotherapy by MAP vaccines derived from mHpa

Immunoprotection and immunotherapy were performed according to the previous report (19). For protective vaccination, 30 eight-week-old C57BL/6 mice were divided into 6 groups randomly, vaccinated by s.c. injection of MAP4-mHpa519, MAP4-mHpa398, mHpa519, mHpa398, negative peptide, or PBS-pulsed DCs 3 times at 7-day intervals. Seven days after the last injection, 5×10^5 B16 cells in 200 μL PBS were s.c. injected to each mouse; mice were sacrificed after 6 weeks, and the tumor

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size was measured with a caliper ruler. As controls, mice were injected with 200 μL PBS- or NYKHCFEI-pulsed DCs.

For immunotherapy study, 30 eight-week-old C57BL/6 mice were divided into 6 groups randomly and injected s.c. with 5×10^5 B16 cells. When the tumor growth was first observed, mice were injected s.c. with MAP-mHpa519-, MAP4-mHpa398-, mHpa519-, or mHpa398-pulsed DCs. Peptide-loaded DCs were injected s.c., 3 times at 1-week intervals, respectively. After 6 weeks, mice were sacrificed and a caliper ruler was used to measure the size of tumor. The mice in negative control group were injected with human influenza virus octapeptide (NYKHCFEI)-pulsed DCs or 100 μL PBS.

Survival rates of tumor-bearing mice after injection of therapeutic vaccine

Thirty mice were randomly divided into 6 groups. B16 cells (2×10^5) were injected s.c. in the back flank. After 7 days, when palpable tumors (about 1 mm in diameter) had developed, MAP4-mHpa519-, MAP4-mHpa398-, mHpa519-, or mHpa398-pulsed DCs were injected s.c. 3 times at 7-day intervals. Control mice were injected with 100 μL PBS- or human influenza virus octapeptide (NYKHCFEI)-pulsed DCs (19). Survival rates of mice bearing tumors were observed over a 90-day period.

Statistics

All experiments were conducted in triplicate; the results are presented as mean ± SD. Statistical analyses were conducted by one-way ANOVA with post Tukey test. $P < 0.05$ was considered statistically significant. All statistical analyses were conducted with the SPSS 11.5 software. Survival analysis was conducted by Kaplan–Meier analysis and log-rank test by Prism 5.0 software (GraphPad).

Results

Synthesis, purification, and identification of H-2K^b–restricted CTL epitope peptides from murine heparanase

We synthesized two 4-branched MAPs according to the amino acid sequence of mMAP4-Hpa398–405 (LSLLFKK) and mMAP4-Hpa519–526 (FSYGFFVI) by SPPS, respectively. Peptide purity was confirmed by analytic reverse-phase HPLC. Peptide purity was 90% or more, which corresponds to the international reference standard for polypeptide experiments. The identification of the peptides was confirmed by mass spectrographic analysis. There was not a conspicuous discrepancy between the observed value and the theoretical value, which indicated that those peptides were the correct peptides.

Heparanase and H-2K^b expression in tumor cell lines

Heparanase mRNA and protein expression in target cells were analyzed by RT-PCR and Western blot analysis, respectively. Results showed that heparanase mRNA and protein expression were detected in B16 melanoma cells, EL-4 lymphoma cells, Lewis lung cancer cells, and P815 mastocytoma cells (Fig. 2A and B). However, H-2K^b expression on the surface of target cells was detected on B16, EL-4, and Lewis lung cancer cells, but not on P815 cells (Fig. 2C).

Identification of mouse bone marrow–derived DCs

Mature DCs were generated from bone marrow–derived cells by using granulocyte macrophage colony-

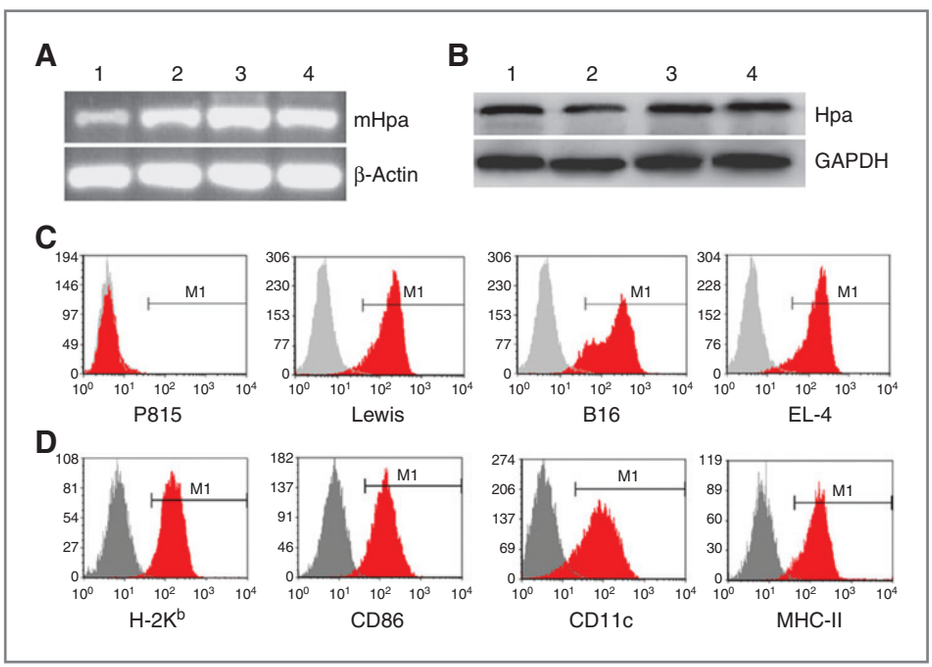

Figure 2. Phenotypes of target cells and mature murine DCs. A, heparanase mRNA expression in different target cells as determined by RT-PCR. 1, B16 melanoma cells; 2, Lewis lung cancer cells; 3, EL-4 lymphoma cells; and 4, P815 mastocytoma cells. B, heparanase protein expression in different target cells as determined by Western blot analysis. 1, B16 melanoma cells; 2, Lewis lung cancer cells; 3, EL-4 lymphoma cells; and 4, P815 mastocytoma cells. C, H-2K^b surface expression on different target cells as examined by flow cytometry. D, mDC phenotypic markers were confirmed by flow cytometry. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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stimulating factor (GM-CSF), IL-4, and TNF-α. The DCs were harvested and the phenotypes of DCs were analyzed by flow cytometry. The results showed that the mature DCs expressing high levels of CD86 (82.5%), CD11c (73.2%), MHC-II (80.1%), and H-2K^b (84.5%; Fig. 2D).

Synthetic peptide induction of CTLs specific for murine heparanase

To detect whether MAP-mHpa vaccines could elicit stronger heparanase-specific CTL responses against various tumor cells than corresponding linear peptides *in vivo*, mDCs were pulsed with MAPs, and their corresponding linear peptides or a negative control peptide. Next, C57BL/6 mice were vaccinated s.c. 3 times at 7-day intervals with the peptide-pulsed DCs as previously described (18). Splenocytes from the vaccinated mice served as effectors. The results indicated that effectors induced by MAP4-mHpa398-, MAP4-mHpa519-, mHpa398-, or mHpa519-pulsed dendritic cells could lyse B16 cells (Hpa$^+$ and H-2K^{b+}), Lewis cells (Hpa$^+$ and H-2K^{b+}), and EL-4 cells (Hpa$^+$ and H-2K^{b+}). Compared with their corresponding linear peptides, heparanase-MAP vaccines elicited increased rates of specific target cell lysis. At the highest E/T ratio, 80:1, the lysis rate was more than 20%. Whereas, the effectors could not lyse heparanase-positive and H-2K^b–negative cells (P815 cells), even with the E/T at 80:1. Effectors generated from the negative peptide failed to lyse target cells (Fig. 3A–D). Taken together, these results indicate that MAP vaccines are capable of eliciting a more powerful heparanase-specific CTL response, compared with that elicited by vaccination with their corresponding linear peptides.

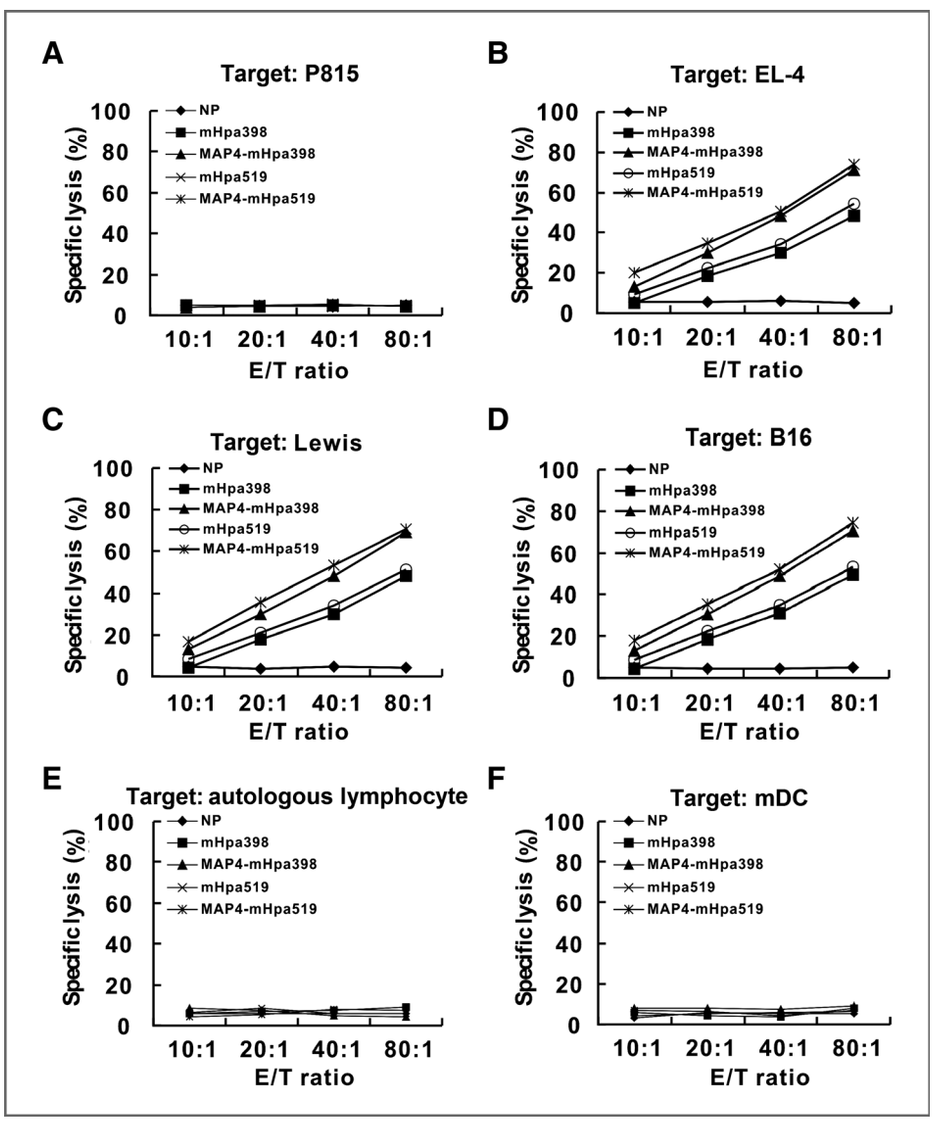

Figure 3. Effectors were induced by DCs loading with peptides. Specific lysis of MAP and linear peptide vaccination induced CTLs on various target cells were determined by standard 4-hour ^{51}Cr-release assay. E/T ratios are presented on the *x*-axis, whereas the *y*-axis represents the percentage of specific lysis. CTLs generated from DCs pulsed with NYKHCFEI served as a negative peptide (NP) control. A, P815 mastocytoma cells; B, EL-4 lymphoma cells; C, Lewis lung cancer cells; D, B16 melanoma cells; E, autologous lymphocytes; and F, DCs.

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Killing effect of murine heparanase–specific CTLs on autologous lymphocytes and DCs

It has been reported that in normal tissue, very low level expression of heparanase could be detected in immunologic cells such as lymphocytes, natural killer cells, DCs, and inflammatory cells. We use heparanase as a target for tumor immunotherapy; *in abstracto* the effectors could recognize heparanase expression in the normal tissue, lyse these cells, and elicit an autoimmune disorder. To investigate the heparanase-specific CTLs on activated lymphocytes and DCs, CTLs induced by murine heparanase–specific peptides were used to lyse autologous lymphocytes and DCs. The autologous lymphocytes, used as target, were prepared by DCs loaded with heparanase-MAP vaccine *ex vivo*. Results indicated that heparanase vaccination had no detectable lysis effect on these cells regardless of MAP vaccination or vaccination with their corresponding linear peptides (Fig. 3E and F).

ELISPOT assay for IFN-γ–producing cells

IFN-γ–producing cells induced by vaccination were quantitated by ELISPOT assay. As shown in Fig. 4, vaccination with MAP4-mHpa398-, MAP4-mHpa519-, mHpa398-, and mHpa519-pulsed DCs generated peptide-specific T-cell responses; MAP vaccinations induced increased frequencies of IFN-γ–producing T cells, compared with vaccination with control peptides ($P < 0.05$). Also, mice vaccinated with MAP4-mHpa398 and MAP4-mHpa519 induced increased numbers of IFN-γ–positive cells than mice vaccinated with their corresponding linear peptides mHpa398 and mHpa519 ($P < 0.05$). We also found that the number of IFN-γ–positive cells was significantly reduced after $CD4^+$ T cells were depleted ($P < 0.05$; Fig. 4A and B). These results suggest that the generation of IFN-γ–producing cells depends on the help of $CD4^+$ T cells.

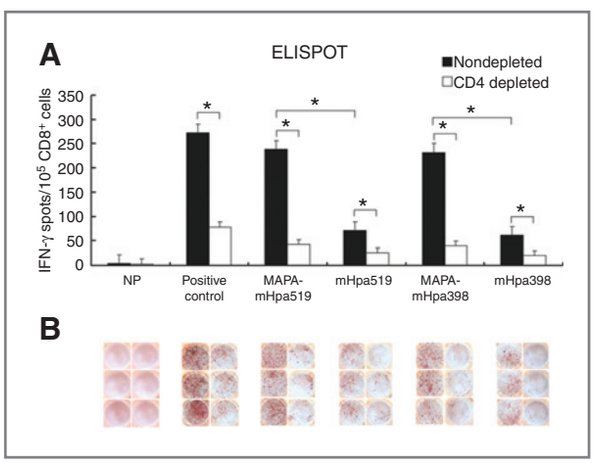

Figure 4. A and B, the IFN-γ–producing cells enumerated by ELISPOT assay before and after $CD4^+$ T-cell depletion. Twelve mice were randomly divided into 6 groups. C57BL/6 mice were treated with anti-mouse-CD4 monoclonal Ab (CD4-depleted) or with saline (nondepleted) once a day for 3 days before vaccination. One week after the third vaccination, splenocytes were cultured with the corresponding peptide at a final concentration of 30 μmol/L. ELISPOT assay was then used to test for IFN-γ–producing cells before and after $CD4^+$ T-cell depletion. Phytohemaglutinnin (PHA) was used as positive control. Column, mean and bars, SE. *, statistically significant values, defined as $P < 0.05$ and determined with one-way ANOVA with post Tukey test.

Induction of immunoprotection and immunotherapy in mice

For immunotherapy, B16 melanoma cells were injected s.c. into the right flank of mice. After 1 week, we made a B16 preexisting tumor–bearing mice model and we vaccinated mice with 3 injections of MAP-mHpa398-, MAP-mHpa519-, mHpa398-, or mHpa519-pulsed DCs once a week for 3 times. PBS- or NYKHCFEI-pulsed DCs were injected as a negative control. The largest diameter of each tumor was measured with a caliper ruler once every 10 days after the injection of B16 cells. Six weeks after the injection of B16 cells, mice were sacrificed and tumors were removed. Tumor size was measured with a caliper ruler. Results showed that MAP4-mHpa398-, MAP4-mHpa519-, mHpa519-, or mHpa398-pulsed DCs inhibited tumor growth. Tumor size was significantly smaller in the MAP-mHpa398, MAP-mHpa519, mHpa398, and mHpa519 therapeutic groups than in the control groups ($P < 0.05$). Furthermore, tumor size was also significantly smaller in the MAP4-mHpa398 and MAP-mHpa519 therapeutic groups than it was in the mHpa519 and mHpa398 groups (Fig. 5A; $P < 0.05$).

To investigate the immunoprotection potential of vaccination with MAP4-mHpa398-, MAP4-mHpa519-, mHpa398-, and mHpa519-pulsed DCs *in vivo*, we vaccinated mice with peptide-pulsed DCs 3 times at weekly intervals. Next, B16 melanoma cells were inoculated to C57BL/6 mice by s.c. injection. PBS- or NYKHCFEI-pulsed DCs were injected as a negative control. The largest diameter of each tumor was measured with a caliper ruler once every 10 days. Six weeks after the injection of B16 cells, mice were sacrificed and the tumors were removed. Tumor size was measured with a caliper ruler. In these experiments, all mice vaccinated with MAP4-mHpa398-, MAP4-mHpa519-, mHpa398-, and mHpa519-pulsed DCs were protected from tumor growth. Tumor size in MAP4-mHpa398, MAP4-mHpa519, mHpa398, and mHpa519 groups was significantly smaller than tumor size in the control groups ($P < 0.05$). Furthermore, tumor size in MAP4-mHpa398 and MAP4-mHpa519 protective groups was also smaller than in mHpa519 and mHpa398 groups (Fig. 5B). Taken together, these data indicate that murine heparanase–derived MAPs offer a strong possibility not only to immunize against tumors but also to treat tumor-bearing hosts successfully.

Survival rates after therapeutic vaccinations

B16 melanoma cells were inoculated by s.c. injection. After the development of palpable tumors (about 1 mm in diameter), mice were vaccinated with injections

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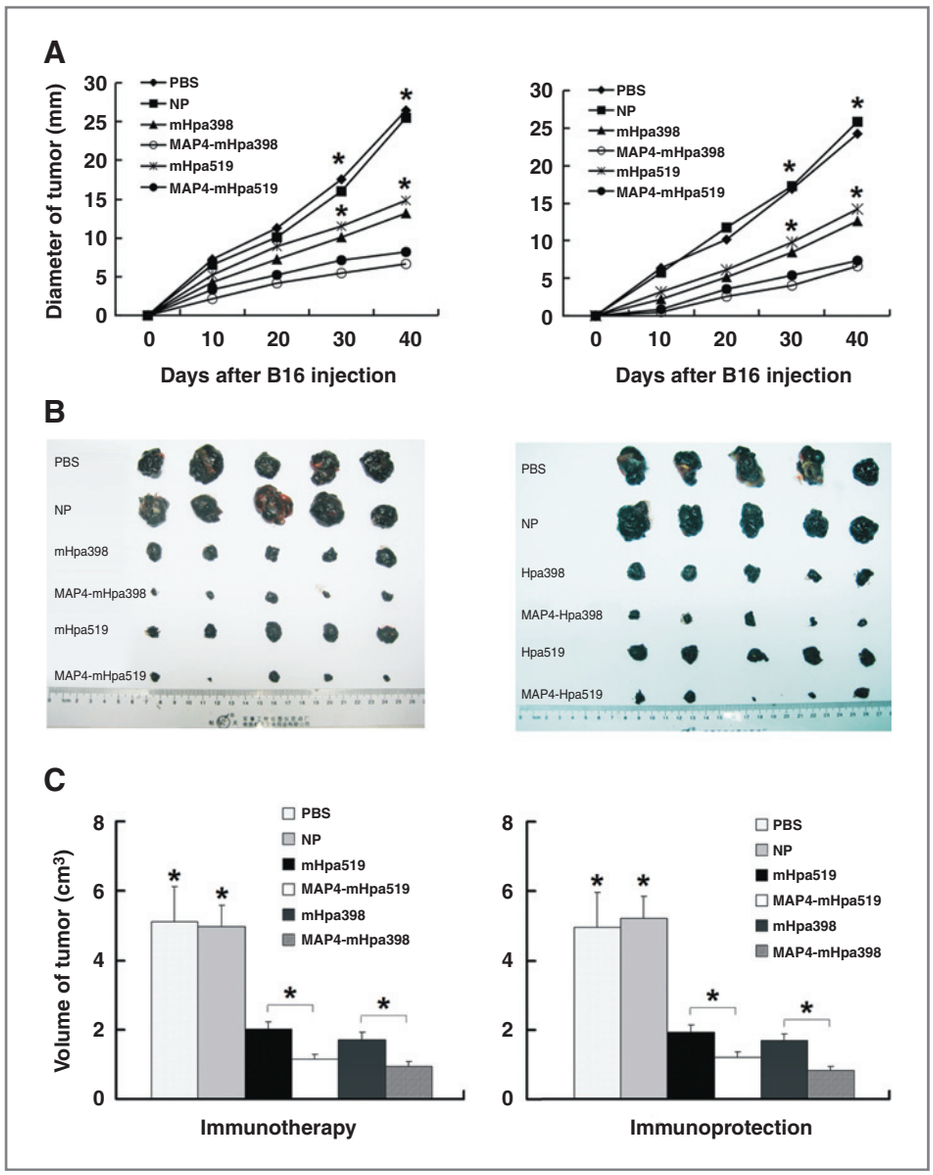

Figure 5. Immunotherapy and immunoprotection generated by murine heparanase-MAP vaccines and their corresponding linear peptides. Immunotherapy (left) or immunoprotection (right) generated by murine heparanase-MAP vaccines and their corresponding linear peptides in C57BL/6 tumor–bearing mice. Mice that received negative peptide (NP)-pulsed DCs or PBS by s.c. injection served as controls. A, dynamic change in the largest diameter of the tumor after immunotherapy (left) or immunoprotection (right) with murine heparanase-MAP vaccines and their corresponding linear peptides. B and C, the tumor volume at time of sacrifice. *, statistically significant values, defined as $P < 0.05$ and determined with a paired Student *t* test, compared with corresponding groups.

of MAP-mHpa398-, MAP-mHpa519-, mHpa398-, and mHpa519-pulsed DCs 3 times at 7-day intervals. PBS- or NYKHCFEI-pulsed DCs were injected as a negative control. The survival of mice bearing tumors was observed for 90 days after the development of palpable tumors. Mice in the negative peptide and PBS control groups started dying 25 days after tumor cell inoculation; all mice in the control groups had died by day 37. Mice that received therapeutic vaccinations with mHpa398- or mHpa519-pulsed DCs began dying at day 48 postinoculation; however, multiple mice from this group survived until the end of the 90-day observation period, resulting in a 40% survival rate. Mice in the MAP4-mHpa398 treatment group began dying at day 85 postinoculation and exhibited an 80% survival rate at the end of the experiment. All mice treated with MAP4-mHpa519–pulsed DCs survived until the end of the observation period, resulting in a survival rate of 100% (Fig. 6).

Discussion

Although peptide-based vaccination has several advantages, including the chemical stability, lack of oncogenic potential, and relatively easy construction and production of the vaccine, the low immunogenicity, easy degradation, and linear structure of peptide-based vaccines make it difficult to induce effective antitumor immune responses *in vivo*. Thus, the widespread use of peptide vaccines has been limited (14). Another possible explanation is the frequent presence of regulatory T cells (Treg) in patients with cancer. Tregs accumulate at the tumor site, where they suppress the effector function of tumor antigen–

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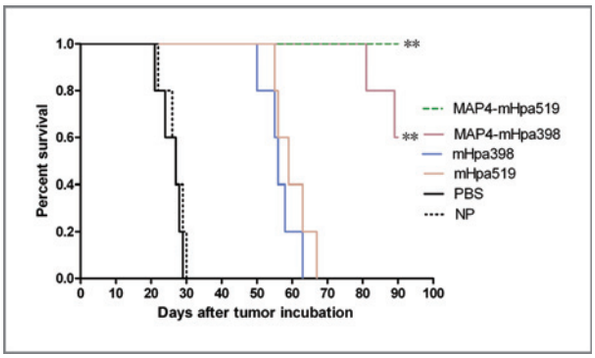

Figure 6 Survival analysis with different vaccination schemes. The *x*-axis displays time expressed as days from initiation of treatment. The Kaplan–Meier test indicated a statistically significant difference between unvaccinated and peptide-vaccinated groups, as well as a statistically significant difference between mice treated with MAP vaccines and mice treated with their corresponding linear peptides. **, the log-rank test is shown compared with unvaccinated and linear peptide–vaccinated groups ($P < 0.01$). NP, negative peptide.

specific T cells, resulting in tumor growth despite the presence of tumor antigen–specific T cells (31, 32). Interestingly, the study by Bonertz and colleagues showed that Tregs against heparanase could not be found in patients with colorectal cancer (33).

In the past several decades, much progress has been made toward the development and structural design of complex polypeptides for use as constituents of a single immunogen. MAP conjugates provide a means to include different stage-specific peptides on one molecule, resulting in a multiepitope and multistage vaccine molecule that potentially improves polypeptide immunogenicity. MAPs, which are based on a small immunologically inert core molecule with radial branching lysine residues that allow for a number of peptide antigens to be anchored, offer an attractive alternative to conventional linear peptide approaches (23, 34). Thus, MAP structure results in a large macromolecule with a unique 3-dimensional configuration that has a high molar ratio of peptide antigen to core molecule and that does not require a carrier protein for elicitation of immune responses. Since invention of these molecules, most MAPs have been used as antigens in serodiagnostic tests and in vaccine discovery (3–5).

Our previous study found that three 4-branched MAP vaccines based on HLA-A2–restricted CTL epitopes of human heparanase elicited stronger immune response against various tumor cell lines than did corresponding linear peptide vaccines both *in vitro* and *ex vivo* (35). Because the mechanisms of antigenic uptake, presentation, and immune response induction *in vivo* are extremely complex, whether these MAP vaccines could generate much stronger immune response *in vivo* remained unknown.

In the present study, we generated two 4-branched MAP vaccines on the basis of previously identified H-$2K^b$–restricted murine heparanase CTL epitopes (17). *Ex vivo* experiments showed that MAP vaccines induced increased heparanase-specific lysis of B16 melanoma cells, EL-4 lymphoma cells, and Lewis lung cancer cells in an MHC-I restricted manner, compared with vaccination with their corresponding linear peptides. More importantly, *in vivo* experiments showed that MAP4-mHpa398 and MAP4-mHpa519 vaccination not only offered more effective immunoprotection but also provided superior immunotherapy in tumor-bearing hosts, compared with vaccination with the corresponding linear peptides. Moreover, MAP vaccines also increased the survival rate and prolonged the life span of tumor-bearing mice. These results suggest that heparanase-derived MAP vaccines have more immunogenicity and are capable of inducing stronger heparanase-specific immune responses against tumors *in vivo*.

Furthermore, the present study revealed that peptide-pulsed DCs stimulated a large amount of IFN-γ–producing cells, thereby increasing the antitumor efficacy of MAP vaccines from heparanase by IFN-γ pathway. In addition, $CD4^+$ T-cell depletion significantly reduced the number of IFN-γ–positive cells induced by vaccination with MAPs or their corresponding linear peptides. Johannsen and colleagues revealed that the T-helper (T_H)1-associated cytokines IFN-γ and IL-2 played key roles in CTL priming by upregulating the chemokine receptor on naive $CD8^+$ T cells (36). IFN-γ induces T_H0 cells to differentiate toward T_Hl cells, promoting the T_Hl cell response and thus regulating the balance of T_Hl/T_H2 (37). In addition, IFN-γ promotes antigen presentation and antigen activation, and it initiates immune responses by strengthening MHC-II expression in the human macrophages considered. Fallarino and colleagues suggest that $CD4^+$ lymphocytes enhance the ability of antigen-presenting cell) to initiate endogenous $CD8^+$ T-cell responses to authentic, poorly immunogenic tumor rejection antigens (38). Our results suggest that the MAP vaccines not only induced heparanase-specific CTLs to lyse various tumor cells but also increased IFN-γ secretion and enhanced the T_H1 immune response, which produced a lethal effect on the tumors. These findings also suggest that heparanase is an ideal broad-spectrum TAA that is suitable for clinical use in the treatment of advanced-stage tumors.

From a safety perspective, the use of heparanase CTL epitopes as TAAs in vaccination poses a risk of autoimmune adverse effects, because heparanase is also expressed by some immune cell populations, including T and B lymphocytes, DCs, macrophages, neutrophils, and mast cells (39). Although our previous study showed that CTLs induced by vaccination with linear polypeptide heparanse could not lyse autologous lymphocytes and DCs expressing heparanase, it was unclear whether heparanase-specific CTLs induced by MAP vaccines, with their enhanced immunogenicity, could lyse autologous immune cells. To this end, MAP vaccine–induced CTLs were used to lyse autologous lymphocytes and DCs. The results indicated that CTLs induced by MAP vaccines did not lyse autologous lymphocytes and DCs. Moreover, we further examined changes in white blood cells in

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immunotherapy and immunoprotection mice before and after vaccination with MAPs or their corresponding linear peptides. These results showed no significant changes in white blood cells (data not shown). These results were similar to the studies by Vonderheide (40, 41). In the experiment carried out by Vonderheide, human telomerase reverse transcriptase (hTERT) served as a universal TAA. He found that hTERT-specific CTLs induced by hTERT-transfected DCs could not lyse hTERT-positive lymphocytes. He explained in his study that the expression of hTERT in lymphocytes was below the threshold, which hTERT-specific CTLs could recognize. In the present study, we also deduced that the level of heparanase expressed in normal cells was below the threshold recognized by these heparanase peptide–specific CTLs. Taken together, our results show that lysis of lymphocytes or DCs do not occur *ex vivo* or *in vivo*. This result suggests that the MAP vaccines based on heparanase are safe and effective.

In summary, we designed 2 MAP vaccines composed of murine heparanase T-cell epitopes (MAP4-mHpa398–405 and MAP4-mHpa519–526) that induced heparanase-specific CTL responses and had distinct effects of immunoprotection and immunotherapy on tumor-bearing mice. MAP vaccines induced stronger responses and better effects, compared with those of vaccination with their corresponding linear peptides. These results suggest that MAP vaccination is an effective design approach for targeting TAAs. Thus, our findings indicate that MAP vaccines based on heparanase T-cell epitopes are suitable immunogens for antitumor immunotherapy and suggest promising potential for clinical applications in humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X.-D. Tang, G.-Z. Wang, M.-H. Lü, S.-M. Yang
Development of methodology: X.-D. Tang, G.-Z. Wang, M.-H. Lü, S.-M. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.-H. Lü, S.-M. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X.-D. Tang, G.-Z. Wang, J. Guo, M.-H. Lü, C. Li, N. Li, Y.-L Chao, C.-Z. Li, Y.-Y. Wu, C.-J. Hu, S.-M. Yang
Writing, review, and/or revision of the manuscript: X.-D. Tang, G.-Z. Wang, M.-H. Lü, S.-M. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X.-D. Tang, G.-Z. Wang, J. Guo, M.H. Lü, C. Li, N. Li, Y.-L. Chao, C.-Z. Li, Y.-Y. Wu, C.-J. Hu, S.-M. Yang
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References

1. Saravanan P, Kumar S, Kataria JM. Use of multiple antigenic peptides related to antigenic determinants of infectious bursal disease virus (IBDV) for detection of anti-IBDV-specific antibody in ELISA–quantitative comparison with native antigen for their use *in sero* diagnosis. J Immunol Methods 2004;293:61–70.
2. Mishima T, Murata J, Toyoshima M, Fujii H, Nakajima M, Hayashi T, et al. Inhibition of tumor invasion and metastasis by calcium spirulan (Ca-SP), a novel sulfated polysaccharide derived from a blue-green alga, *Spirulina platensis*. Clin Exp Metastasis 1998;16:541–50.
3. Liu CJ, Lee PH, Lin DY, Wu CC, Jeng LB, Lin PW, et al. Heparanase inhibitor PI-88 as adjuvant therapy for hepatocellular carcinoma after curative resection: a randomized phase II trial for safety and optimal dosage. J Hepatol 2009;50:958–68.
4. Edovitsky E, Elkin M, Zcharia E, Peretz T, Vlodavsky I. Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst 2004;96:1219–30.
5. Zcharia E, Philp D, Edovitsky E, Aingorn H, Metzger S, Kleinman HK, et al. Heparanase regulates murine hair growth. Am J Pathol 2005;166:999–1008.
6. Mule JJ. Dendritic cell-based vaccines for pancreatic cancer and melanoma. Ann N Y Acad Sci 2009;1174:33–40.
7. Rajagopal D, Paturel C, Morel Y, Uematsu S, Akira S, Diebold SS. Plasmacytoid dendritic cell-derived type I interferon is crucial for the adjuvant activity of Toll-like receptor 7 agonists. Blood 2010;115:1949–57.
8. Cohen E, Doweck I, Naroditsky I, Ben-Izhak O, Kremer R, Best LA, et al. Heparanase is overexpressed in lung cancer and correlates inversely with patient survival. Cancer 2008;113:1004–11.
9. Beckhove P, Helmke BM, Ziouta Y, Bucur M, Dörner W, Mogler C, et al. Heparanase expression at the invasion front of human head and neck cancers and correlation with poor prognosis. Clin Cancer Res 2005;11:2899–906.
10. Ikeguchi M, Fukuda K, Yamaguchi K, Kondo A, Tsujitani S, Kaibara N. Quantitative analysis of heparanase gene expression in esophageal squamous cell carcinoma. Ann Surg Oncol 2003;10:297–304.
11. Ferro V, Dredge K, Liu L, Hammond E, Bytheway I, Li C, et al. PI-88 and novel heparan sulfate mimetics inhibit angiogenesis. Semin Thromb Hemost 2007;33:557–68.
12. Shafat I, Ben-Arush MW, Issakov J, Meller I, Naroditsky I, Tortoreto M, et al. Pre-clinical and clinical significance of heparanase in Ewing's sarcoma. J Cell Mol Med 2011;15:1857–64.
13. Dredge K, Hammond E, Handley P, Gonda TJ, Smith MT, Vincent C, et al. PG545, a dual heparanase and angiogenesis inhibitor, induces potent anti-tumour and anti-metastatic efficacy in preclinical models. Br J Cancer 2011;104:635–42.
14. Naz RK, Dabir P. Peptide vaccines against cancer, infectious diseases, and conception. Front Biosci 2007;12:1833–44.
15. Cai YG, Fang DC, Chen L, Tang XD, Chen T, Yu ST, et al. Dendritic cells reconstituted with a human heparanase gene induce potent cytotoxic T-cell responses against gastric tumor cells *in vitro*. Tumour Biol 2007;28:238–46.
16. Sommerfeldt N, Beckhove P, Ge Y, Schütz F, Choi C, Bucur M, et al. Heparanase: a new metastasis-associated antigen recognized in breast cancer patients by spontaneously induced memory T lymphocytes. Cancer Res 2006;66:7716–23.
17. Tang XD, Wan Y, Chen L, Chen T, Yu ST, Xiong Z, et al. H-2Kb-restricted CTL epitopes from mouse heparanase elicit an antitumor immune response *in vivo*. Cancer Res 2008;68:1529–37.
18. Chen T, Tang XD, Wan Y, Chen L, Yu ST, Xiong Z, et al. HLA-A2-restricted cytotoxic T lymphocyte epitopes from human heparanase as

Published OnlineFirst March 21, 2012; DOI: 10.1158/1535-7163.MCT-11-0607

novel targets for broad-spectrum tumor immunotherapy. Neoplasia 2008;10:977–86.
19. Tang XD, Liang GP, Li C, Wan Y, Chen T, Chen L, et al. Cytotoxic T lymphocyte epitopes from human heparanase can elicit a potent anti-tumor immune response in mice. Cancer Immunol Immunother 2010;59:1041–7.
20. Zhou K, Zhu H, Huang L, Guo Y, Yan Y. Induction of anti-tumor immunity by dendritic cells pulsed with an endoplasmic reticulum retrieval signal modifies heparanase epitope in mice. Cytotherapy 2010;12:735–42.
21. Tacken PJ, de Vries IJ, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from *ex vivo* loading to *in vivo* targeting. Nat Rev Immunol 2007;7:790–802.
22. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol 2005;5:296–306.
23. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci U S A 1988;85:5409–13.
24. Pini A, Falciani C, Bracci L. Branched peptides as therapeutics. Curr Protein Pept Sci 2008;9:468–77.
25. Reynolds SR, Dahl CE, Harn DA. T and B epitope determination and analysis of multiple antigenic peptides for the Schistosoma mansoni experimental vaccine triose-phosphate isomerase. J Immunol 1994;152:193–200.
26. Zhao G, Lin Y, Du L, Guan J, Sun S, Sui H, et al. An M2e-based multiple antigenic peptide vaccine protects mice from lethal challenge with divergent H5N1 influenza viruses. Virol J 2010;7:9.
27. Gomara MJ, Fernandez L, Perez T, Ercilla G, Haro I. Assessment of synthetic chimeric multiple antigenic peptides for diagnosis of GB virus C infection. Anal Biochem 2010;396:51–8.
28. Yang JM, Wang HJ, Du L, Han XM, Ye ZY, Fang Y, et al. Screening and identification of novel B cell epitopes in human heparanase and their anti-invasion property for hepatocellular carcinoma. Cancer Immunol Immunother 2009;58:1387–96.
29. Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int J Pept Protein Res 1990;35:161–214.
30. Iglesias MC, Mollier K, Beignon AS, Souque P, Adotevi O, Lemonnier F, et al. Lentiviral vectors encoding HIV-1 polyepitopes induce broad CTL responses *in vivo*. Mol Ther 2007;15:1203–10.
31. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942–9.
32. Nummer D, Suri-Payer E, Schmitz-Winnenthal H, Bonertz A, Galindo L, Antolovich D, et al. Role of tumor endothelium in CD4+ CD25+ regulatory T cell infiltration of human pancreatic carcinoma. J Natl Cancer Inst 2007;99:1188–99.
33. Bonertz A, Weitz J, Pietsch DH, Rahbari NN, Schlude C, Ge Y, et al. Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. J Clin Invest 2009;119:3311–21.
34. Drijfhout JW, Bloemhoff W. A new synthetic functionalized antigen carrier. Int J Pept Protein Res 1991;37:27–32.
35. Wang GZ, Tang XD, Lü MH, Gao JH, Liang GP, Li N, et al. Multiple antigenic peptides of human heparanase elicit a much more potent immune response against tumors. Cancer Prev Res (Phila) 2011;4:1285–95
36. Johannsen A, Genolet R, Legler DF, Luther SA, Luescher IF. Definition of key variables for the induction of optimal NY-ESO-1-specific T cells in HLA transgene mice. J Immunol 2010;185:3445–55.
37. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. Annu Rev Immunol 2003;21:713–58.
38. Fallarino F, Grohmann U, Bianchi R, Vacca C, Fioretti MC, Puccetti P. Th1 and Th2 cell clones to a poorly immunogenic tumor antigen initiate CD8+ T cell-dependent tumor eradication *in vivo*. J Immunol 2000;165:5495–501.
39. McKenzie EA. Heparanase: a target for drug discovery in cancer and inflammation. Br J Pharmacol 2007;151:1–14.
40. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 1999;10:673–9.
41. Vonderheide RH, Anderson KS, Hahn WC, Butler MO, Schultze JL, Nadler LM. Characterization of HLA-A3-restricted cytotoxic T lymphocytes reactive against the widely expressed tumor antigen telomerase. Clin Cancer Res 2001;7:3343–8.

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Molecular Cancer Therapeutics

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